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Contribution of the macrophage migration inhibitory factor superfamily of cytokines in the pathogenesis of preclinical and human multiple sclerosis: In silico and in vivo evidences

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ABSTRACT

Macrophage migration inhibitory factor (MIF) is a cytokine with pleiotropic actions involved in the pathogenesis of autoimmune disorders, including Multiple Sclerosis (MS). We have first evaluated in silico the involvement of MIF, its homologue D-DT, and the receptors CD74, CD44, CXCR2 and CXCR4 in encephalitogenic T cells from a mouse model of MS, the Experimental Allergic Encephalomyelitis (EAE), as well as in circulating T helper cells from MS patients. We show an upregulation of the receptors involved in MIF signaling both in the animal model and in patients. Also, a significant increase in MIF receptors is found in the CNS lesions associated to MS. Finally, the specific inhibitor of MIF, ISO-1, improved both ex vivo and in vivo the features of EAE. Overall, our data indicate that there is a significant involvement of the MIF pathway in MS ethiopathogenesis and that interventions specifically blocking MIF receptors may represent useful therapeutic approaches in the clinical setting.

1. Introduction

The Macrophage Migration Inhibitory Factor (MIF) is a pro-inflammatory cytokine produced by virtually every tissue (Stosic-Grujicic et al., 2009). MIF is constitutively expressed and secreted from intracellular pools. Among its several biological properties, MIF activates the macrophages and induces the synthesis of nitric oxide, the secretion of matrix metalloproteinases and the production of pro-inflammatory cytokines and chemokines. MIF also promotes Th1 polarization, by stimulating IL-2 and IFN-gamma production from T cells, and regulates antibody production and cytotoxic T cell responses (Cvetkovic and Stosic-Grujicic, 2006).

The transduction of MIF signaling seems to be mainly regulated by the type II transmembrane protein, CD74 (Leng et al., 2003). Leng and collaborators have demonstrated that MIF binds to the extracellular domain of CD74 and, in turn, activates the ERK1/2 pathway (Leng et al., 2003). The phosphorylation of MAPK, upon engagement of CD74, requires the coexpression of CD44, a known activator of the Src tyrosine kinase (Shi et al., 2006). Moreover, following endocytosis, MIF binds the intracellular protein JAB1, thus inhibiting its downstream pathways, represented by the activation of AP-1 and the degradation of p27Kip1 (Kleemann et al., 2000). Finally, MIF exhibits chemokine-like properties via non-cognate binding to the chemokine receptors, CXCR2 and CXCR4 (Bernhagen et al., 2007).

MIF has been identified as a key player in the etiopathogenesis of a variety of both organ-specific and systemic autoimmune diseases, both in rodent models and humans (Cox et al., 2013; Cvetkovic et al., 2005; Cvetkovic and Stosic-Grujicic, 2006; Denkinger et al., 2003; Nakajima et al., 2006; Nicoletti et al., 2005; Onodera et al., 2007; Powell et al., 2005; Stosic-Grujicic et al., 2008, 2009).

Multiple sclerosis (MS) is a chronic immunoinflammatory diseases of the central nervous system, with autoimmune etiopathogenesis, that leads to demyelination and neurodegeneration. Symptoms are variable and may change during time, even in the same patients. A body of data suggests a key role for MIF in the pathogenesis of MS. It has been shown that MIF-deficient mice are protected from the adoptive transfer model of experimental autoimmune encephalomyelitis (EAE) and that intraspinal injection of MIF reconstitutes the ability of MIF-deficient mice

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to develop EAE pathology (Cox et al., 2013). The inhibition of MIF, via anti-MIF antibodies, has also proven to be therapeutically effective in ameliorating EAE (Denkinger et al., 2003; Powell et al., 2005). In clinical studies, augmented levels of MIF were observed in serum and in cerebrospinal fluid (CSF) of patients with active MS. In particular, MIF protein levels in CSF of MS patients undergoing relapses were found significantly elevated as compared to control samples. However, no significant differences were found in CSF levels of MIF among non-relapsed MS patients and other non-inflammatory neurological disease patients (Niino et al., 2000). Later on, the finding was partly confuted, as MIF levels were found to be augmented in CSF samples from MS patients (Khaibullin et al., 2017). In agreement with these findings, Cox et al. observed that MIF is highly expressed in human active MS lesions (Cox et al., 2013).

Genetic polymorphism studies also support the role of MIF in MS pathogenesis. The MIF-173 GC genotype was associated with a higher EDSS score in MS. In another study, patients with MIF-173 CC genotype exhibited significantly lower age of onset compared with those with the MIF-173 CG and MIF-173 GG genotypes (Akcali et al., 2010; Victor et al., 2015).

In 1993, a new member of the MIF protein superfamily has been characterized, the D-dopachrome tautomerase (D-DT), located on Chromosome 22, adjacent to the MIF gene (Odh et al., 1993). In humans, D-DT and MIF amino acid sequences share a 34% similarity (Merk et al., 2011, 2012). Similarly to MIF, D-DT binds the receptor complex CD74-CD44, although with a dissociation rate 11-fold higher as compared to MIF (Merk et al., 2011, 2012). However, despite the structural and biochemical similarities shared by MIF and D-DT, the biological functions of these two genes need to be fully deciphered. Since MIF and D-DT seem to synergize in different biological settings (Coleman et al., 2008), the identification of D-DT has attracted attention on the possibility that this cytokine could also play a pathogenic role in MS. It has recently been demonstrated that MIF and D-DT levels are augmented in males with progressive disease as compared with relapsing-remitting males (RRMS) and female MS subjects, with increased levels of CD74 in females vs. males with high MS disease severity (Benedek et al., 2017). MIF and D-DT levels in males with progressive disease significantly correlated with the presence of two high-expression promoter polymorphisms located in the MIF gene, a -794CATT5-8 microsatellite repeat and a -173 G/C SNP (Benedek et al., 2017). Conversely, mice lacking MIF or D-DT developed less-severe signs of MOG-induced EAE, thus indicating that these two genes act as co-pathogenic contributors (Benedek et al., 2017). Hence, this study suggests that MIF and D-DT are sex-specific disease modifiers and raises the possibility that tailored anti-MIF and -D-DT treatment of clinically isolated syndrome or RR-MS males with a high-expressor genotype, might slow or prevent the onset of progressive MS. Additionally, selective targeting of MIF/D-DT:CD74 signaling might provide an effective, trackable therapeutic approach for MS subjects of both sexes.

Along this line of research, we have adopted an in silico approach to further evaluate the role of MIF and D-DT signaling in the pathogenesis of both EAE and MS. Subsequently we evaluated the in vitro effects of the small molecule inhibitor of MIF, ISO-1 (Al-Abed and Vanpatten, 2011), on autoantigen-induced pro-inflammatory cytokines secretory capacity and proliferation of splenocytes from mice with MOG-induced EAE. Our in silico analysis evidences a variable expression of MIF receptors in CD4+ T cells and CNS lesions, that seems to be more robust in the mouse models than in humans. In agreement with the known pathogenic contribution of MIF to EAE pathogenesis, ISO-1 down-regulated the pro-inflammatory secretory capacity and autoantigen-induced proliferation of encephalytogenic CD4+ T cells from mice with MOG-induced EAE and ameliorated the course of the disease when administered in vivo.

