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Role for Macrophage Migration Inhibitory Factor in Multiple Sclerosis

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Sommario

Introduction

Macrophage Migration Inhibitory Factor (MIF)

The Macrophage Migration Inhibitory Factor (MIF) is a pro-inflammatory cytokine expressed by a variety of cell types including epithelial, endothelial and immune cells (Stosic-Grujicic, 2009). Unlike other cytokines, MIF is constitutively expressed and stored in intracellular pools and does not require de novo protein synthesis before secretion . Therefore, it does not require protein synthesis prior to secretion in the extracellular environment (Bacher, 1996). In 1993, Flieger et al. have shown that MIF production takes place through a different pathway to protein export, suggesting the involvement of ATB (A3) conveyor (Flieger, 2003). More recently, Merk et al. have demonstrated that the Golgi-associated protein P115, is an intracellular MIF binding partner, and they also have shown the P115-dependent release of MIF from human monocyte/macrophages (Merk, 2009). MIF possesses properties of cytokine, enzyme, endocrine molecule and chaperon-like protein and in 1994, it was also found that MIF also acts as a hormone. Bernhagen et al. identified MIF as a major secreted protein released from the anterior pituitary cells in response to a septic shock induced by the administration of LPS (Bernhagen, 1993). The production of MIF at the level of corticotrophic and thyrotoxic cells of the anterior pituitary gland and its collocation in secretory granules with adrenocorticotrophic hormone (ACTH) or the thyroid stimulating hormone (TSH) further supported the role of MIF in the axis of ' Hypothalamic-pituitary-adrenaline (HPA)(Bernhagen, 1993). MIF derived from pituitary gland enters the bloodstream after infectious or stressful stimulation to the body, leading to an increase in circulating MIF concentrations. MIF expression in various mouse organs showed MIF release 6 hours after administration of LPS, followed by mRNA MIF induction, and 24 hour restoration of intracellular MRI immunoreactive (Bacher, 1997). In human serum, the physiological concentration of MIF is between 1 and 15 ng / ml, while concentrations up to 350 ng / ml are reached for inflammatory stimulation (Bernhagen et al, 1993).

MIF is a homotrimer of 12.5 kDa subunits, with oxidoreductase and tautomerase activity (Stosic-Grujicic, 2009). Substrates for the enzymatic activity of MIF are represented by phenylpyruvic-acid, p-hydroxyphenylpyruvic-acid, 3,4-dihydroxyphenylaminechrome, and norepinephrinechrome. Because of its homotrimeric structure, there are multiple binding sites for potential inhibitors of MIF that could disrupt its tertiary structure and inhibit its enzymatic activity or the binding to CD74 and other ligands.

The roles of MIF in immunologic response regulation are different. As a product of cells of the innate immune system, MIF acts through enhancement of TLR4 expression, phagocytosis, intracellular

killing, nitric oxide, H2O2 and TNF-α production in macrophages, thus representing an important factor in the protection of the host against various infectious agents. Through induction of IL-12 and inhibition of IL-10 synthesis, MIF favors Th1 immune response (Cvetkovic, 2006).

A regulatory role of MIF has been observed in MIF gene deficient cells. It has been shown that MIF deficiency attenuates leukocyte–endothelial cell interactions (Gregory, 2004), as well as the expression and function of IL-1 and TNF receptors (Toh, 2006), thus providing further molecular evidence for the critical role of MIF in autoimmune and inflammatory states.

Several data suggest a key role of MIF in the pathogenesis of Multiple Sclerosis (MS). In mice with EAE, an animal model of MS, MIF was found to be upregulated in the affected tissue (Baker, 1991). Immunoneutralization or genetic depletion of MIF reduced the severity of the disease by impairing the migration of autoreactive T cells to the CNS and downregulating the inflammatory cytokine production (Denkinger, 2003; Powell, 2005). Moreover, intraspinal microinjection of MIF resulted in the upregulation of inflammatory mediators in microglia, which was sufficient to restore EAE-mediated inflammatory pathology in MIF-deficient mice (Cox, 2013).

The inhibition of MIF actions by usage of neutralizing anti-MIF antibodies has also proven therapeutically effective (Denkinger, 2003; Powell, 2005). Indeed, MIF blockade decreases the expression of VCAM-1 in the CNS, and impairs the homing of neuroantigen-specific T cells to this site. Moreover, MIF blockade reduces the clonal size of the autoantigen-specific Th1 cells, and increases their activation threshold (Denkinger, 2003).

In clinical studies, enhanced levels of MIF were observed in serum and in cerebrospinal fluid of patients with active/relapsed MS (Niino, 2005). In particular, Niino and collaborators found that the concentration of MIF in CSF samples was significantly elevated in relapsed cases of MS compared with control samples. In addition, Cox et al. observed that MIF is highly expressed in human active MS lesions (Cox, 2013).

