Immunobiology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Tumor necrosis factor stimulates expression of CXCL12 in astrocytes

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A R T I C L E I N F O

Article history:
Received 8 December 2014
Received in revised form 13 January 2015
Accepted 14 January 2015
Available online xxx

Keywords:
Experimental autoimmune encephalomyelitis
Tumor necrosis factor
Astrocyte
CXCL12
Neuroinflammation

A B S T R A C T

It has been increasingly appreciated that tumor necrosis factor (TNF) performs various protective and anti-inflammatory functions in multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE). Recently, CXCL12 has been identified as a key inhibitor of leukocyte entry into the central nervous system (CNS) and as a regulator of inflammation resulting from the invasion. Here, a positive correlation between expression of TNF and CXCL12 in the CNS samples of EAE rats is presented. Also, it is shown that TNF potentiates CXCL12 expression in astrocytes. These results contribute to a view that TNF produced within the CNS plays a protective role in neuroinflammation.

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Introduction

Autoimmune response against the central nervous system (CNS) plays a dominant role in pathogenesis of a chronic inflammatory and demyelinating disease multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE) (Sospedra and Martin, 2005). Autoreactive CD4⁺ T lymphocytes (T helper, Th) of Th1 and Th17 phenotype are major pathogenic cells in MS and EAE (Petermann and Korn, 2011), while various populations of immune cells and CNS-resident cells contribute to the pathogenesis through divergent effector mechanisms including cytokine generation (Sospedra and Martin, 2005). Tumor necrosis factor (TNF) is a pluripotent cytokine that mediates deleterious inflammatory effects on the CNS tissue, but that also contributes to neuroprotection and remyelination in MS and EAE (Taoufik et al., 2008). Such pluripotency seems to stem from different activities of soluble form (sTNF) and transmembrane form (tmTNF) of the cytokine, as well as from functional diversity of its receptors TNFRI and TNFRII. TNF was detected in the inflamed CNS of MS patients, both in immune cells and in nonhematopoietic cells (Hofman et al., 1989; Selmaj et al., 1991). Its levels in CSF of MS patients correlate with the disease activity and blood–brain barrier disruption (Sharief and Thompson, 1992; Drulović et al., 1997) and it has recently been implied that TNF causes excitotoxic neurodegeneration in primary progressive MS (Rossi et al., 2014). Still, treatment of MS patients with lenercept (TNFRI fusion protein) or infliximab (anti-TNF antibody) led to exacerbation of the disease in clinical trials (reviewed in Dendrou et al., 2013). Also, anti-TNF treatment of patients with rheumatoid arthritis, Crohn's disease and psoriasis has been repeatedly associated with demyelination and other neurological adverse events (Kaltsonoudis et al., 2014). Finally, it has recently been shown that a single nucleotide polymorphism (rs1800693) in TNFR1 gene that leads to formation of soluble, i.e. blocking, TNFR1 is a genetic risk for MS (Gregory et al., 2012). Thus, while it is clear that TNF has a dual role in MS pathogenesis, its disease-promoting and beneficial effects in MS are still uncompletely understood.

Abbreviations: AO, Albino Oxford; c.s., clinical score; CFA, complete Freund's adjuvant; CNS, central nervous system; d.p.i., days post immunization; DA, Dark Agouti; DLN, draining lymph node; DLNC, draining lymph node cells; EAE, experimental autoimmune encephalomyelitis; FCS, fetal calf serum; IFN, interferon; IL, interleukin; MBP, myelin basic protein; MS, multiple sclerosis; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffer saline; PCR, polymerase chain reaction; RDV, relative densitometry values; RT, reverse transcription; SC, spinal cord; SCH, spinal cord homogenate; SD, standard deviation; SDS, sodium dodecyl sulfate; TGF, transforming growth factor; Th, helper T cells; TNF, tumor necrosis factor; TNFR, TNF receptor.

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http://dx.doi.org/10.1016/j.imbio.2015.01.007
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Comparison of autoimmune reaction directed against the CNS in Albino Oxford (AO) and Dark Agouti (DA) rats that are at the opposite poles of susceptibility to EAE induction has been a valuable tool for studying neuroinflammation (Momcilović et al., 2012). AO rats are almost completely resistant to EAE induction as they do not express clinical signs of EAE in response to harsh immunization protocols that are efficient in other relatively resistant rat strains (Miljkovic et al., 2006). On the contrary, DA rats readily develop clinically manifested EAE even if subjected to mild immunization, e.g. immunization without an adjuvant (Stosic-Grujicic et al., 2004). The comparison of these two strains of rats has recently allowed us to demonstrate an importance of a chemokine CXCL12 for regulation of neuroinflammation (Miljković et al., 2011a) in agreement with reports from other groups that indicate important role of CXCL12 in MS pathogenesis. Namely, it was reported that CXCL12 prevents leukocyte migration into the CNS and that it promotes immunoregulatory mechanisms within the CNS (reviewed in Momcilović et al., 2012). Astrocytes play important role in MS and EAE pathogenesis. These cells are major components of blood–brain barrier, they are potential antigen-presenting cells and they are able to produce various anti- and pro-inflammatory soluble mediators, including cytokines and chemokines (Miljković et al., 2011b). Notably, astrocytes are among the most prominent producers of CXCL12 in the inflamed CNS (Ambrosini et al., 2005).