2. Materials and methods

2.1. Microarray analysis

2.1.1. Analysis of CNS-infiltrating CD4+ T cells from a preclinical model of MS

The GSE57098 microarray dataset was selected to evaluate the transcriptional levels of genes of interest in encephalitogenic T cells from a preclinical model of MS. GSE57098 included data from CD4+ T cells isolated from mice with experimental allergic encephalomyelitis, induced by immunization with MOG35-55-peptide. Naive CD4+ T cells were isolated from B6.2d2 transgenic mice with MOG-specific T cell receptors. Briefly, mononuclear cells were first isolated from brain and spinal cords using a 40–70% Percoll gradient and then, CD4+ T cells were enriched by positive sorting using anti-CD4+ beads, reporting a purity of > 93%. Remaining cells were largely CD11b-expressing cells (CD11c+, CD8+, CD45R+ cells < 1%). The Agilent-026655 Whole Mouse Genome Microarray 4x44K v2 platform was used for the generation of the transcriptional profile of the samples. Details on the experimental design can be retrieved form the relative publication (Hoppmann et al., 2015).

2.1.2. Analysis of circulating blood cells from MS patients

Gene Expression Profiling of Resting and Activated CD4+ T Cells from patients with MS and healthy donors was obtained from the GSE78244 dataset. Data on genes of interest were evaluated from unstimulated cells and following 24 h incubation with anti-CD3/CD28 antibodies. Data from 14 RR (Relapse Remitting) - MS patients and 14 control subjects are included. All the patients were women and none of them had received immunomodulatory or immunosuppressive treatment for at least two months prior to sampling, except for one patient that received intravenous immunoglobulin 15 days before sampling. Purity of cells was > 96%. Complete demographic data are available in the relative publication (Hellberg et al., 2016). The Agilent-039494 SurePrint G3 Human GE v2 8x60K Microarray platform was used.

Data obtained from the GSE15245 dataset were used to evaluate the possible relationship between expression levels of genes of interest and the time to relapse. The transcriptional profile from 62 drug-naïve patients with definite MS were generated using the Affymetrix Human Genome U133A 2.0 Array (Gurevich et al., 2009) and data were used to predict MS relapses by transcription levels of MIF and its receptors in PBMCs. Patients had a mean age of 38.5 ± 1.4, and a mean EDSS score of 2.4 ± 0.2 (Gurevich et al., 2009). Complete demographic data are available in the relative publication (Gurevich et al., 2009). Sample population was sorted based on the expression levels of MIF, DDT, CD74, CD44,CXCR2 and CXCR4. Log-rank test was applied to evaluate differences in the percentage of patients developing acute relapses in a 1500-day time-frame.

2.1.3. Dataset selection for the analysis of oligodendrocyte damage and remyelination processes in lumbar spinal cords of EAE affected mice and cortical lesions of MS patients

For the analysis of oligodendrocyte damage and remyelination processes, the datasets GSE60847 and GSE48872 were chosen. GSE60847 included data from homogenized lumbar spinal cord tissue isolated from EAE-affected mice and sham control mice. Each sample consisted of lumbar spinal cord tissue pooled from 3 animals and the Illumina MouseWG-6 v2.0 R2 expression beadchip platform was used (Schmitz et al., 2014). The spinal cords of the EAE-affected mice were collected during the flare of the disease, at day 16 post-immunization. GSE48872 included gene expression profiles from adult Oligodendrocytes Precursor Cells (aOPCs) isolated from the brain of postnatal (day 1 to day 5) and 2-month-old mice, while adult OPCs in demyelinating conditions (activated aOPCs) were isolated from the brain of mice that had been treated for 5 weeks with cuprizone, that induces demyelinating lesions as compared with normal cortex of age matched

controls. Adult oligodendrocytes (OLs) were obtained from brains of 2-month-old mice (Moyon et al., 2015). The dataset GSE32645 was used to evaluate the levels of genes of interest in active cortical MS lesions as compared to normal cortex of age matched controls. In addition, cortexes of patients with other neurological diseases, including chronic tuberculous meningitis, neurodegenerative lesions of Alzheimer's disease were also available. Data from three patients per group were available. The Agilent-014850 Whole Human Genome Microarray 4x44K platform was used for this study (Fischer et al., 2013).

2.2. In vitro, ex vivo and in vivo analysis

2.2.1. Animals

Female 8 to 10 week-old C57BL/6 mice were purchased by ENVIGO RMS srl (San Pietro al Natisone, Udine, Italy). The animals were kept at the Department of Biomedical and Biotechnological Sciences, University of Catania, Italy. Animals were kept under standard laboratory conditions (non-specific for pathogens) with ad libitum access to food and water and were allowed to adapt to their environment for a week before commencing the study. The protection of animals used in the experiment complies with Directive 86/609/EEC, implemented by D.Lgs. 26/2014.

2.2.2. Induction of EAE induced by MOG in C57BL/6 mice and in vivo treatment with ISO-1

MOG35-55 was synthesized by Genemed Synthesis Inc. (San Francisco CA). The animals were immunized via subcutaneous injection of 200 μg of MOG emulsified in CFA containing 1 mg of Mycobacterium tuberculosis H37RA (Difco, Detroit, MI, USA). Emulsion was injected in a volume of 0.2 ml divided in two sites draining into the axillary lymph nodes. Pertussis toxin (Calbiochem, Nottingham, UK) was administered i.p. at the dose of 200 ng/mouse on days 0 and 2 post-immunization (Mangano et al., 2014). Mice were observed daily by measuring their body weights and clinical signs of EAE. Clinical evaluation was performed using the following score: 0 = no sign of disease; 0.5 = Partial paralysis of the tail; 1 = paralysis of the tail; 1.5 = tail paralysis + unilateral frontal paralysis; 2 = tail paralysis + back weakness or partial paralysis back; 2.5 = tail paralysis + partial paralysis of the back; 3 = tail paralysis + complete paralysis back; 3.5 = tail paralysis + complete back paralysis + incontinence; 4 = tail paralysis + paralysis backward + partial paralysis or partial paralysis of the forelimbs; 5 = dying or dead.

ISO-1, synthetized as described elsewhere (Al-Abed et al., 2005), was dissolved in a solution of 5% DMSO/95% H_2O and administered i.p. to mice daily, from day 8 until day 46, at the dose of 40 mg/kg. This dose of ISO-1 has been chosen on the basis of previous studies indicating its capacity to ameliorate the development of immunoinflammatory diabetes induced in mice and of a model of Experimental Allergic Neuritis (Cvetkovic et al., 2005; Nicoletti et al., 2005). Dexamethasone 0.3 mg/kg was used as positive control.

2.2.3. Ex vivo restimulation of splenocytes with MOG35–55

At day 14 post-immunization, spleens were collected from vehicle-treated mice with clinical score ≥ 2 (n = 5, not included in the in vivo study) and cell suspensions were prepared by grinding the organs and suspending them in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 mg/ml of penicillin/streptomycin (complete medium). Splenocytes were treated with an ACK Lysis Buffer (Invitrogen, Monza, Italy) to remove red blood cells.

2.2.4. Determination of cytokines in cell culture supernatants

Splenocytes were plated at a concentration of 2×10^6 cells/well in 24-well microtiter plates, stimulated with $MOG_{35–55}$ (40 μg/ml) and incubated for 48 h along with scalar concentration of the MIF inhibitor, ISO-1, or vehicle. At the end of the incubation period, supernatants were collected for determination of TNF-alpha, IFN-gamma, IL-17 and IL-4 concentrations by sandwich ELISA, using a commercially available kit (R&D systems, Minneapolis, MN, USA).

