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The motorized RhoGAP myosin IXb (Myo9b) in leukocytes regulates experimental autoimmune encephalomyelitis induction and recovery

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ABSTRACT

Myo9b regulates leukocyte migration by controlling RhoA signaling. Here we assessed its role in active experimental autoimmune encephalomyelitis (EAE). Myo9b$^{-/-}$ mice show a delay in the onset of EAE symptoms. The delay in disease onset was accompanied by reduced numbers of Th1 and Th17 cells in the CNS. Myo9b$^{-/-}$ mice showed no recovery from disease symptoms and exhibited elevated numbers of both Th17 cells and CD11b+ macrophages. Bone marrow chimeric mice demonstrated that the absence of a leukocyte source of Myo9b was responsible for the delayed leukocyte infiltration into the CNS, delayed EAE onset and lack of recovery.

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1. Introduction

Multiple sclerosis (MS) is a complex chronic autoimmune disease that is characterized by inflammation and demyelination of the central nervous system (CNS) (Compston and Coles, 2008). EAE is widely used to study the pathogenic pathway that leads to the development of MS and serves as a general model for autoimmune diseases (Miller et al., 2010). Upon activation by antigen presenting cells (APCs) in the periphery, naïve CD4+ T cells can differentiate into Th1 cells and Th17 cells, respectively. These activated T cells first expand and then extravasate into the CNS through the blood–brain barrier (BBB). CNS infiltrating T cells are reactivated by myelin epitopes presented by endogenous APCs and further interact with other immune cells within the CNS. Although many studies have proven that T cells are essential and sufficient for induction of EAE, blood-derived macrophages and resident microglia can also contribute to the development of EAE, including the initiation and recovery stages (Brosnan et al., 1981; Heppner et al., 2005; Ponomarev et al., 2005). Activated autoreactive T cells facilitate the recruitment and migration of other inflammatory cells into the CNS, including autoreactive B cells, macrophages and T cells. The convergence of these various cell types within the CNS parenchyma culminates in the destruction of oligodendrocytes and neurons, and the development of disease symptoms characterized by progressive paralysis (Goverman, 2009).

Many factors can impact on immune cell trafficking into the CNS. Of those, Rho GTPases are likely to play an important role in cell recruitment since these G proteins can regulate cell protrusion, contractility and adhesion. For example, signal transduction pathways of Rho GTPases, such as RhoA and Rac1, are critical in facilitating transendothelial migration of T lymphocytes (Etienne et al., 1998; Adamson et al., 1999).

Rho GTPases act as molecular switches, cycling between a GTP-bound, active form and a GDP-bound, inactive form. The Rho GTPases are switched on by guanine nucleotide exchange factors (GEFs) and switched off by GTPase activating proteins (GAPs) (Rossman et al., 2005; Tcherkezian and Lamarche-Vane, 2007). In addition, guanine nucleotide-dissociation inhibitors sequester and block Rho GTPases (Dransart et al., 2005). Mammalian class IX myosins, consisting of myosin IXa (Myo9a), previously called myr 7, and myosin IXb (Myo9b), previously called myr 5, are single headed molecular motors. In their tail domains, they harbor a Rho GTPase activating protein (Rho-GAP) domain, which can increase the rate of GTP hydrolysis by Rho, switching it to the GDP-bound inactive form (Reinhard et al., 1995; Muller et al., 1997; Chieregatti et al., 1998; Post et al., 1998). The two myosin IX isoforms, Myo9a and Myo9b, differ in their expression and localization. Myo9a is expressed abundantly in brain and testis. In the brain, Myo9a was observed in ependymal cells at all developmental stages (Chieregatti et al., 1998; Gorman et al., 1999; Abouhamed et al., 2009). Myo9b is expressed abundantly in tissues of the immune system, like lymph nodes, thymus, and spleen and in several immune cells including dendritic cells, macrophages and CD4+ T cells (Wirth et al., 1996). Macrophages and dendritic cells deficient for Myo9b (Myo9b$^{-/-}$) exhibit reduced migration velocity and impaired chemotaxis (Hanley et al.,

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2010; Xu et al., 2014). Myo9b was shown to be a key negative regulator of RhoA activity in these cells as its loss caused a significant enhancement of RhoA activity (Hanley et al., 2010). Genetic variations in the gene encoding Myo9b heavy chain have been linked with different inflammatory bowel diseases (IBD), such as celiac disease, Crohn's disease and ulcerative colitis (Monsuur et al., 2005; van Bodegraven et al., 2006; Cooney et al., 2009). Furthermore, genetic variations in the gene encoding the Myo9b heavy chain are associated with a higher risk for several autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus and type 1 diabetes (Sanchez et al., 2007; Santiago et al., 2008).

In the present study, we investigated the function of Myo9b during EAE development. The obtained results indicate that Myo9b plays an important role in the propagation of the inflammatory response in the CNS, a finding with potential therapeutic relevance in autoimmune neuroinflammatory diseases such as MS.

2. Material and methods

2.1. Mice

Myo9b deficient mice (Myo9b$^{-/-}$) and WT littermates (backcrossed to C57BL/6 at least 10 generations) were used in active EAE experiments (Hanley et al., 2010). C57BL/6-Ly5.1 (CD45.1) mice were used in bone marrow chimera experiments. All procedures and protocols met the guidelines for animal care and experiments in accordance with national and European (86/609/EEC) legislation.

