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Characterization of migration parameters on peripheral and central nervous system T cells following treatment of experimental allergic encephalomyelitis with CRYAB

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ABSTRACT

CRYAB, a small heat shock protein, was previously shown to decrease neuroinflammation in experimental allergic encephalomyelitis (EAE). We investigated whether the expression of cell adhesion molecules and chemokine receptors on peripheral and spinal cord T cells, that could possibly affect their migration to the central nervous system, was altered following EAE CRYAB treatment. Less LFA-1+ lymphocytes and lower levels of iTAC, MCP-5 and MIG were observed in spinal cords of CRYAB-injected EAE animals. In addition, fewer blood T cells expressed CCR6, CXCR4 and CCR7 and *in vivo*-derived CRYAB EAE CD4+ lymphocytes were less migratory towards a MIP-3alpha gradient *in vitro*.

1. Background

Multiple sclerosis (MS) and its animal model, experimental allergic encephalomyelitis (EAE), are characterized by infiltration of myelin-, and possibly neuronal, auto-reactive $CD4^+$ and $CD8^+$ T cells into the central nervous system (CNS). In general, peripheral T cells are primed in the secondary lymphoid organs (spleen and lymph nodes) and migrate into the blood en route to the CNS (Rossi et al., 2011). Multiple cell adhesion molecules and chemokine receptors on these immune cells facilitate their trafficking from peripheral niches to their central destination (Flugel et al., 2001; Charo and Ransohoff, 2006; Bromley et al., 2008; Ransohoff, 2009; Holman et al., 2011; Rossi et al., 2011). In terms of cell adhesion molecules, L-selectin, which is expressed on myeloid cells, naïve T cells and some activated and central memory lymphocytes, mediates homing to inflammatory sites (Ley and Kansas, 2004; Rossi et al., 2011) while the integrins Lymphocyte Function-Associated Antigen 1 (LFA-1) and Very Late Antigen-4 (VLA-4) promote firm adhesion and/or arrest of leukocytes in the blood vessels of lymphoid organs or at sites of inflammation (Melton et al., 2010; Rossi et al., 2011). VLA-4 and LFA-1 also play a role in controlling leukocyte entry into the CNS and blocking them improves clinical disease in EAE (Yednock et al., 1992; Cayrol et al., 2008; Acharya et al., 2010; Melton et al., 2010; Rossi et al., 2011) and MS (Cayrol et al., 2008; Larochelle et al., 2011). In addition, CD6, which is expressed by most T cells and binds to Activated Leukocyte Cell Adhesion Molecule-1, mediates leukocyte extravasation into the inflamed CNS in EAE (Bowen et al., 1995; Cayrol et al., 2008).

Along with adhesion molecules, multiple chemokines and their receptors have been implicated in the trafficking of leukocytes to secondary lymphoid organs and the CNS under physiological and inflammatory conditions such MS and EAE. For example, in the periphery, $CCR7^+$ dendritic cells and naïve and memory $CD4^+$ T cells have been found to follow a CCL19 and CCL20 chemokine gradient from blood to lymph nodes and from tissues into lymphatic organs (Charo and Ransohoff, 2006; Holman et al., 2011). Further, in MS patients, CCL20 signaling through CCR6 on Th17 T cells was shown to be important for these immune cells to be able to enter the CSF through the choroid plexus (Reboldi et al., 2009) while increased expression of CCR1, CCR2 and CCR5 is seen in

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perivascular leukocytes of MS patients that may mediate chemotaxis along the CCL3, CCL4, CCL5 and CCL7 gradients produced by astrocytes and microglia (Ambrosini and Aloisi, 2004; Muller et al., 2004).

A role for chemokines and their receptors has also been observed in EAE. CCL2, CCL3, CCL4, CCL5 and CXCL10 are secreted by leukocytes in the perivascular space and parenchymal lesions of EAE mice (Fischer et al., 2000; Ubogu et al., 2006) and, abluminal expression of CXCL12 at the CNS vasculature appears to attract and retain CXCR4-expressing immune cells to perivascular spaces (McCandless et al., 2009; Holman et al., 2011). Further, EAE severity is reduced in CCR1 and CCR2 knockout mice (Izikson et al., 2000; Rottman et al., 2000). Overall, a plethora of cell adhesion molecules and chemokines and their receptors (Bauer et al., 2009) orchestrate the trafficking of T cells from the periphery to the CNS in MS and EAE. As a consequence, alteration in their expression that may disrupt their migration has been proposed as one therapeutic intervention as validated by Natalizumab, a monoclonal antibody against VLA-4 that suppresses CNS inflammation and reduces relapses in some MS patients (Miller et al., 2003).

CRYAB, a small heat shock protein, is the most highly upregulated gene expressed in acute lesions in MS brains (Chabas et al., 2001) with localization to astrocytes and oligodendrocytes (van Noort et al., 1995). Multiple studies have demonstrated protective functions of this heat shock protein in a variety of cells including chaperoning (Bhat and Nagineni, 1989; Iwaki et al., 1991; Kato et al., 1991a, 1991b; Benjamin et al., 1997; Horwitz, 2003; Ito et al., 2003; Rajasekaran et al., 2007), pro-survival (Aoyama et al., 1993; Mehlen et al., 1995; Andley et al., 2000; Kamradt et al., 2001; Ray et al., 2001; Alge et al., 2002; Liu et al., 2004; Morrison et al., 2004), anti-neurotoxic (Masilamoni et al., 2005a, 2005b, 2006) and anti-inflammatory (Ousman et al., 2007). We, and colleagues, also previously showed that recombinant human (rhu)-CRYAB was therapeutic in EAE by ameliorating clinical signs when administered intravenously during ongoing disease (Ousman et al., 2007). The suppression of clinical disease was associated with reduced splenocyte and astrocyte activation, less demyelination and, a decrease in the number of inflammatory infiltrates present in the brains and spinal cords of rhu-CRYAB-treated EAE animals as compared to vehicle EAE controls (Ousman et al., 2007). Here, we assessed whether the reduced CNS inflammation was associated with alterations in expression of cell adhesion molecules and chemokine receptors on T cells in the blood [peripheral blood mononuclear cells (PBMCs)], spleen, lymph node and spinal cord compartments of EAE animals injected with the small heat shock protein.