Biology of the Macrophage Migration Inhibitory Factor

MIF is a cytokine, the term cytokine describes a functional class of protein mediators, which are produced in a regulated fashion to affect the activation and differentiation of the immune response. Once released, they usually act in an autocrine or paracrine manner leading to an ensuing activation of the innate (dendritic cell, monocyte/macrophage) or the adaptive (T and B cell) immune response

characterized by further production of an array of immunoregulatory cytokines. Cytokines have the cardinal properties of pleiotropism, synergy, antagonism, and redundancy, aggravating the understanding of their precise function in different physiologic and pathologic contexts. Discovered in the early 1960s as one of the first cytokines to be described, MIF was first considered a T cell cytokine, regulating the activation of T cells induced by mitogenic or antigenic stimuli (Calandra, T. et al.,1998; Bacher, M. et al.,1996). Monocytes and macrophages that had previously been considered to be the target of MIF action were observed to be a significant source of MIF. Upon pro-inflammatory stimuli, macrophages were reported to produce TNFα and nitric oxide (NO) (Calandra, T.,1994; Thiele, M.,2005). Subsequently, more cell types, such as eosinophil granulocytes, B cells and mast cells, were identified to be involved in MIF action (Rossi, A. G. et al., 1998; Takahashi, A. et al., 1999; Bacher, M. et al., 1997). Even gastrointestinal colorectal adenoma cells and prostatic adenocarcinoma cells showed increased MIF levels, suggesting protumorigenic activity of MIF (Javeed A. et al., 2008; Simons D. et al., 2011). Consistent with its definition as a cytokine, MIF has been shown to exhibit various molecular modes of action relevant to antimicrobial host defense. MIF binding to the CD74/CD44 receptor complex leads to ERK-1/2 activation, which results in downstream PGE2 production via cPLA2 and COX-2 activation. Moreover, phosphorylation of ERK-1/2 indirectly upregulates TLR4, the cell surface receptor for gram-negative bacteria, through stabilization of Ets transcription factor. This results in the upregulation of immune response genes and, thus, production of pro-inflammatory cytokines and adhesion molecules. MIF also protects cells from apoptosis by inhibiting p53 via COX-2 (Merk M. et al., 2011).

Receptors for Macrophage Migration Inhibitory Factor

The CD74 receptor plays a very important role in our research. CD74 (MHC invasive chain class II, Ii) is a non-polymorphic type II transmembrane glycoprotein. In addition to being a Class II MHC chaperone, CD74 plays different biological, and physiological functions and is involved in some pathologies, plays important roles in many inflammatory diseases, such as liver fibrosis, type I diabetes, systemic lupus erythematosus, and Alzheimer disease. In addition, CD74 is a high affinity membrane receptor for macrophage migration Inhibitor Factor (MIF), D-dopachrome tautomerase (DDT / MIF-2) and bacterial proteins.

MIF is a key cytokine closely involved in autoimmune and inflammatory diseases. MIF attracts and subsequently retains activated immune cells from the periphery to the inflamed tissues (Javeed A. et al.,2008). The biological effects of MIF are predominately mediated through its primary receptor,

CD74 (Simons D. et al.,2011). The comprehensive analysis recently shows that MIF controls the activation of CD74 (Pantouris G. et al.,2015). MIF inhibits the directed migration of monocytes to chemokines, such as monocyte chemoattractant protein. MIF promotes the arrest of monocytes and T cells in vitro (Bernhagen J. et al.,2007). MIF increases the secretion of proinflammatory cytokines like IL-1, IL-2, IL-6, IL-8, INF-c, and TNF-a, and the expression of adhesion and inflammatory molecules such as iNOS (Gregory JL,Liu YH,2008). All these actions of MIF are mediated through CXCR2 and CXCR4 which are closely magnified by CD74 (Simons D. et al.,2011; Bernhagen J. et al.,2007). MIF counteracts glucocorticoid inhibition of proinflammatory cytokine secretion in response to lipopolysaccharide in macrophages (Calandra T. et al.,2000; Bach JP. Et al., 2008). T cells and macrophages release MIF in response to glucocorticoid and autocrine MIF then overrides glucocorticoid inhibition of T-cell proliferation and cytokine secretion (Bacher M. et al.,1996; Calandra T. et al.,1995).