2.2. Antibodies

The antibodies used in immunofluorescence staining were directed against pan-laminin (455) (Agrawal et al., 2006) and CD4 (H129.19; BD Pharmingen). The antibodies used in flow cytometry were as follows: CD45.2 (104; eBioscience), CD45.1 (A20; BD Pharmingen), CD11b/MAC-1 (M1/70; BD Pharmingen), CD4 (H129.19; BD Pharmingen), CD8 (53–6.7; eBioscience), B220 (RA3-6B2; BD Pharmingen), CD25 (7D4; BD Pharmingen), Foxp3 (FJK-16S; eBioscience), IL-17 (TC11-18H10.1; BD Pharmingen), and IFN-γ (XMG1.2; BD Pharmingen).

2.3. Active EAE

6–8 week-old female mice were used for active EAE induction by subcutaneous immunization with $MOG_{35–55}$ peptide as described previously (Agrawal et al., 2006). The animals were examined daily for neurological defects with clinical grading as follows: stage 1 — limp tail; stage 2 — hind limb weakness; stage 3 — severe hind limb weakness; stage 4 — hind quarter paralysis; and stage 5 — immobilization or death.

2.4. Bone marrow chimeric mice

Recipient Myo9b$^{-/-}$ or WT mice were lethally irradiated (11 Gy) and reconstituted with either WT or Myo9b$^{-/-}$ bone marrow cells or a 1:1 mixture of WT to Myo9b$^{-/-}$ bone marrow cells. Polymorphic lineage determinants (CD45.1/CD45.2) were used for tracking donor versus host derived immune cells. Mice with >95% donor cell engraftment as defined by flow cytometry were employed in EAE (approximately 6–8 weeks after bone marrow transfer).

2.5. Cytometric bead assay (CBA)

Immunized WT and Myo9b$^{-/-}$ CNS samples were snap frozen in liquid nitrogen and homogenized on ice in radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% Na-desoxycholate, 0.1% SDS, 50 mM Tris HCl, pH 8, and protease inhibitor mixture (Sigma-Aldrich)). Cytokine concentrations in the samples were determined using a CBA kit according to the manufacturer's instructions (BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit).

2.6. Flow cytometry

Mice were perfused with PBS before spleens, lymph nodes (LNs), spinal cords and brains were excised. Cells were isolated by cell straining (70 μm for spleens and LNs, 100 μm for brains). Erythrocytes were lysed in spleen preparations with 1× lysis buffer (BD Pharm Lyse). Leukocytes from the blood were isolated using a Ficoll gradient (Cedarlane Laboratories). Brain homogenates were separated into neuronal and leukocyte populations by discontinuous density gradient centrifugation using isotonic Percoll (Amersham Biosciences). For intracellular cytokine staining, isolated leukocytes were stimulated with PMA (10 ng/ml) (Sigma-Aldrich) in the presence of 1 μg/ml Ionomycin and 10 μg/ml Brefeldin A (Sigma-Aldrich) at 37 °C for 6 h. An intracellular staining kit (eBioscience) was used to stain for Foxp3 and intracellular cytokines after permeabilization and fixation of cells. Flow cytometry analysis was performed using a FACSCalibur (BD Biosciences) with the antibodies listed above.

2.7. Histological analysis of spinal cord sections

Mice were intracardially perfused with 4% paraformaldehyde in ice cold PBS, and spinal cords were collected. Paraffin-embedded sections from the thoracic, lumbar and sacral spinal cord were stained with hematoxylin and eosin. Inflammatory foci were quantified as focal areas with ≥10 lymphocytes.

2.8. In vitro T cell proliferation assay

CD4+ T cells were isolated from draining LNs (dLN) of $MOG_{35–55}$ immunized mice at day 10 after immunization using magnetic (MACS) beads (Miltenyi Biotech). Cells were cultured in 96-well flat-bottom microculture plates at a density of 2–5 × 10^4 cells/well in 200 μl RPMI/5%FCS and 0.05 mM β-mercaptoethanol. Splenic DCs (isolated using magnetic beads from non-immunized mice) were treated with Mitomycin C and co-cultured with CD4+ T cells at a density of 1–2 × 10^4 cells/well. In addition, different concentrations of $MOG_{35–55}$ (0–100 μg/ml) were used to stimulate the proliferation of CD4+ T cells for 3 days. T cell proliferation was then determined by the ATPlite assay system (Bittner et al., 2013).

2.9. Ex vivo recall assay

The cells were isolated from the draining LNs of mice at day 10 after immunization with $MOG_{35–55}$ peptide. After lysis of erythrocytes, lymphocytes were counted and cultured in 96-well plates at a density of 5 × 10^5 cells/well in 200 μl RPMI/5%FCS and 0.05 mM β-mercaptoethanol. Cells were cultured for 72 h at 37 °C in the presence of different concentrations of $MOG_{35–55}$ peptide. After 72 h, culture supernatants were collected and the concentrations of IL-1α, IFN-γ, IL-2, IL-6, GM-CSF, IL-4, IL-5, IL-10, TNF-α and IL-17 were measured using a CBA kit according to the manufacturer's instructions (BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit).

2.10. Determination of MOG-specific IgG

Sera were collected from WT and Myo9b$^{-/-}$ mice and stored at 4 °C. ELISA plates were coated with $MOG_{35–55}$ (0–100 μg/ml) either overnight at 4 °C or for 2 h at room temperature. Plates were then washed with 1× PBS/0.05% Tween 20 and blocked with 100 μl 1× PBS/1%BSA/0.05% Tween 20 for 1 h at room temperature. Plates were washed and 100 μl diluted sera (1:50, 1:100, 1:200) were added in triplicate and incubated at room temperature for 1 h. Plates were washed, and plate-bound antibody was detected with anti-mouse IgG conjugated to HRP

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