2. Materials and methods

2.1. Animals, EAE induction and recombinant human (rhu)-CRYAB treatment.

EAE was induced in 8–12 week old female 129S6 mice (Taconic Farms) with 100 μg myelin oligodendrocyte glycoprotein peptide 35–55 (MOG_{35-55}) (Stanford Pan Facility) in complete Freund's adjuvant (CFA) (volume ratio 1:1) containing 4 mg/ml of heat-inactivated *Mycobacterium tuberculosis* H37Ra (Difco Laboratories). Animals were also injected intravenously (i.v.) on the day of, and two days following immunization, with 400 ng of *Bordetella pertussis* toxin (List Biologicals). For treatment, animals were injected i.v. every day from the day of immunization with 10 μg of rhu-CRYAB (USBiologicals) in 100 μl saline or 100 μl saline as control until day 9 or days 15–18 post-immunization. EAE scores were: 0 = normal, 1 = limp tail, 2 = weak hind limbs, 3 = complete hindlimb paralysis, 4 = complete hindlimb paralysis with some forelimb weakness, 5 = moribund/death. All procedures were carried out in accordance with the guidelines of the Canadian Council of Animal Care and have received approval by the University of Calgary Animal Resources and Ethics Committee. Mice (n = 8–10 per group) were examined and scored daily for clinical signs of EAE.

2.2. Spleen, lymph node and peripheral blood mononuclear cell isolation

Spleens and, axial and inguinal lymph nodes were crushed through a 70 μm mesh cell strainer while blood samples were obtained by cardiac puncture under ketamine:xylazine (200 mg/kg: 10 mg/kg) anesthesia. Red blood cells in the spleen and blood samples were lysed with ACK lysis buffer.

2.3. CNS immune cell isolation

Following perfusion with cold phosphate buffered saline (PBS), spinal cords were crushed though a 70 μm mesh cell strainer, mixed with a Percoll:10× PBS solution at a density of 1.123 g/l and underlaid with a 1.08 g/l Percoll:1× PBS solution. Cells at the 1.123:1.08 g/l interphase were harvested following centrifugation at 1200 g for 30 min at 20 °C without brakes.

2.4. Fluorescence-activated cell sorting (FACS)

0.5–1 × 10^6 cells were mixed with rat anti-mouse Fc block (CD16, Clone 2.4G2, BD Biosciences) at room temperature and then incubated with a combination of fluorescently tagged antibodies at a concentration of 2.5% in the dark for 30 min at 4 °C. The viability dye Sytox Blue (Sigma) was added just prior to acquisition (1:200). The FACS antibodies used were all rat anti-mouse (BD Biosciences, eBiosciences or R&D Systems): CD45 (Clone 30-F11), CD3 (Clone 145-2C11), CD4 (Clone RM4-5), CD8 (Clone 53–6.7), CD49d (VLA-4) (Clone eBioHMb1-), CD11a (LFA-1) (Clone M17/4), CD6 (Clone IM348), CD62L (L-selectin) (Clone MEL-14), CD196 (CCR6) (Clone 140706), CD184 (CXCR4) (Clone 2B11), and CD197 (CCR7) (Clone 4B12). All FACS experiments were performed on individual animals with each group (rhu-CRYAB and PBS) consisting of 3–5 mice. 50,000 (spinal cord)-100,000 (blood, spleen, lymph node) cells were acquired and gated on lymphocytes (Fig. 2A). The data is expressed as percentages (Figs. 1–4) and absolute numbers (Supplementary Figs. 1–4, 6). Since absolute numbers will change depending on how many cells the experimenter acquires, we believe that expressing the data as a percentage is more representative of any changes and thus these are shown in the primary figures.

2.5. Chemokine protein array

Total proteins lysates were obtained from spinal cords in a solution containing 50 mM Tris–HCl pH 7.4, 1% NP-40, 10% glycerol, 1 mM ethylenediaminetetraacetic, 1 mM Na_3VO_4, 1 mM sodium fluoride, 1 mM dithiothreitol, 4.5 mM sodium pyrophosphate, 10 mM beta-glycerophosphate, and a protease inhibitor cocktail tablet (Roche Diagnostics). The supernatants were collected after centrifugation at 14,000 rpm at 4 °C for 30 min, and protein content determined with a spectrophotometer using absorption at 280 nm. Levels of chemokines were assessed using a Quantibody mouse chemokine array (Ray Biotech Inc., Cat # QAM-CHE-1) that was processed according to the manufacturer's instructions. Briefly, 500 μg of protein in 100 μl was applied to the arrays and incubated overnight at 4 °C. The arrays were then incubated with a detection antibody followed by Cy3 equivalent dye-conjugated streptavidin and the fluorescence detected using a Perkin Elmer Scan Array 5000 machine. Each chemokine array was performed on individual mice with 4 mice per rhu-CRYAB and PBS groups.

2.6. Migration assay

At day 9 post-immunization, CD4+ T cells were isolated from PBMCs from PBS and rhu-CRYAB-treated EAE mice using positive selection (MACS-Miltenyi) and resuspended in RPMI 1640 media supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.5 μM beta-mercaptoethanol, 10% fetal calf serum, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate. 5 × 10^5