2.2.5. RNA isolation and real-time RT-PCR

Total RNA was extracted from splenocytes stimulated with $MOG_{35–55}$ (40 μg/ml) and incubated for 72 h along with scalar concentration of the MIF inhibitor, ISO-1, or vehicle using TRIzol reagent (Life Technologies, Monza, Italy). Two micrograms of RNA were retrotranscribed and cDNA used for the determination of TBX21, RORC, GATA3 and FoxP3 by real-time RT-PCR using the FastStart SYBR Green Master (Roche, Monza, Italy). Primer sequences were in house-designed or obtained from the PrimerBank database (http://pga.mgh.harvard.edu/primerbank/).

Gene expression was calculated using the formula: $2-\Delta\Delta Ct$, where $\Delta\Delta Ct$ = (Ct, target gene-Ct, beta-actin) treated group – (Ct, target gene-Ct, beta-actin) control group.

2.2.6. Determination of (auto)antigen-induced proliferation

Splenocytes were plated in triplicate in 96-well microtiter plates at a concentration of $4*10^5$ cells/well, in a volume of 50 μl per well in supplemented media, and incubated at 37 °C and 5% CO_2 for 72 h. At the end of the incubation period, MTT was added to cultures to a 0.5 mg/ml working concentration in the medium, from a 5-mg/ml stock solution in PBS (Phosphate-Buffered Saline). Following 4 h of incubation, precipitated dye was resuspended in 0.1 N HCl in isopropyl alcohol and absorbance was measured at 570 nm. Absorbance was quantified as relative percentages compared to control condition.

2.3. Statistical analysis

Data are shown as Mean ± S.D. and statistical analysis was performed using either the Student's *t*-test or One-Way ANOVA, followed by Bonferroni multiple test correction. Survival analysis was performed using Log-rank test. GraphPad Prism software were used for the statistical analysis and the generation of the graphs.

3. Results

3.1. Evaluation of transcriptomic levels of MIF and its receptors in encephalitogenic T helper cells

The expression levels of MIF, and of the homologue DDT, was first evaluated on CD4+ T cells isolated from the CNS of mice with MOG-induced EAE. As compared to naive CD4+ T cells, isolated from B6.2d2 transgenic mice with MOG-specific T cell receptors, the activated encephalitogenic T cells expressed significantly higher levels of MIF ($p < 0.01$) (Fig. 1). In contrast, no significant differences were observed in the levels of DDT (Fig. 1). Analysis of the transcriptional levels of CD74 and CD44, revealed a strong and significant upregulation of both genes in CNS-isolated T cells ($p < 0.01$ and $p < 0.001$, respectively) (Fig. 1). Similarly, the two co-receptors, CXCR2 and CXCR4, were markedly upregulated in the CNS-infiltrating cells, entailing a strong statistical significance ($p < 0.01$ for both genes) (Fig. 1).

3.2. Analysis of circulating CD4 T cells in MS patients and healthy people

Evaluation of the transcriptional levels of MIF, DDT, CD74, CD44 and of the co-receptors, CXCR2 and CXCR4, in CD4 T cells isolated from PBMCs of MS patients and healthy controls revealed that no differences between the two groups of people, with the only exception for CD44 (Fig. 2) that was found to be significantly higher in the peripheral CD4 T cells from MS patients ($p < 0.05$) (Fig. 2).

In addition, upon stimulation of the cells with anti-CD3 and anti-CD28, both MIF and CD74 were upregulated in a superimposable fashion both in MS patients and healthy controls ($p < 0.01$ and $p < 0.001$, for MIF and CD74, respectively) (Fig. 2). On the other

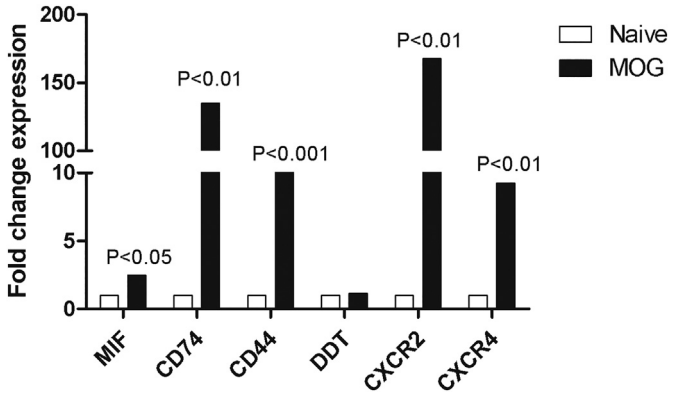

Fig. 1. Analysis of CD4 T cells from a preclinical model of MS. The transcriptional levels of MIF, D-DT, CD74, CD44, CXCR2 and CXCR4 was evaluated in encephalitoenic T cells isolated from experimental autoimmune encephalitomyelitis (EAE)-affected animals upon immunization with MOG35-55-peptide. Naive CD4+ T cells were isolated from B6.2d2 transgenic mice with MOG-specific T cell receptors. Data were retrieved from the freely accessible GSE57098 microarray dataset.

hand, CD44 levels slightly decreased following T cell activation, reaching the statistical significance only in the MS group ($p < 0.05$) (Fig. 2). No modulation could be observed for DDT, CXCR2 and CXCR4 (Fig. 2).

3.3. Prediction of MS relapses by transcription levels of MIF and its receptors in PBMCs

We wanted to determine whether different expression levels of MIF and its receptors in PBMCs from MS patients could either promote or confer protection from acute relapses. Data from 51 untreated patients (16 males and 35 females) with definite MS were included. No significant sex-related differences were found for any of the genes analyzed (data not shown). Patients population was divided into two groups based on the expression level of each of the genes of interest (referred as High and Low expression) and survival curves generated for an observational period of 1500 days. MIF, DDT, CD74, CD44, CXCR2 and CXCR4 were considered in the analysis, but none of these genes resulted to be potential predictor of relapses (Fig. 3). Unexpectedly a trend to protection from relapses ($p = 0.079$) was observed in the patients expressing higher levels of D-DT and lower levels of CD44 ($p = 0.1029$) (Fig. 3).

3.4. Evaluation of transcriptomic levels of MIF and its receptors in MS-associated CNS lesions

We wanted to determine the expression levels of MIF and related genes in the central nervous system (CNS) from patients and animal models of MS. To this aim, we interrogated the three microarray datasets, GSE60847, GSE48872 and GSE38010.

In lumbar spinal cords of EAE-affected mice, no significant modulation in MIF and DDT expression was observed, as compared to healthy control mice (Fig. 4). A significant increase in CD74 ($p < 0.01$), CD44 ($p < 0.01$) and CXCR4 ($p < 0.05$) was observed in EAE spinal cord as compared to control samples. A similar trend of increase was observed for CXCR2, but the statistical significance was not reached (Fig. 4).

Along the same lines, CD74 and CD44 were significantly increased ($p < 0.05$ and $p < 0.01$, respectively) in adult oligodendrocyte precursors from cuprizone-challenged animals with respect to control adult oligodendrocyte precursors, as detected in GSE48872 (Fig. 5).

No significant differences for the analyzed genes were observed in the cortical lesions of MS patients, although a trend of increase could be observed for MIF, CXCR2 and CXCR4 as compared to healthy control cortex (Fig. 6).