The binding of MIF to its receptor complex CD74/CD44 leads to the activation of the extracellular signal regulated kinase (ERK) 1 and 2 in the mitogen-activated protein kinase (MAPK) pathway, and the PI3K/Akt/SRC signal transduction cascade (Lue H. et al., 2007; Shi X. et al.,2006), which, in turn, increase cell proliferation, decrease cell apoptosis, and enhance cell migration (Meyer-Siegler KL. Et al., 2006; Lee CY. Et al., 2012;). Inhibition of MIF activity or MIF expression reduces microbial products-induced phosphorylation of p38 and ERK1/2 MAPKs and secretion of cytokines. High doses of MIF counter-regulate adenosine and prostaglandin E2-mediated inhibition of ERK1/2 activation and TNF-a production in newborn monocytes exposed to Escherichia coli (Roger T. et al., 2016;). In contrast, other studies show that MIF could activate the AMP-activated protein kinase (AMPK) pathway to decrease cell proliferation, cell viability, and metastatic ability in some cancers (Kim HS. et al., 2012; Lee CW. et al., 2012). Overexpression of CD74 leads to upregulation of NF-kB dependent genes encoding cytokines in macrophages. CD74 controls B-cell differentiation and maturation in the spleen of mice (Matza D. et al., 2002; Becker-Herman S. et al 2005;). Activation of CD74 by MIF or activating antibodies results in a signaling cascade in B cells that involved Syk tyrosine kinase, PI3K, and Akt and leads to CD74 intramembrane cleavage and CD74 intracellular domain (CD74-ICD) release, NFkB activation, Bcl-xL upregulation, and cell survival (Starlets D. et al., 2006;). CD74 ectodomain undergoes a first proteolytic cleavage in the endocytic compartment and a secondary intramembrane domain cleavage by the c-secretase-presenilin complex to liberate CD74-ICD from the lipid bilayer into the cytosol. Transport to the endocytic compartment is essential for CD74 processing and intramembrane cleavage. CD74-ICD then translocates to the nucleus and activates NF-kB p65/RelA homodimer and the B-cell-enriched coactivator, TAFII105 (Matza D. et

al., 2002; Becker-Herman S. et al., 2005;) The signal is terminated by degradation of the active CD74-ICD fragment (Binsky I. Et al., 2007;).

In addition to activating the type-II receptor CD74. (MIF) exhibits chemokine-like activities through non-cognate interactions with the chemokine receptors CXCR2 and CXCR4.

The activation of MIF-CXCR2 and -CXCR4 axes promotes recruitment of leukocytes and contributes to the promotion of other biological activities. As for the MIF-CXCR2 interaction it has been found to commit a pseudo-ELR and an N-like motif, with respect to the interaction of CXCR4 and MIF nothing has been discovered about it.

MIF activity also manifests itself through the binding of three receptors: the CD74-CD44, CXCR2 and CXCR4 complex. The role of MIFs with these three different receptors is the answer to the various biological activities associated with MIF. MIF is also unique because it is the only protein that activates both an ELR + and ELR-chemocin receptor. The relationships leading to the biological function between MIF and CXCR2 or CD74 (Kraemer, S.et al,2011; Pantouris, G.2015; Weber, C.2008) have been studied, but interactions with CXCR4 are not yet known.

D-dopachrome tautomerase (DDT): a MIF homologue gene

As we already know, the importance of MIF has been widely studied be a relevant parameter in severe conditions such as sepsis and septic shock (Rex S,2014; Pohl J, 2016; Lehmann LE et al., 2009;), cardiac arrest syndrome (Pohl J. et al., 2016;), kidney failure (Luedike P. et al., 2015;), heart failure (Mueller KA., 2016;) and cancer. Numerous experimental and clinical studies have suggested a prognostic role for MIF in these severe conditions. However, the relevance of the newly-characterized MIF superfamily, ddopachrome Tautomerase (DDT or MIF-2) (HeLJ et al,2015;) is still unknown. In 1990, a new protein (DDT or MIF-2) was obtained by purification of mouse melanocytes during an investigation into the regulation of mammalian melanogenesis. This molecule has been defined as dopachrome tautomerase according to its tautomerase activity on dopachrome (Aroca, P. et al.,1990). It has been reported that dopachrome tautomerase increases the amount of melanin formed by tyrosin-1-tyrosine melanoma. Subsequently, two melanin-forming cells (Tsukamoto, K. et al,1992; Winder, A. J. et al,1993) have been defined as two l-isomers linked to dopachrome tautomerase membrane, tyrosinase-related protein-1 and -2 (TRP-1, TRP-2). Both enzymes catalyze the isomerization of L-dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA). These isomers were obtained by purification of a specific enzyme for the tautomerization of D-dopachrome, which was called D-

