Immunobiology 220 (2015) 845–850

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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ARTICLE INFO

Article history:
Received 8 December 2014
Received in revised form 13 January 2015
Accepted 14 January 2015
Available online 22 January 2015

Keywords:
Experimental autoimmune encephalomyelitis
Tumor necrosis factor
Astrocyte
CXCL12
Neuroinflammation

ABSTRACT

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.

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
0171-2985/

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 × *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 × *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

densitometry values (RDV), i.e. ratio of TNF and beta-Actin bands densitometry values.

Reverse transcription – real time polymerase chain reaction

In order to determine cytokine gene expression real time PCR was performed. Total RNA was isolated 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′; TNF forward primer 5′-TCG AGT GAC AAG CCC GTA GC-3′; TNF reverse primer 5′-CTC AGC CAC TCC AGC TGC TC-3′; CXCL12 forward primer 5′-GAT TCT TTG AGA GCC ATG TC-3′; CXCL12 reverse primer 5′-GTC TGT TGT TGC TTT TCA GC-3′. Accumulation of PCR products was detected in real time and the results analyzed with 7500 System Software (AB) and presented as 2^{-dCt}, dCt being the difference between Ct values of a gene of interest and the endogenous control (β-actin).

Statistical analysis

The results are presented as mean + SD of values obtained in repeated experiments. A Student's *t* test (two-tailed) was performed for statistical analysis of differences between two groups. ANOVA followed by Tukey test was performed for comparison of mean values of multiple groups. Pearson's test was used for the correlation analysis. A *p* value less than 0.05 was considered statistically significant.

Results

TNF mRNA expression in spinal cords of DA rats and AO rats

TNF mRNA expression was determined in the CNS samples of the immunized and non-immunized AO and DA rats. In DA rats TNF expression increased in spinal cord homogenates after immunization, but this increase was not statistically significant. Actually, there was no significant difference in TNF expression in spinal cord homogenates of DA rats between any of the time points examined (Fig. 1A). On the contrary, in AO rats TNF mRNA expression was significantly increased in spinal cord homogenates upon immunization, reaching the highest values on 9–11 d.p.i., and then significantly decreased on 18–22 d.p.i. (Fig. 1A). When the strains were compared, TNF expression in the homogenates was consistently higher in AO than in DA rats, both in samples of non-immunized animals and in samples obtained at the time of onset and peak of EAE in DA rats (Fig. 1A). Similar results were obtained with immune cells isolated from spinal cords (isolates): TNF mRNA expression was significantly higher in AO isolates than in DA isolates on 12–15 d.p.i. and on 18–22 d.p.i. (Fig. 1B). Also, TNF mRNA expression was higher in $CD4^+$ T cells purified from the isolates of AO rats on 12–15 d.p.i. (Fig. 1C). Thus, relatively high mRNA expression of TNF is a characteristic of EAE-resistant rats.

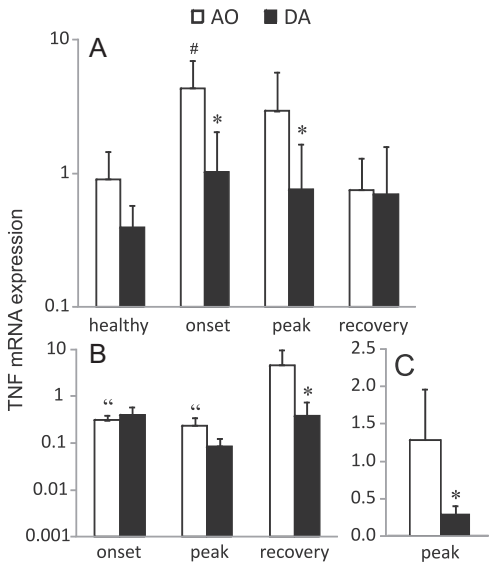

Fig. 1. TNF mRNA expression in the spinal cords of AO and DA rats. RNA was obtained from spinal cord homogenates (A), immune cells isolated from the spinal cords (B) or $CD4^+$ T cells purified from the immune cells (C) at the indicated time points of different phases of EAE in DA rats. Subsequently, real time RT-PCR was performed. The results are presented as mean + SD of values from at least 6 samples per group obtained in at least 3 independent experiments. *$p < 0.05$ refers to statistically significant difference between AO and DA rats. #$p < 0.05$ refers to statistically significant difference to samples of non-immunized rats. "$p < 0.05$ refers to statistically significant difference to recovery.