3.5. Effects of ISO-1 on MOG-specific encephalitogenic cells

In order to evaluate the effects of specific MIF inhibition on encephalitogenic cells, we immunized C57Bl/6 mice with $MOG_{35–55}$ and at overt disease, splenocytes were collected and re-stimulated with antigenic peptide, in the presence of scalar concentrations of the MIF inhibitor, ISO-1. ISO-1 treatment was associated to dose-dependent reduction in MOG-specific proliferation, reaching a ~40% reduction at the concentration of 100 nM, as compared to vehicle-treated MOG-stimulated splenocytes (Fig. 7A).

ISO-1 significantly downregulated the expression of the Th1- and Th17-specific transcription factors, TBX21 and RORC, but negligible effects were observed for the Th2 specific transcriptor factor, GATA3, and for FoxP3, that is a phenotypic and functional hallmark of regulatory T cells (Fig. 7B).

MOG-restimulation of splenocytes from EAE-affected mice was associated to a significant upregulation of TNF-alpha production, IFN-gamma, IL-17, and IL-4, as detected by ELISA. ISO-1 treatment dose-dependently inhibited the secretion of the proinflammatory cytokines, TNF-alpha, entailing a strong statistical at all the three tested concentrations (Fig. 7C). A significant reduction in INF-gamma and IL-17 levels could also be observed upon ISO-1treatment at the concentrations of 100 and 50 nM. No significant modulation was, on the contrary, observed in the IL-4 supernatant levels upon ISO-1 treatment, at the three tested concentrations (Fig. 7C).

3.6. Effects of ISO-1 on EAE

In order to evaluate the in vivo effects of ISO-1 in the development and progression of EAE, mice were immunized with $MOG_{35–55}$ and treated with ISO-1 40 mg/kg in a late prophylactic treatment, starting from day 8 post-immunization, when the mice had not yet developed overt clinical signs of EAE. The kinetic and severity of EAE development in control mice was similar to that we have previously reported in our animal house in previous publications (Donia et al., 2010; Mangano et al., 2010). At the end of the experimental period, vehicle-treated mice showed a cumulative score of 54.9 ± 9 and a duration of disease of 29.6 ± 1.8. As expected and in agreement with our previous studies (Donia et al., 2010) treatment with Dexamethasone significantly reduced both cumulative score (6.7 ± 10.9) and duration of disease (5.4 ± 9.1). Treatment with ISO-1 significantly reduced the cumulative score (24.6 ± 21.3, $p = 0.0006$ vs. vehicle) as compared to vehicle-treated mice, and the duration of the disease (15.9 ± 11.5, $p = 0.0052$ vs. vehicle) (Fig. 7D). Mean daily score in ISO-1-treated mice was significantly lower starting from day 16 post-immunization, as compared to vehicle control mice (Fig. 7D).

4. Discussion

The main findings of our in silico study is the consistent, though variable, expression of the receptors involved in MIF/D-DT signaling in T cells and CNS lesions from both EAE-affected mice and MS patients.

As regard to MIF and D-DT receptors, when analyzing CNS-infiltrating CD4+ T cells from EAE-affected mice, a strong and significant upregulation of MIF, CD74, CD44 and the two co-receptors CXCR2 and CXCR4 was noticed, as compared to cells obtained from control mice. A first point of attention should be payed to the unexpected increase in CD74 in 2D2 Tg T cells, as it is generally believed that CD4+ T cells are class II negative and do not express the CD74 receptor. Although, we cannot exclude the contamination of monocyte/macrophages, it is however unlikely that this contamination could explain the marked increase in CD74 expression observed. Also, it has been previously demonstrated that CD4 T cells express the CD74 receptor and that it is

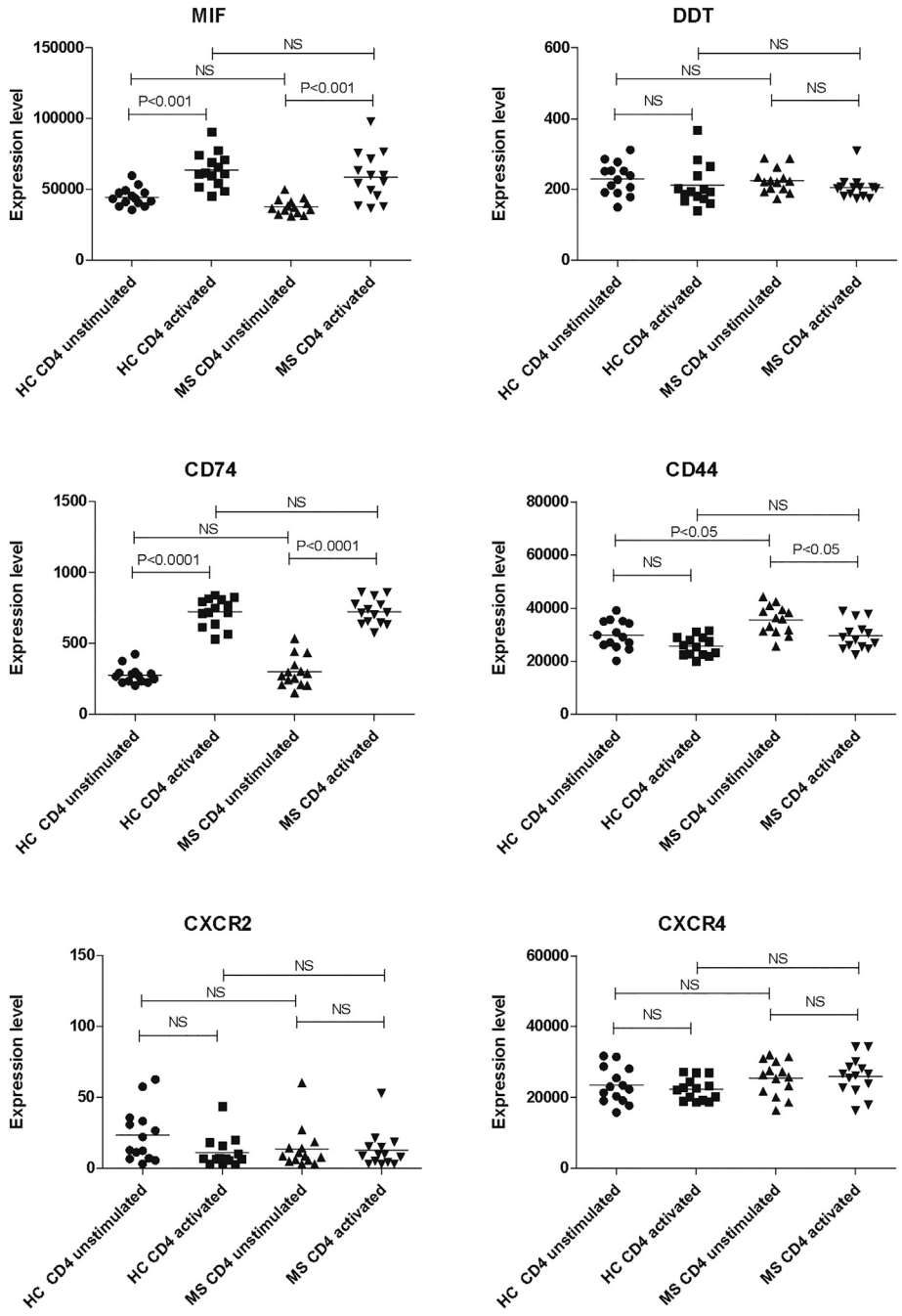

Fig. 2. Analysis of circulating blood cells from MS patients. Gene Expression Profiling of Resting and Activated CD4+ T Cells in Patients with Multiple Sclerosis and healthy donors was obtained from the GSE78244 dataset. Genes of interest were evaluated from unstimulated cells and following 24 h incubation with anti-CD3/CD28 antibodies.

biologically active (Doherty et al., 2017; Gaber et al., 2011). Doherty and collaborators have indeed shown that a small percentage of CD4+ Teff cells express CD74 and can passively transfer inflammatory joint swelling (Doherty et al., 2017), while Gaber et al. showed that under normoxic conditions, the proportion of CD25-expressing CD4+T cells upon PHA stimulation was significantly increased by anti-CD74 treatment (Gaber et al., 2011).

