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High interleukin-10 expression within the central nervous system may be important for initiation of recovery of Dark Agouti rats from experimental autoimmune encephalomyelitis

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ABSTRACT

Dark Agouti (DA) rats are highly susceptible to induction of experimental autoimmune encephalomyelitis (EAE), still they completely recover from the disease. Here, we were interested to determine contribution of major anti-inflammatory cytokines transforming growth factor (TGF)-β and interleukin (IL)-10 to the recovery of DA rats from EAE. To that extent we determined CNS expression of these cytokines in DA rats at different phases of EAE and compared data to those obtained in EAE-resistant Albino Oxford (AO) rats. Higher expression of TGF-β was persistently observed in the CNS of AO rats, even if rats were not immunized. This implied that high TGF-β within the CNS is important for resistance of AO rats to EAE induction. On the contrary, IL-10 expression was consistently higher in DA than in AO rats and it culminated at the peak of EAE. Methylprednisolone suppressed EAE and expression of IL-10 in spinal cord homogenates, while IL-10 was increased in CNS-infiltrating immune cells. This implied that IL-10 might have a significant role in recovery of DA rats from the disease. Thus, we next explored effects of IL-10 on astrocytes, glial cells that largely contribute to control of CNS inflammation. IL-10 stimulated astrocytic expression of an important regulator of neuroinflammation, CXCL12. Thus, IL-10 might contribute to recovery of DA rats from EAE through induction of CXCL12 expression in astrocytes.

Introduction

Experimental autoimmune encephalomyelitis (EAE) is an animal model of multiple sclerosis (MS), chronic inflammatory and demyelinating disease of the central nervous system (CNS). Autoreactive $CD4^+$ T cells (T helper, Th) of IFN-γ-generating Th1 and IL-17-producing Th17 phenotype are major pathogenic populations in EAE and MS (El-behi et al. 2010; Petermann and Korn 2011). Albino Oxford (AO) and Dark Agouti (DA) rats are at the opposite poles of susceptibility to EAE induction. AO rats do not develop EAE in response to harsh immunization protocols that are efficient in other relatively resistant rat strains (Miljkovic et al. 2006), while DA rats develop EAE after mild immunization in the absence of any adjuvant (Stosic-Grujicic et al. 2004). Therefore, we have investigated discrepancies in anti-CNS immune response between AO and DA rats in order to determine cellular and molecular factors that are important for EAE pathogenesis, and potentially relevant for MS. In this way, we have recently demonstrated that a chemokine CXCL12 has a profound anti-inflammatory role in EAE (Miljković et al. 2011). Additional reports from other groups indicate that CXCL12 might have important role in MS pathogenesis (reviewed in Momcilović et al. 2012). Astrocytes are glial cells that play important role in relation between CNS and cells of immune system in MS and EAE. They are constituents of blood brain barrier, they produce various anti-inflammatory mediators and they actively contribute to remyelination of axons (reviewed in Miljković et al. 2011). Importantly, astrocytes are major producers of CXCL12 in the inflamed CNS (Ambrosini et al. 2005).

Abbreviations: AO, Albino Oxford; c.s., clinical score; CFA, complete Freund's adjuvant; CNS, central nervous system; ConA, concanavaline A; ConASn, cell free supernatant collected from 48 h culture of rat splenocytes stimulated with ConA; d.p.i., days post immunization; DA, Dark Agouti; DLN, draining lymph node; DLNC, draining lymph node cells; EAE, experimental autoimmune encephalomyelitis; IFA, incomplete Freund's adjuvant; IFN, interferon; IL, interleukin; MBP, myelin basic protein; MOG, myelin oligodendrocyte protein; MP, methylprednisolone; MS, multiple sclerosis; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffer saline; SC, spinal cord; SCH, spinal cord homogenate; TGF, transforming growth factor; Th, helper T cells.