TNF production in spinal cords of DA rats and AO rats

TNF concentration in supernatants of spinal cord homogenates subjected to high speed centrifugation was without significant difference in AO and DA samples on 9–11 d.p.i. and on 12–15 d.p.i. (Fig. 2A). Also, immunoblot of spinal cord homogenates have shown that there is no difference in transmembrane (26 kD) or soluble (17 kD) TNF isoform in samples of AO and DA rats at the time of EAE peak in DA rats (Fig. 2B). Still, TNF level was higher in supernatants of AO isolates than in corresponding DA samples on 12–15 d.p.i. (Fig. 2C). Thus, production of TNF was similar in EAE-resistant and EAE-prone rats.

Correlation of TNF and CXCL12 mRNA expression in the CNS of AO and DA rats

TNF and CXCL12 mRNA expression correlation was determined in spinal cord homogenates of the immunized DA rats. Samples from non-immunized rats were included in the analysis, as well. A strong positive correlation ($r = 0.870$, $p < 0.01$) between expression of the two genes was demonstrated in the spinal cord homogenates (Fig. 3A). Accordingly, a strong positive correlation between TNF and CXCL12 mRNA expression was observed in spinal cord homogenates isolated from AO rats ($r = 0.758$, $p < 0.01$). This result implied that there is a link between TNF and CXCL12 mRNA expression regulation in the CNS during EAE.

TNF stimulates CXCL12 expression in astrocytes

Effects of TNF on CXCL12 expression in astrocytes were investigated. In order to mimic neuroinflammation, astrocytes isolated from DA rats were stimulated with IFN-γ and IL-17 and treated with TNF. Concentrations of IFN-γ and IL-17 that were used in our experiments have been previously shown to be effective in individual or cooperative stimulation of astrocytes to produce inflammatory mediators, e.g. nitric oxide (Trajkovic et al., 2001). However,

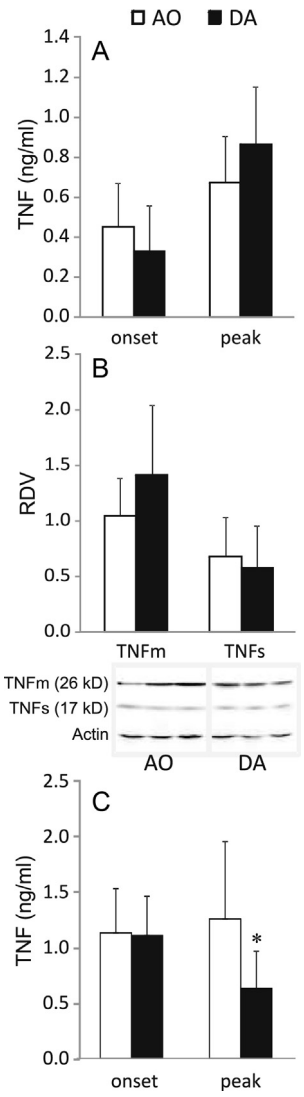

Fig. 2. TNF production in the spinal cords of AO and DA rats. (A) Spinal cord homogenates were centrifuged and supernatants were collected for testing by ELISA. (B) Relative expression of transmembrane and soluble TNF (TNFm and TNFs, respectively) in spinal cord homogenates on 12–15 d.p.i. determined by immunoblot. A representative blot showing 3 AO and 3 DA samples is also presented. Relative densitometry value (RDV) is normalized to AO TNFm mean densitometric value that was arbitrarily given value of 1. C) Immune cells isolated from spinal cords were grown in cell culture for 24 h. Cell-free supernatants were tested by ELISA. The results are presented as mean + SD of values from at least 6 samples per group obtained in at least 3 independent experiments. $^{*}p < 0.05$ refers to statistically significant difference between AO and DA rats.

they were not efficient in inducing CXCL12 in astrocytes (Fig. 4A). On the contrary, TNF induced CXCL12 gene expression in astrocytes on its own and in the presence of IFN-γ and IL-17 (Fig. 4A). Further, DA astrocytes were treated with ConASn that contains various cytokines, including TNF (>200 pg/ml). In response to ConASn CXCL12 expression was markedly elevated in astrocytes, while an inhibitor of TNF activity, etanercept significantly reduced CXCL12 expression in ConASn-stimulated astrocytes (Fig. 4B). Finally, in order to determine if the observed effect of TNF on CXCL12 mRNA expression in astrocytes was strain specific, astrocytes isolated from AO rats were treated with TNF. Here again, TNF alone or in cooperation with IFN-γ and IL-17 stimulated CXCL12 expression (Fig. 4C). Thus, TNF is a potent stimulator of CXCL12 expression in astrocytes.