The observation of the increased levels of CD74 after the activation of peripheral CD4 T cells with anti-CD3/CD28 antibodies is also noteworthy. Although this observation could potentially be accounted for by secreted factors from the T cells that affected other cells (e.g., monocytes) contaminating the CD4+ preparation from PBMC, however our internal data do no show significant changes in CD74 expression in monocytes stimulated under M1 conditions (data not shown), that could explain the significant increase in CD74 that we observed in GSE7824.

In contrast to the data from the murine cells, when peripheral CD4+ T cells from MS patients were studied, the only gene significantly upregulated in MS patients vs healthy controls was CD44. A role for the augmented expression of CD44 in MS can be further hypothesized with preliminary and clear caution on the basis of the trend toward protection against MS relapses, that was observed in PBMCs from MS patients with “low” expression of CD44. These two observations seem to fit in well together for possible pathogenic contribution of CD44 in MS and warrant further studies for the better understanding and eventual therapeutic implications of selective CD44 antagonism in

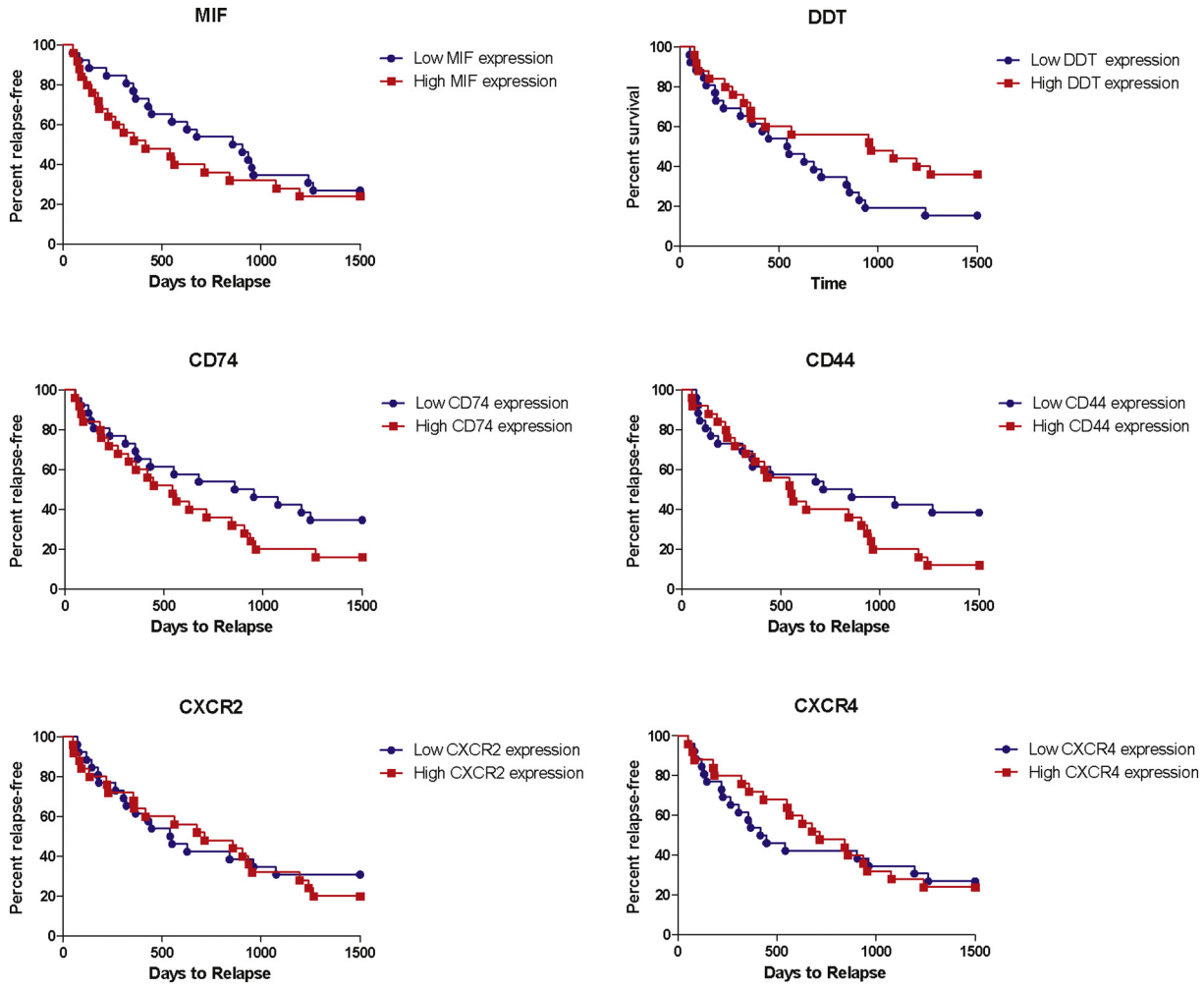

Fig. 3. Prediction of MS relapses by transcription levels of MIF and its receptors in PBMCs. Patients population was divided into two groups based on the expression level of each of the genes of interest (referred as High and Low expression) and survival curves generated for an observational period of 1500 days. MIF, DDT, CD74, CD44, CXCR2 and CXCR4 were considered in the analysis. Data were retrieved from the freely accessible GSE15245 microarray dataset.

the clinical setting. It should also be taken into consideration that the lack of comparable data in CD4+ T cells from males with RR MS impedes drawing firms conclusions.

A convergent point between murine EAE and human MS in the in silico analysis is the lack of significant upregulation of D-DT in either the CD4+T cells or in the CNS lesions of murine and human disease. In addition, higher levels of D-DT in PBMC from patients with RR MS exhibited a trend to protection from MS relapses. These findings are somehow unexpected in light of the key role for D-DT in rodent and human MS, that has recently been reported by Benedek et al. (2017). However, the proper comparisons of our analysis with the study of Benedek et al. is hampered by differences in the population of patients recruited. Indeed, while the latter study has reported augmented circulating levels of D-DT in males with progressive MS, our data originate from female RR MS patients (GSE78244) (Hellberg et al., 2016). In this context, although the trend to protection from relapses in those MS patients that expressed high D-DT levels in their PBMCs does not achieve statistical significance this observation might further underline eventual variable roles of D-DT in different clinical forms of MS. Overall, these apparently conflicting results warrant additional studies aimed at evaluating the role of endogenous D-DT in patients with different forms of MS, as this could facilitate comprehension of pathogenic mechanisms in human MS and suggest the possible adoption of tailored therapies aimed at targeting D-DT in certain subset of MS patients. Further caution should also be exercised in view of the fact that the T cell transcriptome data were generated with bulk T cells and not with myelin-specific T cells. Therefore, this may potentially underestimate the contribution of MIF/D-DT produced by autoreactive T cells to the disease process in the CNS. Ex vivo studies on CNS myelin autoreactive T cell clones from MS patients are in progress to extend and confirm these findings.