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Anti-inflammatory cytokines TGF-β and IL-10 are very important for regulation of immune response (Li and Flavell 2008). TGF-β is produced by virtually all cell types and mice deficient for this cytokine succumb to multifocal inflammatory disease mediated by autoreactive T cells (Yaswen et al. 1996). Because of its omnipresence, TGF-β is believed to act continuously to prevent development and reactivation of self-reactive cells (Germain 2012). IL-10 is produced by various cells of adaptive and innate immune system, including those that are relevant for MS and EAE pathogenesis, i.e. T cells, macrophages, dendritic cells and B cells (Harris and Fabry 2012). Both IL-10 and TGF-β are considered to be protective in neuroinflammation (Link 1998; Baabe et al. 2002).

By using our system of DA vs. AO comparison, we determined that AO rats express more TGF-β in the CNS, while IL-10 expression is higher in DA rats CNS at the peak of EAE. Suppression of EAE by methylprednisolone upregulated IL-10 in CNS-infiltrating immune cells of DA rats. In addition, IL-10 stimulated expression of CXCL12 in astrocytes. Thus, pattern of IL-10 expression indicates that this cytokine might promote recovery of DA rats from EAE. Its effect on CXCL12 generation in astrocytes might contribute to the process.

Methods

Experimental animals, EAE induction and evaluation

AO and DA rats – 2–3 months of age, sex matched in each experiment – were maintained in the animal facility of the Institute for Biological Research "Sinisa Stankovic". Animal experiments were approved by the local ethics committee (Institute for Biological Research "Sinisa Stankovic", No. 2-22/10). EAE was induced with rat spinal cord homogenate (SCH) in phosphate buffer saline (PBS, 50% w/v) mixed with equal volume of complete Freund's adjuvant (CFA, Difco, Detroit, MI), or with MBP (50 μg/rat, guinea pig MBP, kind gift from Professor Alexander Fluegel, University of Goettingen, Germany) emulsified with equal volume of CFA. The animals were injected intradermally into their right hind footpad with 100 μl of SCH + CFA or with 100 μl of MBP + CFA, as previously described (Miljkovic et al. 2006). The rats were monitored daily for clinical signs (c.s.) of EAE, and scored according to the following scale: 0, no clinical signs; 1, flaccid tail; 2, hind limb paresis; 3, hind limb paralysis; 4, moribund state or death. Rats were sacrificed at different time points, i.e. days post immunization (d.p.i.). Draining lymph node (DLN) were isolated from rats in the inductive phase (6 d.p.i.). Spinal cords (SC) were removed from rats and homogenized for further analysis at the onset (9–11 d.p.i.; c.s. 1), at the peak (12–14 d.p.i.; c.s. 2–4) and at the recovery (18–22 d.p.i.; c.s. 1 or less) of EAE in DA rats. Rats were extensively perfused with cold PBS through left ventricule before spinal cord isolation and homogenization.

For the treatment with methylprednisolone (MP), DA rats were immunized with SCH + CFA. Beginning on the day when first neurological signs appeared, DA rats were injected daily for 3 days with MP (Hemofarm,Vršac, Serbia) (i.p. 50 mg/kg body weight) or vehicle (PBS). Three hours after the last injection of MP animals were sacrificed. Rats were extensively perfused with cold PBS through left ventricule before spinal cord isolation and homogenization.