dopachrome tautomerase (Odh, G. et al,1993). The D-DT enzymatic activity has been found in the male rat, localized in the liver, kidneys, spleen and also in the human at the level of melanoma cells, liver cells and ultimately in the blood (Bjork, P. et al.,1996). Similar to MIF, MIF-2 is strongly expressed in most tissues (Merk M et al.,2011). These two proteins work cooperatively and it has been shown that neutralizing MIF-2 in vivo leads to significantly decrease of inflammation (Merk M et al.,2011). MIF as MIF-2 is also stored in cytosol in preformed storage pools that allows rapid release of both proteins on different stress stimuli (Burger-Kentischer A., 2002;Merk M.2012). It is not common that the MIF-2 secretion mode is caused by the so-called non-classic path for secrecy and is based on the lack of a sequence of signals that usually average Golgi-dependent secretion (H. Sugimoto, M. et al., 1999). There are currently several stimuli known to stimulate MIF and MIF-2 secretion, such as LPS, inflammation, hypoxia, hyperoxia, ischemia and reperfusion, hormone, surgical stress and mitogenic factors (M. Merk, et al. 2011). Rapid secretion from MIF and MIF-2 cellular pools is therefore useful for serving as indicators in a wide range of critical illnesses.

D-DT and MIF are considered homologous as they share the identity of 35% (M. Merk, et al. 2011). From a structural point of view, the D-DT topology resembles that of MIF and bends to form an homotrimer with extended contacts between the subunits through the lateral bands of the beta leaf . The link between D-DT and CD74 also exhibits high affinity (D-DT: KD = 5.42 109 M vs MIF: KD = 1.40 109 M) (M. Merk, et al. 2011) and competes with MIF to bind to the receptor, suggesting that the two homologues could share the same binding site on CD74. As a MIF, D-DT is able to activate ERK1 / 2 MAP kinase activation in similar signaling responses.

Despite the structural and functional knowledge of MIF and D-DT, molecular parameters that regulate the interaction of these two cytokines with their shared receptor are not fully understood.

From a genomic point of view, the macrophage migration inhibitory factor gene MIF, located on 22q11.2, encodes a multifunctional cytokine, MIF. However, the MIF-antisense transcription, called MIF-AS, is a novel unknown lncRNA. Studies on the biological function of MIF-AS and its role have not yet been reported. Single nucleotide polymorphism (SNP), caused by a single nucleotide variation, mainly refers to the genomic DNA sequence polymorphism. Recent testimonies have confirmed that SNPs in lncRNAs (long non coding RNAs) can influence its biological mRNA formation processes, which can lead to the aberration of its interactors (Zhu Z. et al., 2012; Li L. et al., 2013)

MIF Inhibitors

MIF proinflammatory function studies showed a direct association between the catalytic site surrounding pro-inflammatory actions of Pro1 and MIF. The mutagenic analysis of the catalytic site around Pro1 has led to the conclusion that it is involved in the counterregulation of glucocorticoids (Lubetsky, J. B. et al.,2002), in the formation of neutrophils (Swope M. et al., 1998) and in the increase in the regulation of metalloproteinases-1 and -3 in the synovial fibroblast's Arthritis and Cancer (Onodera S. et al., 2000). Therefore, it has been argued that molecules targeting this site may be useful in inhibiting MIF actions in vitro and in vivo. These small molecules act as catalytic inhibitors of MIF's enzymatic activity by adhesion to the amino-terminal protein tautomerase region. Among these molecules, the most important are N-acetylbenzocinone-imine (NAPQI) (126), (S, R) -3- (4-hydroxyphenyl) -4,5-dihydro-5-isoxazole methyl acetic acid (ISO- 1) E 4-iodo-6-phenylpyrimidine (4IPP). The reaction between the metabolism of the aminotinophene NAPQI and MIF of iminochinone creates a covalent bond and produces a modified NAPQI species which inhibits the activity of tautomerase and consequently decreases the cell binding capability and reduction of effective Immunosuppressive (Senter, P. D. et al., 2002). ISO-1 reversibly binds to MIF and not only inhibits MIF's tautomerase activity but also has a counter-regulatory activity of glucocorticoid, producing TNFα, PGE2 and COX-2 (Lubetsky, J. B. et al., 2002) and protecting from death in an in vivo mouse model of endotoxic shock (Merk, M. et al., 2008). Using computational virtual screening, 4-IPP has been identified as suicidal substrate for MIF, causing Pro covalent modification. It has been shown that this compound is about 5 to 10 times more potent of ISO-1 in blocking MIF-dependent catalysis, cell migration and in vitro independent growth (Lubetsky, J. B. et al., 2002). Further differences in potential inhibitors of these small molecule inhibitors are evident when comparing the half-maximum concentration inhibition (IC50) determined by the D-dopachrome tautomerase assay; ISO-1 has an IC 50 of ~ 50 μM, ~ 40 μM NAPQI and ~ 5 μM 4-IPP (Merk, M. et al., 2008). In 2011, Al-Abed and others reported the first potential endogenous MIF antagonist, thyroid hormone (T4) . The authors suggested that T4 may influence inflammatory responses mediated by MIF (eg survival in mice with severe sepsis) by inhibiting its terminal amino tautomerase region (Al-Abed, Y. et al., 2011).