In light of these points and since the augmented expression of D-DT in PBMC from MS patients only exhibited a trend to protection from relapses that did not reach statistical significance much caution should be exercised when interpreting the potentially protective role of endogenous D-DT in female with RR MS vs the pathogenic role it has been shown to play in progressive forms of MS in males. Nonetheless, of particular relevance in the context of gender-dependent pathways operating in MS is a recent observation suggesting a sex-related action of IFN- β in MS (Contasta et al., 2012). In particular, the Authors found that IFN-β restored the homeostasis of regulatory T cells and retarded progression to neurological disability through regulation of IL-6 in women and IFN-γ in men and they also identified gender-specific biomarkers for IFN-β in MS (Contasta et al., 2012). In light of this study, and along with the previous work and our present preliminary observation, additional studies are warranted on the potential gender-biased role of D-DT in MS as this could be of diagnostic and clinical relevance in designing tailored therapeutic approaches.

How D-DT could mechanistically exert a beneficial role in females with RR-MS remains to be studied. However, although both MIF and D-

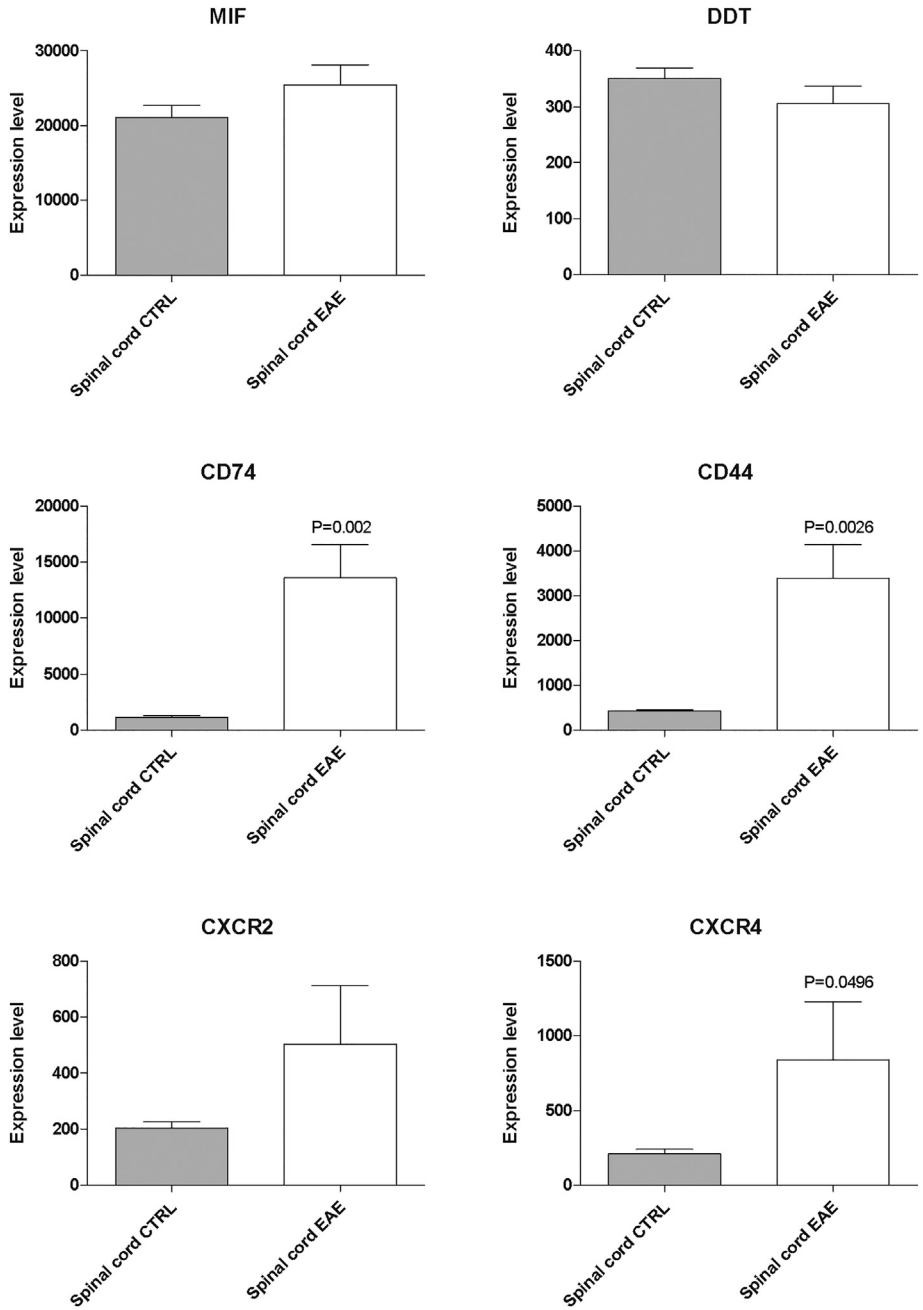

Fig. 4. Analysis of EAE-affected spinal cord. Gene expression was evaluated on homogenized lumbar spinal cord tissue isolated from EAE-affected mice and sham control mice. Data were retrieved from the freely accessible GSE60847 microarray dataset. The spinal cords of the EAE-affected mice were collected during the flare of the disease, at day 16 post-immunization.

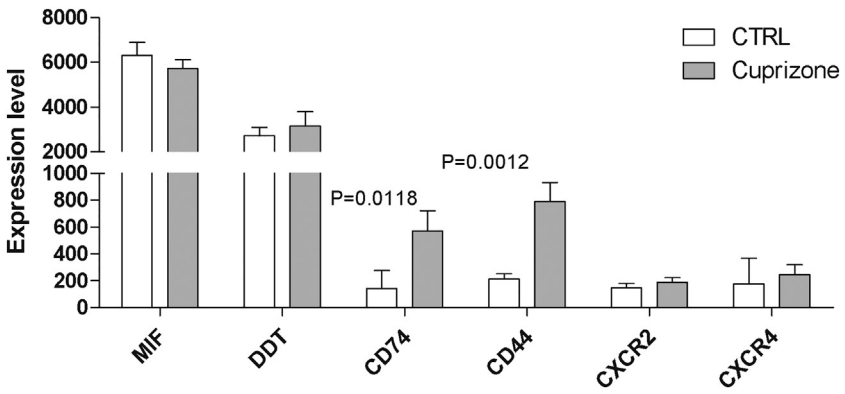

Fig. 5. Analysis of oligodendrocyte damage and remyelination process. Expression levels of MIF, DDT, CD74, CD44, CXCR2 and CXCR4 were evaluated in adult oligodendrocyte precursor cells (OPCs) isolated from the brain of postnatal (day 1 to day 5) and 2-month-old mice, and in adult OPCs in demyelinating conditions (activated aOPCs) isolated from the brain of mice previously treated for 5 weeks with cuprizone (0.2%). Data were retrieved from the freely accessible GSE48872 microarray dataset.