Discussion

In the present paper, it is shown that relatively high CNS TNF mRNA expression is a feature of EAE resistant AO rats. The observed pattern of the CNS TNF expression in AO and DA rats is similar to that previously observed for CXCL12 (Miljković et al., 2011a). Furthermore, a strong positive correlation has been determined between mRNA expression of TNF and CXCL12 in spinal cord homogenates of AO and DA rats. Importantly, low expression of TNF and CXCL12 coincides with high number of immune cells infiltrating the CNS in EAE-susceptible DA rats, while high expression of these molecules concurs with low number of the infiltrating cells in EAE-resistant AO rats (Miljković et al., 2011a). Thus, it could be assumed that TNF induces CXCL12 that limits immune cell invasion into the CNS. Indeed, TNF is shown able to induce and stimulate CXCL12 expression in astrocytes that are important source of this chemokine within the CNS.

TNF contributes to MS pathogenesis through induction of cytokine and chemokine expression in the CNS, disruption of blood–brain barrier, promotion of inflammatory cell infiltration and direct induction of neurodegeneration (Caminero et al. 2011; Sibson et al., 2002; Rossi et al., 2014). However, it also promotes remyelination and has neuroprotective effects (Taoufik et al., 2008). The dual role of TNF in neuroinflammation can be explained by divergent effects that this cytokine exerts through TNFRI and TNFRII. For instance, it has been shown that TNFRI (p55) is necessary for infiltration of immune cells into the CNS parenchyma (Gimenez et al., 2006), while TNFRII (p75) is essential for engagement and induction of regulatory T cells (Tsakiri et al., 2012). Accordingly, TNFRI−/− mice were partially protected from MOG(35-55)-induced EAE, whereas TNFRII−/− mice exhibited exacerbated EAE (Suvannavejh et al., 2000; Eugster et al., 1999). As sTNF has higher affinity for TNFRI, while tmTNF has higher affinity for TNFRII it is proposed that blockade of sTNF/TNFRI axis, while preserving tmTNF/TNFRII axis might be a right approach for treatment of MS (Van Hauwermeiren et al., 2011). This idea is firmly supported by the reports of beneficial effects of a selective sTNF blocker XPro1595, but not of a general TNF inhibitor etanercept in EAE in mice (Brambilla et al., 2011; Taoufik et al., 2011). Still, we did not detect higher tmTNF or lower sTNF production in EAE-resistant AO rats. This result suggests that potential beneficial effects of TNF in AO rats are not a consequence of elevated expression of tmTNF.

Importantly, although sTNF has been reportedly indicated as a proinflammatory molecule in EAE and MS, its positive effect on CXCL12 expression in astrocytes has been observed in our study. Influence of TNF on CXCL12 production in astrocytes was not determined in our study. Still, our previous data implied that CXCL12 production was dominantly regulated at transcriptional level within the CNS (Miljković et al., 2011a). In the same paper CXCL12 was identified as a key protective molecule in rat EAE. Importantly, application of an inhibitor of CXCL12 activity – AMD3100, to AO rats is one of the rare treatments that have been efficient in overcoming the resistance of these rats to EAE induction (Miljković et al., 2011a). As astrocytes are among the principal producers of this chemokine in neuroinflammation (Ambrosini et al., 2005), our results about the positive influence of TNF on CXCL12 generation in astrocytes indicate that TNF could be an important stimulator of CXCL12 expression in neuroinflammation. Besides astrocytes, endothelial cells are the most prominent producers of CXCL12 in the CNS (Momcilović et al., 2012). As a matter of fact, irregular CXCL12 expression in blood–brain barrier endothelial cells was shown responsible for initiation and propagation of the CNS inflammation in EAE in mice (McCandless et al., 2006). This data contributes to the understanding of the importance of the complex net of the CNS endothelial cell adhesion and chemotactic molecule production in MS and EAE (Engelhardt, 2008).