Multiple Sclerosis: Introduction

Multiple Sclerosis (SM), often referred to as plaque sclerosis, sclerosis, or polisclerosis, is a chronic autoimmune demyelinating disease affecting the central nervous system causing a wide range of signs and symptoms. As we know, the nervous impulse is transmitted through the nerve cells through electrical signals, which pass through long nerve fibers defined as axons, completely coated by the myelin sheath.

During the disease, the patient's immune system attacks and damages this nerve cell shell, making communication between the brain and the spinal cord difficult as the patient's immune system attacks and damages this sheath. When this happens, axons are no longer able to transmit signals effectively.

MS has a prevalence ranging from 2 to 150 cases per 100.000 individuals, depending on the country or specific population (Rosati G. et al., 2001). It is estimated that the disease affects about three million people in the world, half a million in Europe and about 68.000 in Italy; the most affected Italian region is Sardinia. A study found that in the United States, in 1990, there were around 250.000 to 350.000 cases of multiple sclerosis (DW. Anderson et al., 1992).

Population and geographic-based epidemiological studies have been widely used to increase the knowledge of the disease and have led to the proposal of various etiologic theories.

The condition is most common in females, with a ratio of about 3 to 1 compared with males. In children sex-related incidence is greater, while in subjects over 50 years the disease affects men and women in almost equal proportions.

It was found a north-south gradient in the northern hemisphere and a south-north gradient in the southern hemisphere. People living near the equator are less affected by multiple sclerosis. At least in part to explain the existence of this gradient, it has been suggested that climate, lack of sunlight and vitamin D reduction may be factors responsible for the disease. However, there are important exceptions to this model, with variations in prevalence rates experienced over time, and in general this trend may disappear. This indicates that other factors, such as the environment or genetics, need to be taken into account to explain the origin of MS. It is also more common in regions with northern Europe populations.

A central mission in multiple sclerosis research was to determine the sequence of manifestations that developed inflammatory plaque. It has generally been stated that this histopathological sign derives from a violation of the blood-blooded integrity of a person genetically predisposed to the disease. A hypothesis suggests that some forms of systemic infection can cause upregulation of adhesion molecules on the brain and spinal cord endothelium, allowing leukocytes to accommodate and cross the vessel walls to enter the normally immunologically privileged central nervous system. If lymphocytes programmed to recognize myelin antigen exist within the cell infiltrate, they can trigger a cascade of events resulting in inflammatory lesion and acute demyelination. These lesions typically develop in white matter, where the main goals are the myelin sheath and the myelin cell, the oligodendrocyte. However, lesions of gray-matter, where the primary objective is also myelin, are known to occur.

Pathology and clinical diagnosis of MS

Multiple Sclerosis is a disease that manifests its severity depending on which areas of the central nervous system have been affected. Therefore the evolution of the disease is very variable and the symptoms can change gravity and duration even in the same patient. Even if the symptoms and manifestation of the disease are very varied, there are more frequent clinical patterns. Most patients show a sudden onset of the disease with subsequent relapses and remittances leading to slow deterioration and incomplete functional recovery. Only a minority has a rapidly progressive clinical deterioration. The negative outcome of the disease is the result of two processes that characterize the course of the disease, a deterioration caused by the partial recovery of acute lesions and a slow and gradual deterioration that mainly characterizes progressive forms. In the clinical field, four subcategories generally recognized in the MS have been differentiated :

Relapsing-remitting MS (RRMS): The recurrent-remitting form is the most common and affects about 85% of cases. In some patients it can enter a transitional phase after some years. In this case, the attacks repeat at close frequency almost never entering the state of retirement. The interval between relapse is very short and usually the attacks tend to have the same target. This stage represents the most difficult and challenging condition as it is a constant deterioration of the patient's condition, which is at high risk of developing progressive secondary multiple sclerosis

Secondary progressive MS (SPMS): The secondary progressive form has a recurrent-remitting initial pattern followed by a progressively secondary form that occurs with or without superimposed

recurrences and with possible phases of relative remission and stabilization. In the event of a fallback, the recoveries are incomplete and there is a progression of the deficits even in the periods between one fallout and the next.

Primary progressive MS (PPMS): Affecting 8-10% of patients, the disease starts from the beginning with a progressive course characterized by possible stages of relative improvement and stabilization

Progressive-relapsing MS (PRMS): The least common form, is characterized by continuous progression from onset, with recurrences sometimes followed by recovery. The intervals between one relapse and the following are characterized by a continuous progression of the disease, unlike the recurrent-remitting form where the interval between two relapses is progressive, affecting less than 5% of the patients.