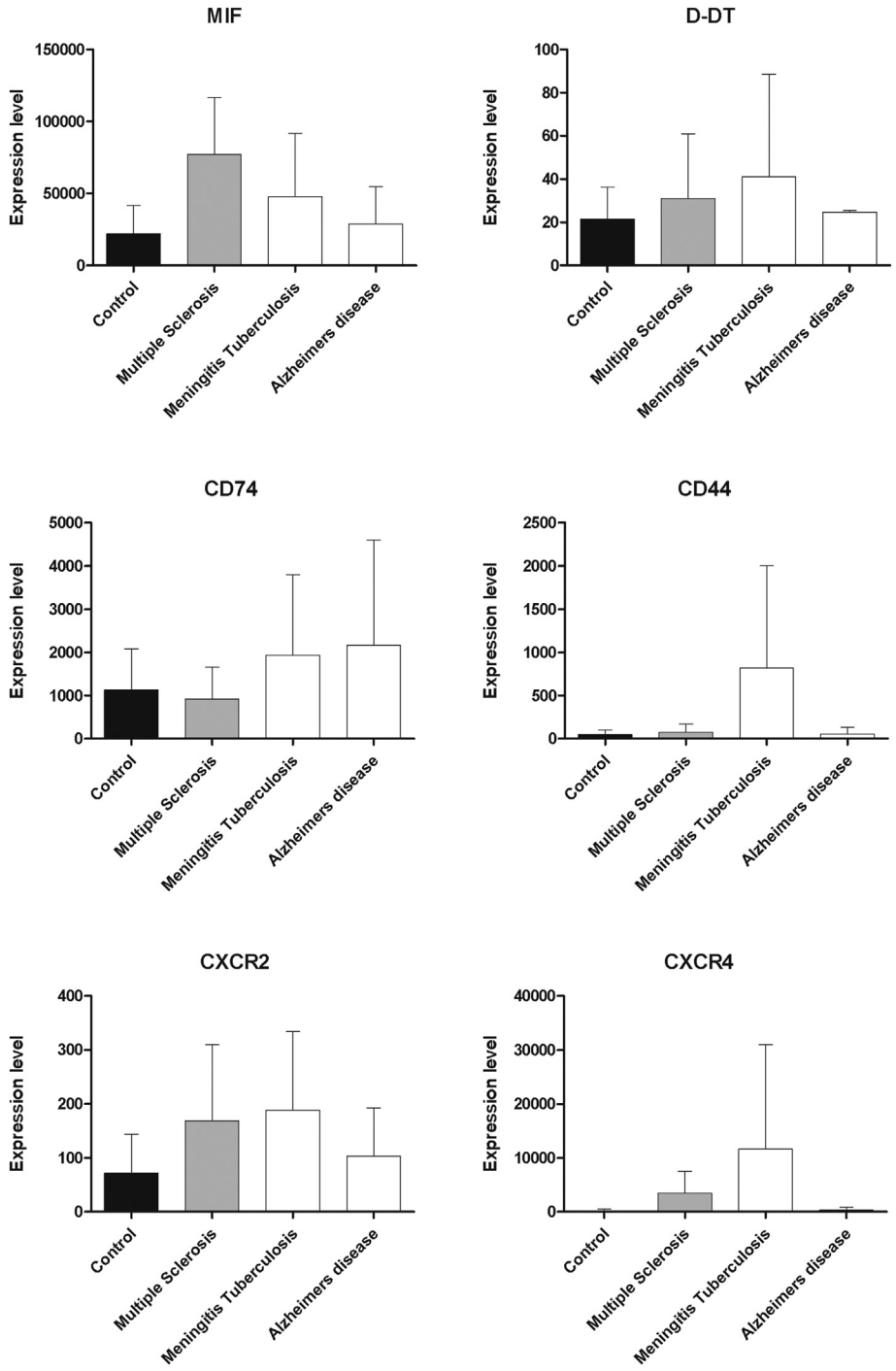

Fig. 6. Analysis of demyelinated MS lesions. Expression levels of MIF, DDT, CD74, CD44, CXCR2 and CXCR4 were evaluated in active MS lesions, inflammatory lesions of chronic tuberculous meningitis, neurodegenerative lesions of Alzheimer's disease and with normal cortex of age matched controls. Data were retrieved from the freely accessible GSE32645 microarray dataset.

DT are primarily known to exert proinflammatory functions via multiple pathways (Stosic-Grujicic et al., 2009), recent findings also demonstrate that they also share the capacity to exert anti-inflammatory responses, via activation of the AMPK pathway, that is an endogenous down-regulator of inflammatory responses (Antonioli et al., 2016; Heinrichs et al., 2011; Martin et al., 2009; Miller et al., 2008).

The anti-inflammatory action of both endogenous and exogenous MIF has also been recently demonstrated in a model of acute kidney injury, in spite of the apparently paradox that this model is prevented by glucocorticoids (GC) and that MIF is a known antagonist of GC (Choi et al., 2013; Stoppe et al., 2018).

The dichotomic action of cytokines in different models is well known, and we and others have previously demonstrated dichotomic effects of IFN-gamma and TNF-alpha in models of SLE, rheumatoid arthritis and type 1 diabetes (Boissier et al., 1995; Nicoletti et al., 2000; Yang et al., 1994). In addition, although MIF and D-DT have been described to primarily exert synergistic actions on promotion of oncogenesis and immunoinflammatory responses (Mangano et al., 2018; Rajasekaran et al., 2014), recent evidence have demonstrated that these homologous cytokines may also display opposite effects in certain settings, including adipose tissue and wound repair and myocardial ischemic reperfusion injury. In the first condition, the harmful effect