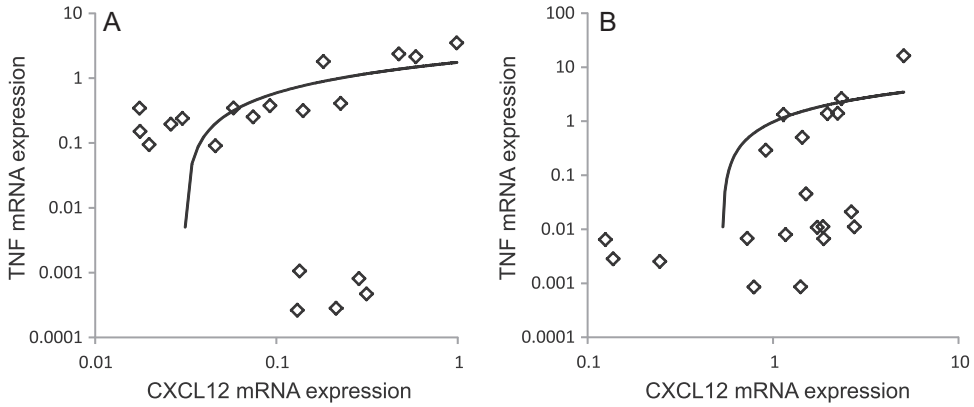

Fig. 3. Correlation between TNF and CXCL12 expression in spinal cord homogenates of rats. Spinal cords were isolated from non-immunized rats or from the immunized rats at the time of onset, peak and recovery in DA rats ($n = 20$, $n = 5$ for each time point). Correlation between expression of TNF and CXCL12 mRNA expression in DA (A) and AO (B) rats was determined. Curves on the graphs represent trendlines of correlation.

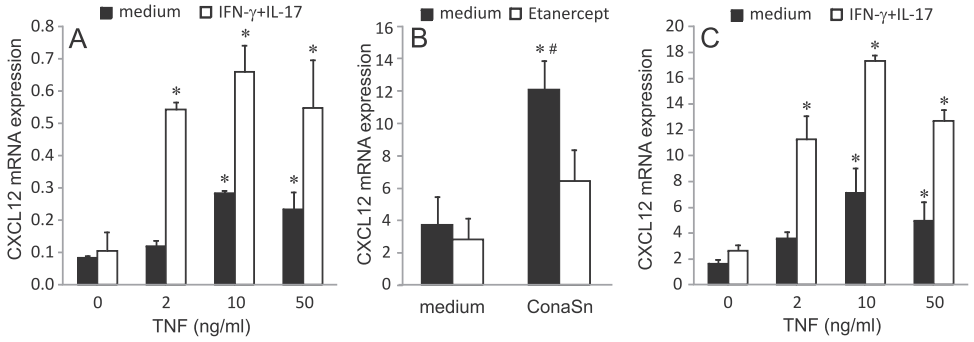

Fig. 4. The influence of TNF on CXCL12 mRNA expression in astrocytes. Astrocytes were isolated from DA rats (A, B) or AO rats (C) and they were treated with TNF in the absence (medium) or presence of 10 ng/ml IFN-γ and 50 ng/ml IL-17 (A, C) or they were stimulated with ConASn (20%) in the absence or presence of etanercept (250 ng/ml) (B). After 24 h of cultivation, RNA was isolated from the cells and real time RT-PCR was performed. The results are presented as mean + SD from three experiments. $^{*}p < 0.05$ refers to statistically significant difference in comparison to untreated cultures. $^{\#}p < 0.05$ refers to statistically significant difference in comparison to cultures treated with etanercept.

Endothelial cell regulation of T cell migration across the blood–brain barrier is counteracted by locally produced TNF (Yang et al., 2013). On the contrary, TNF could contribute to the regulation through stimulation of CXCL12 expression in endothelial cells. Therefore, it would be important to determine the effect of TNF on CXCL12 expression in the CNS endothelial cells. Importantly, high expression of TNF might be responsible for high expression of CXCL12 in the CNS of EAE-resistant AO rats. It was previously shown that CXCL12 expression is higher in AO rats only at the time of peak, but not at the time of onset of EAE in DA rats (Miljković et al., 2011a). Accordingly, here it is presented that TNF production by the immune cells isolated from spinal cord of the immunized rats follow the same time pattern. This pattern is not observed if total TNF release within the CNS is determined. Thus, it is tempting to speculate that TNF produced by the inflammatory immune cells has the major role in stimulation of CXCL12 expression within the CNS. Worthy of notion, other factors besides TNF determine level of this chemokine in the inflamed CNS. We have recently demonstrated that IL-10 stimulates CXCL12 expression in astrocytes (Blaževski et al., 2013). Also, nitric oxide, which is abundantly produced in DA, but not AO rats CNS after EAE immunization seems to be a key inhibitor of CXCL12 expression in neuroinflammation (Petković et al., 2013).

Conclusions

It has been previously shown that CXCL12 and TGF-β are amply expressed in the CNS of AO rats and that they could contribute to the resistance of these rats to EAE (Miljković et al., 2011a; Lukic et al., 2001; Blaževski et al., 2013). Here, relatively high expression of TNF in the CNS of AO rats, even when these animals are not immunized is demonstrated. Also, the ability of TNF to induce CXCL12 expression in the CNS is demonstrated. Thus, the present report adds TNF to the list of potential candidate molecules contributing to resistance of AO rats to EAE induction. In general, our data support a view of beneficial role of TNF in neuroinflammation.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgement

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (173035, 175038 and 173013).

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