RRMS usually manifests with disorders related to sensory apparatus, unilateral optic neuritis, internal ophthalmoplegia, Lhermitte's sign (trunk and limb artifacts evoked by neck flexion), weakness of the limbs, unconsciousness, transient ataxia and symptoms of bladder and the intestine neurogenic. In the second part of RRMS, many patients described fatigue and increased body temperature. The onset of symptoms and symptomatic worsening with increased body temperature (Uhthoff's symptom) and fever pseudo-acerbations suggest diagnosis. Some patients have recurrent, short and stereotyped phenomena (paroxysmal pain or paresthesia, trigeminal neuralgia, episodic unconsciousness or dysarthria and tonal posture) that are highly suggestive of multiple sclerosis. Cortical signs (aphasia, apraxia, recurrent convulsions, visual field loss and early dementia) and extrapyramidal phenomena (chorea and stiffness) are critical to delineating the clinical picture. Finally, cognitive dysfunction, depression, emotional elasticity, dysarthria, dysphagia, dizziness, progressive quadriceps and loss of sensation, atassial tremors, pain, sexual dysfunction, spasticity and other manifestations of dysfunction of the central nervous system can become problematic. Patients with primary progressive multiple sclerosis are often present with a higher upper leg motor syndrome that develops slowly ("chronic progressive myelopathy"). Usually, this form manifests itself in a gradual deterioration, and can develop quadriceps, cognitive decline, visual loss, cerebral and cerebellar stem syndrome, intestines, bladder and sexual dysfunction. Diagnosis is based on established clinical criteria and, if necessary, in the laboratory. The advent of the magnetic resonance imaging (MRI) and advances in cerebrospinal fluid analysis, however, greatly facilitated the diagnosis (and as a result of early treatment) of MS.

Forms of recovery are considered clinically defined when neurological dysfunction becomes "widespread in space and time". Primary progressive multiple sclerosis may be clinically suggested

by a progressive course lasting more than six months, but it is recommended for study laboratories to obtain evidence and support for research to exclude other potentially treatable diseases. For example, structural or metabolic myelopathy can be identified by appropriate laboratory studies, including the MRI spinal.

The cerebrospinal fluid analysis has often highlighted an increase in intrathecal synthesis of limited specificity of immunoglobulins (oligoclonal groups or the synthesis of IgGs) with moderate lymphocytic pleocytosis.

Abnormalities detected by experimenting with potential somatosensors and spinal resonance can best explain the diagnosis in patients who only show optic neuritis or seasonal anomalies in the brain and are therefore suspected of being affected by multiple sclerosis

MS Etiology

The key role in the etiology of the disease is dictated by genetic predisposition and environmental factors combined with a failure of immunity tolerance mechanisms to suppress and effectively abolish the auto-reactive cell.

Specific genes have been identified and make some people susceptible to MS manifestation, particularly the HLA complex, which probably contains the most susceptible gene for MS (Jersild et al., 1973). it was recognized in 1973 that the presence of HLA-DR2 allele significantly increased the risk of multiple sclerosis. HLA class II haplotypes particularly HLA-DR2 (DRβ1 * 1501). DQ6 (DQP1 * 0602), DR3 (DRβ1 * 0301). DQ2 (DQP1 * 0201) and DR4 (DRβ1 * 0401). DQ8 (DQP1 * 0302) demonstrated stronger association with susceptibility to MS. In addition, HLA polymorphism and sunlight UVR exposure are two possible associated causes that contribute to the predisposition of a MS (Bastardo Blanco et al., 2014).

Role of infectious Agents in MS

The activation of reactive T cells can be caused by the structural similarity between infectious antigens and proteins of the myelin sheath (molecular mimicry), leading to the recognition of peptides derived from infectious and autoantigen agents. In-depth research into possible MS's infectious triggers had the goal of demonstrating that T cell specific clones for an immunodominant MBP

epitope also recognize viral peptides. These peptides have been recognized in some viruses such as Epstein Barr virus (EBV), influenza A virus, herpes simplex virus, human papilloma virus, or virus herpes virus-6 (Riedhammer and Weissert , 2015).

Immuno-Pathogenesis of MS

Genetic and environmental factors, such as viral infection, bacterial lipopolysaccharides, reactive metabolites and oxidative stress, could activate the auto-reactive T cells, thus involving a variety of other immune cells: macrophages, B-cells, NK cells, cytotoxic T-cells and microglial cells (Weissert, 2013).

Following the observations made on an animal model of MS, allergic / autoimmune encephalomyelitis (EAE), MS was considered a Th1-guided disease (Sriram and Steiner, 2005). However, it was later demostrated that IL-12 knockout mice (unable to generate Th1 cells) are still susceptible to EAE, while IL-23 knockout mice are not (Langrish et al., 2005). This led to the discovery of Th17, a new subset of Th cells, which secrete IL-17, IL-6 and TNF-α . Th17 cells and also IFN-γ and IL-17 co-expressing Th cells have been detected in cortical lesions of human MS patients, especially the active ones (John et al., 2008).