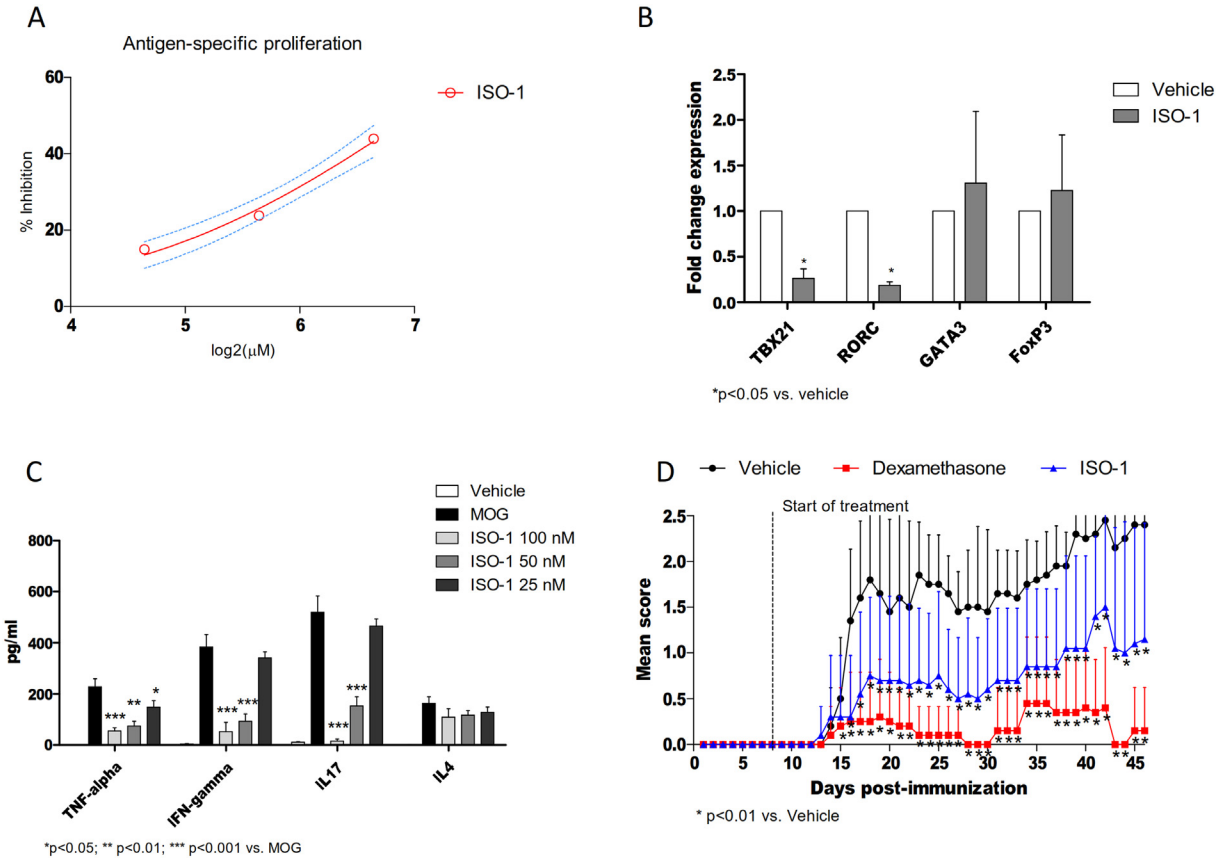

Fig. 7. Effects of ISO-1 on MOG-induced EAE. In order to evaluate the effects of MIF inhibition on encephalitogenic cells, we immunized C57Bl/6 mice with MOG35-55 and at overt disease, splenocytes were collected and re-stimulated with antigenic peptide in the presence of scalar concentrations of the MIF inhibitor, ISO-1. At 72 h of incubation, antigen-specific proliferation was evaluated by MTT assay (A), RT-PCR for TBX21, RORC, GATA3 and FoxP3 (B) and cytokine levels (C) were determined. The in vivo effect of ISO-1 (40 mg/kg), administered from day 8 to day 46 post-immunization, was determined in MOG-immunized mice (D).

exerted from supernatants from inflammatory adipose tissues on the viability of human dermal fibroblast was reduced by D-DT, in a synergistic fashion with anti-MIF antibody, thus indicating an opposite role of MIF and D-DT in this setting (Kim et al., 2017). On the other hand, in patients with myocardial ischemia reperfusion damage, an increased MIF levels during cardiac surgery predicted organ-protective properties, while high MIF-2 levels increased the likelihood of the development of organ dysfunction (Stoppe et al., 2015).

Differences between murine EAE and human MS were also noticed when the expression of these genes were studied in lumbar spinal cords from EAE affected mice and active cortical lesions of MS patients. In EAE mice, we observed significantly higher levels of CD74, CD44 and CXCR4, as compared to control samples, along with no changes in MIF, D-DT and CXCR2 expression. These findings confirm previous data obtained by Benedek and coworkers in EAE induced in DR2-Tg mice showing CD74 changes in spinal cord as a function of EAE severity (Benedek et al., 2013). In particular, the Authors showed that CD74 expression in the spinal cord was significantly increased in resting and activated microglia and in infiltrating monocytes only after the EAE clinical score reached a disease severity score of at least 1.5. Along the same lines, CD74 and CD44 were significantly increased in adult oligodendrocyte precursors from cuprizone-challenged animals with respect to control adult oligodendrocyte precursors, as detected in GSE48872. In contrast, when cortical MS lesions were studied, only a trend of increase could be found for MIF, CXCR2 and CXCR4, as compared to healthy control cortex from MS. This is in partial concordance with Cox et al., who observed the absence of MIF in cortical lesions, although a strong positivity for MIF at the edge of active white matter lesions of MS patients was observed by this author (Cox et al., 2013). Unfortunately, our analysis is performed on only three samples from patients with SPMS. Therefore, the low number of replicates does not allow for a reliable evaluation of the differences between male and female subjects. Hence, no conclusion on the possible influence of sex for the observed differences can be drawn.

The finding from our in silico study suggests that, as compared to healthy controls, MIF expression is not upregulated in resting or (anti-CD3 + anti-CD28 activated) peripheral CD4+ T cells from MS patients. This appears to conflict with the augmented levels of MIF found from another group in the blood and/or CSF of MS patients suffering from MS (Niino et al., 2000). It is possible that these differences are due to different clinical activity of MS of the patients studied, as available clinical data of the patients we employed for the in silico analysis seems to indicate that the patients recruited were in stable RR MS, while augmented levels of MIF in the blood and CSF were only observed during relapses by Niino et al. (2000). A more recent study however, shows that increased MIF levels can also be observed in CSF of RR MS patients that appear to be in stable disease (Khaibullin et al., 2017). The Authors hypothesize that like Th1 cytokines, MIF plays a role in MS pathogenesis, via local secretion in CSF that may be also due to secretion by astrocytes (Khaibullin et al., 2017). If so, this would be consistent with the lack of upregulated synthesis of MIF from peripheral CD4+ T cells from the MS patients that we have observed in our analysis.

In agreement with data showing an involvement of MIF and their receptors in the pathogenesis of EAE/MS, we have presently shown for the first time that the small molecule and specific MIF inhibitor, ISO-1, that represents the prototypic small molecule inhibitor of MIF (Al-Abed et al., 2005), profoundly downregulated the functional activity of these

cells upon ex vivo conditions, including their capacity to secrete proinflammatory cytokines, that plays a key pathogenetic role in both rodent EAE and human MS, such as IFN-gamma and IL-17 (Palle et al., 2017), as well as to proliferate upon auto-antigenic stimulation. Also, in vivo treatment of EAE-affected mice with ISO-1 markedly ameliorated the course of the disease. This is in agreement with previous observations proving that different means of specific blockade of the function of endogenous MIF including knocking out MIF, neutralizing antibody and CD74 antagonist through HLA-DRα1 constructs (Meza-Romero et al., 2014), all improve the course of EAE in vivo (Cox et al., 2013; Denkinger et al., 2003). The recent emergence of dual antagonists of MIF and D-DT, such as 4-IPP (Nobre et al., 2017; Xu et al., 2013) strongly warrants additional preclinical and clinical studies aimed at dismantling the eventual co-pathogenic contribution of D-DT in different forms of rodent EAE and human MS and evaluating the feasibility of tailored therapeutic strategies, aimed at blocking either one of the two homologs or their co-receptors such as CD74 and CD44. Also, the use of therapeutic administration of ISO-1 in EAE is currently under investigation, along with more in depth mechanistic studies for the role of the MIF/CD74 axis in MS.

In addition, as MIF inhibits glucocorticoid activity (Fingerle-Rowson et al., 2003; Lerch et al., 2014), inhibition of MIF could enhance anti-inflammatory actions of glucocorticoids, with consequent reduction of the doses of corticosteroids that are used for the treatment of relapses of MS. Of particular relevance could be the use of MIF- and, eventually D-DT- inhibitors, for those MS patients that develop resistance to steroids treatment. Recent preclinical studies support the potential important role for MIF inhibitors to rescue steroid resistances in MS patients by demonstrating that MIF promotes resistance to glucocorticoid treatment in EAE (Ji et al., 2015).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

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