Isolation of cells and cell cultures

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood subjected to centrifugation in lymphocyte separation medium (ICN Biomedicals, Solon, OH) according to the manufacturer's instructions. DLN cells (DLNC) were isolated from popliteal lymph nodes. $CD4^+$ T cells were purified from DLNC using biotin conjugated antibody specific for CD4 (eBioscience, San Diego, CA) and IMag SAv particles plus (BD Biosciences, San Diego, CA). Obtained purity of $CD4^+$ T cells was more than 97% as determined by flow cytometry. CNS inflammatory cells were obtained from spinal cords of rats perfused with sterile PBS. Spinal cords were homogenized by passing the tissue through 40-μm stainless steel mesh in 5 ml PBS on ice. 50 μl of homogenate samples were taken for RNA isolation and the rest of homogenates was centrifuged (500 g, 3 min, +4 °C) and the pellet was resuspended in 3 ml of 30% Percoll (Sigma–Aldrich) and overlaid on 3 ml of 70% Percoll gradient. Following centrifugation at 870 g for 50 min the cells were recovered from the 30%/70% Percoll interface and washed in RPMI medium (Sigma–Aldrich). Afterwards, the cells were kept on ice until counted and used for RNA isolation or cell culturing. Spleens were isolated from untreated DA rats and mechanically disrupted, passed through 40-μm nylon mesh filter and the resulting suspension was collected by centrifugation. Spleen cells (5×10^6/ml) were stimulated with concanavalin A (ConA, Sigma–Aldrich, 5 mg/ml) for 48 h and subsequently cell culture supernatants (ConASn) were collected. Cells were grown in RPMI-1640 medium supplemented with 5% fetal calf serum (FCS) (both from PAA Laboratories, Pasching, Austria). Astrocytes were isolated from mixed glial cell cultures prepared from brains of newborn DA rats as previously described (McCarthy and de Vellis 1980). Astrocytes were grown in the culture medium supplemented with 4 g/l glucose and they were purified by repetition of trypsinization (0.25% trypsin and 0.02% EDTA, both from Sigma–Aldrich) and replating. The cells used in these experiments were obtained after third passage and were >95% positive for glial fibrilar acidic protein (GFAP) and <3% positive for CD11b, as deduced by cytofluorimetric analysis. Astrocytes were seeded at 1.5×10^5/ml/well of 24-well plates (Sarstedt, Nümbrecht, Germany) and were stimulated with ConASn (20%) or with cytokines IL-10 (20 ng/ml, Peprotech, Rocky Hill, NJ), IFN-γ (10 ng/ml, Sigma–Aldrich) and IL-17 (50 ng/ml, R&D Systems, Minneapolis, MN) and/or treated with a neutralizing anti-IL-10 antibody (R&D Systems).

ELISA

IL-10 concentration in cell culture supernatants was determined by sandwich ELISA using MaxiSorp plates (Nunc, Rochild, Denmark) and anti-cytokine paired antibodies according to the manufacturer's instructions (BD Biosciences). Samples were analyzed in duplicates and the results were calculated using standard curves made on the basis of known concentrations of the recombinant rat cytokines (Peprotech, Rocky Hill, NJ).

Reverse transcription – real time polymerase chain reaction

In order to determine cytokine gene expression real time PCR was performed. First, total RNA was isolated from DLNC, $CD4^+$ T cells, homogenized SC tissue or from SCC immediately after isolation using a mi-Total RNA Isolation Kit (Metabion, Martinsried, Germany) and reverse transcribed using random hexamer primers and MMLV (Moloney Murine Leukemia Virus) reverse transcriptase, according to the manufacturer's instructions (Fermentas, Vilnius, Lithuania). Prepared cDNAs were amplified by using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) according to the recommendations of the manufacturer in a total volume of 20 μl in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Thermocycler conditions comprised an initial step at 50 °C for 5 min, followed by a step at 95 °C for 10 min and a subsequent 2-step PCR program at 95 °C for 15 s and 60 °C for 60 s for 40 cycles. The PCR primers (Metabion) were as follows: β-actin forward primer 5′-GCT TCT TTG CAG CTC CTT CGT-3′; β-actin reverse primer 5′-CCA GCG CAG CGA TAT CG-3′; IFN-γ forward primer 5′-TGG CAT AGA TGT GGA AGA AAA GAG-3′; IFN-γ-reverse primer 5′-TGC AGG ATT TTC ATG TCA CCA T-3′; IL-10