Here, kinetics of TNF expression in rat EAE is studied and strong positive correlation between expression of TNF and CXCL12 in the CNS of EAE rats is determined. It is demonstrated that TNF stimulates CXCL12 expression in astrocytes.

Materials and 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). The animals were injected, as previously described (Miljković et al., 2011a). 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. DA rats had EAE onset on 9–11 days post immunization (d.p.i) (c.s. 1), peak on 12–14 d.p.i. (c.s. 2–4) and recovery on 18–22 d.p.i. (c.s. 1 or less).

Isolation of cells, cell culturing and generation of supernatants

Rats were extensively perfused with cold PBS through left ventricle before spinal cord isolation and homogenization. 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 (100 g, 10 min, +4 °C) and the pellet was resuspended in 3 ml of 30% Percoll (Sigma–Aldrich, St. Louis, MO) 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 (PAA Laboratories, Pasching, Austria). Afterwards, so obtained immune cells (isolates) were kept on ice until counted and used for RNA isolation or cell culturing. Isolates were seeded at 2.5×10^6/ml of 24-well plates (Sarstedt, Nümbrecht, Germany). Alternatively, spinal cord homogenates were centrifuged at $12{,}000 \times g$ for 20 min at 4 °C and supernatants were collected for detection of TNF. $CD4^+$ T cells were purified from the isolates 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. 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, PAA Laboratories). Astrocytes were isolated from mixed glial cell cultures prepared from brains of newborn AO or DA rats as previously described (McCarthy and de Vellis, 1980). They 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 re-plating. 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,). ConASn, recombinant rat TNF (Peprotech, Rocky Hill, NJ), recombinant rat IFN-γ (Sigma–Aldrich), recombinant mouse IL-17 (R&D Systems, Minneapolis, MN) and a TNF inhibitor etanercept (fusion protein of TNFRII and human IgG1) (Amgen-Wyeth, Thousand Oaks, CA,) were used for treatment of astrocytes.

ELISA

TNF concentration in cell culture supernatants and supernatants of homogenates was determined by sandwich ELISA using MaxiSorp plates (Nunc, Rochild, Denmark) and anti-cytokine paired antibodies according to the manufacturer's instructions (BD Biosciences, San Diego, CA). 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).

Immunoblot

Spinal cord homogenates were prepared in a lysis solution (25 mM Tris HCl buffer pH 7.4, 1 mM EDTA-Na_2, 150 mM NaCl, 0.1% SDS, 1% NP-40). Supernatants were collected after centrifugation ($16{,}000 \times g$, 20′) and protein concentration in the supernatants was measured by Lowry protein assay. The volume of the supernatants containing 50 μg of total proteins was mixed with appropriate volume of a gel-loading buffer (62.5 mM Tris–HCl (pH 6.8, 2% w/v sodium dodecyl sulfate (SDS), 10% glycerol, 50 mM dithiothreitol (DTT), 0.01% (w/v) bromophenol blue, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg/ml aprotinine, 2 mM EDTA). The samples were electrophoresed on a 12% SDS-polyacrylamide gel. The samples were electro-transferred to polyvinylidene difluoride membranes at 5 mA/cm^2, using semi-dry blotting system (Fastblot B43, Biorad, Muenchen, Germany). The blots were blocked with 5% (w/v) bovine serum albumin in PBS 0.1% Tween-20 and probed with specific antibodies to TNF (Abcam, Cambridge, UK or Santa Cruz, Dallas, TX) and beta-Actin (Abcam, Cambridge, UK) followed by incubation with secondary antibody at 1:10,000 dilution (ECL goat anti-rabbit or anti-mouse horseradish peroxidase (HRP)-linked, GE Healthcare, Buckinghamshire, UK). Detection was performed by the chemiluminescence (ECL, GE Healthcare) and photographs were made by X-ray films (Kodak, Rochester, NY). Densitometry was performed with Scion Image Alpha 4.0.3.2 (Scion Corporation, Frederick, MD). Results are presented as relative

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