Many elements are involved in the process of immune response developing to myelin antigens with a consequent immune process leading to demyelination. The elements contributing to such processes are the T helper cells, predominantly Th1 and Th17, the cytotoxic T cells, B cells, macrophages, microglia and cytokines that secrete.

Histologically, chronic multiple sclerosis is easily recognizable by the presence of a demyelinated plaque, characterized by a well-demarcated hypocellular area showing myelin loss, partial axonal conservation and astrocytic scarring. These lesions are characterized by areas that show myelin and oligodendrocyte loss along with infiltrates of inflammatory cells such as lymphocytes and macrophages. Inflammatory cells are located close to the blood vessels but may infiltrate the diffused parenchyma. In conclusion, in chronic multiple sclerosis, remyelination is minimal, while plaques in acute and early multiple sclerosis may have a great remyelination (Mouzaki et al., 2015).

Possible Mechanisms of Injury and Repair in Multiple Sclerosis

The CD4 + T cells are adherent to the luminal surface of the venous cells of the venules in the CNS and will move to the CNS when there will be the breakdown of the blood–brain barrier. After the recognition of target antigens on antigen-presenting cells with subsequent amplification of the immune response, adhesion molecule involved in the process are intercellular adhesion molecule 1 (ICAM-1), vascular-cell adhesion molecule 1 (VCAM-1) and E-selectin are involved factors that determine and also facilitate the entry of T cells into the CNS. Proteases, such as matrix metalloproteinases, may further enhance the migration of active immune cells by degrading macromolecules of the extracellular matrix.

The demyelination process also includes various myelin and non-myelin multiple "sclerosis" antigens, including myelin basic protein, myelin-associated glycoprotein, myelin oligodendrocyte glycoprotein, proteolipid, crystalline B , phosphodiesterase and Protein S-100, and other factors, including autoantibodies or cytokines

The damage caused by MS lesions in patients differs considerably in the structural and immunological characteristics, different pathogenic mechanisms are involved (Lucchinetti et al., 1996): lesions mediated by oligodendrocytes and myelin cytokines; digestion of superficial myelin antigens from macrophages; binding of antibodies to myelin and oligodendrocytes (i.e. antibody-dependent cytotoxicity); mediated accidents; and direct injury of oligodendrocytes from CD8 + T cells.

The result of the damage to the myelinic membrane is the presence of denuded axons which are no longer capable of efficiently transmitting the action potentials within the CNS due to the loss of saltatory conduction. This slow-down or blocking of the action potentially results in the production of neurological symptoms. The axons without the myelin sheath may be susceptible to further injuries caused by soluble injury mediators (i.e., cytokines, chemochins, complement proteins and proteases), resulting in irreversible axonal damage such as axonal transection and terminal axon ovoids.

Treatment options in Multiple Sclerosis

The diagnosis is made largely on clinical evidence, often employing a combination of magnetic resonance imaging (MRI), lumbar puncture (LP), serologic testing, and evoked potentials.

It is important to distinguish clinical relapses from the transient worsening of symptoms that may accompany an increase in body temperature or fatigue. Findings of recent disease activity do not invariably indicate an unfavorable long-term prognosis. Patients should limit their exposure to viral illnesses because infections may trigger relapses.

In 1993, the United States Food and Drug Administration (FDA) approved the first disease modifying drug (DMD) for the treatment of RRMS. Interferon beta-1b was a major milestone in MS therapy, serving as the first step in almost three decades of MS drug development. There are now thirteen FDA-approved DMDs for the treatment of RRMS, with several more agents in various stages of development. Interferons (Interferon ß) have been shown to reduce the rate of recurrence in one year (ARR) and the reduction of new MRI lesions by 50-80% in RRMS patients. This drug determines common side effects such as abnormal liver function, leukopenia, depression, flu-like symptoms, and site injection reactions (Paty et al., 1993). Another drug is Glatiramer acetate, approved by the FDA in 1996, has been shown to reduce recurrence rates by about one-third over a two-year period. A common adverse event associated with glatiramer acetate is allergic reactions in the injection site (20-40%). (Johnson et al., 2001). monoclonal antibodies have been remarkably successful, Natalizumab, the recombinant humanized monoclonal antibody, targets the alpha-4 subunit of beta-7 integrins, interfering with the migration of leukocytes through the blood-borne barrier. It has been shown to reduce 60% ARR (Polman et al., 2006; Rudick et al., 2006). Another humanized monoclonal antibody is Alemtuzumab, its mechanism of action is the binding to the membrane protein known such as CD52 present on lymphocytes B, resulting in cellular lysis followed by slow repopulation by non-influenced hematopoietic precursor cells. (Wiendl et al., 2013)

Among the drugs used, those that have been most successful are Interferons (Interferon ß). They have been shown to reduce the annualized recurrence rate (ARR) and the new 50-80% MRI lesions in RRMS patients. Interferons (Interferon ß) have been shown to reduce the annualized recurrence rate

(ARR) and the new 50-80% MRI lesions in RRMS patients among the most successful drug users. Common side effects were abnormalities of liver function, leukopenia, depression, flu-like symptoms, and injection site reactions (Paty et al., 1993). In 1996 the FDA approved the Glatiramer acetate, recidivism rates were reduced by about a third for a two-year period. A common adverse event associated with the treatment with glatiramer acetate was injection site reactions (20-40%). (Johnson et al., 2001). Natlizumab is a recombinant humanized monoclonal antibody, target site is the alpha-4 subunit of beta-7 integrins, interfering with the migration of leukocytes through the blood-brain barrier. It has been shown to reduce the 60% ARR (Polman et al., 2006; Rudick et al., 2006). Alemtuzumab is a humanized monoclonal antibody, its mechanism of action is the binding of the CD52 membrane protein with the resulting rupture of the lymphocyte cell, it is associated with a rapid decrease in circulating B and T lymphocytes, followed by slow repopulation of hematopoietic precursor cells not influenced. (Wiendl et al., 2013).

Among the drugs for oral use, Fingolimod and S1PR1 are a receptor of spingosine-1-phosphate (S1P), including S1PR1. The S1PR1 molecular pathway is essential for cell migration processes, particularly of lymphocyte subtypes expressing the CCR7 homing receptor such as the T Naïve cell and central memory, Th17 cells and B cells. Fingolimod interacts with S1PR1 , S1PR3 and S1PR5 expressed by several cells: neurons, astrocytes, oligodendrocytes and microglia, promoting neuroprotective and regenerative events such as migration of stem cell neurons, repair of receptor neurons, increased endogenous BDNF (*Brain-derived neurotrophic factor*) , progenitor oligodendrocytes and removal Gajofatto et al., 2015). Another drug, approved by the FDA in 2012, is Teriflunomide, it inhibits lymphocyte proliferation by blocking the new pyrimidine synthesis and blocking the cell cycle, thus exerting a cytotoxic effect on proliferation of T and B cells (Loffler et al. Ruckemann et al., 1998). Dimethyl fumarate modulates the activity of the immune system by stimulating the expression of NRF2, is a transcription factor that regulates the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation, approved by the FDA in 2013 for relapsing MS therapy. (Bomprezzi et al., 2015) .

Experimental Allergic Encephalomyelitis (EAE): a rodent model for MS

The complex pathophysiology of MS-characterized by CNS inflammation, demyelination and axonal injury-has made its modeling in experimental systems particularly problematic. Moreover, the evidence that MS does not naturally occur in other species has further complicated MS preclinical studies. Through the years, several MS in vivo models have been developed. The most common

experimental model for MS is ithe experimental autoimmune encephalomyelitis (EAE), that share with the human disease a variety of immunopathological and neuropathological mechanisms: inflammation, dementia, axonal loss and gliosis. Even in the EAE model all inflammatory response mechanisms such as the remielation process are manifested, which can also serve as a model for these processes. Thanks to the studies carried out on EAE models, several drugs have been developed. There is a great heterogeneity in the susceptibility to induction, the induction method, and the response to various immunological or neuropharmacological interventions. Although EAE has been instrumental in understanding the molecular events which take place upon neuroinflammation, not all MS hallmarks can be efficiently shaped within this conceptual frameshift. Thus, alternative models of CNS demyelination have been characterized, either based on viral infection or neurotoxin administration. However imperfect, these models have been able to recapitulate several clinical features of MS and greatly improved our knowledge about immune system functions in health and disease. On the other side, their intrinsic distance from MS has often led to misinterpreting and overestimating the data gleaned from these experimental systems. The fundamental difference between MS and EAE is that of the latter It requires an external immunization phase to be developed, while In humans, self-sensitization is not obvious Artificially induced (Gran et al., 2007).

Immunization with myelin antigens typically occurs through the use of adjuvant, Usually they contain bacterial components highly capable of activating the innate immune system through the Recognition Receptors Patterns (Libbey & Fujinami, 2010).

Aim of the study

Many of the clinical, histological, neurobiological and pharmacological characteristics of human MS can be observed in rodent EAE, a useful tool to investigate emerging therapeutic approaches (Mix E et al., 2008). Similar to human MS, EAE is an heterogeneous disease since various forms of EAE can be induced in susceptible animals depending on the immunizing neuroantigen, the species and the genetic background. For example, the Lewis rat EAE more closely mirrors acute and self-remitting immunoinflammatory events in the CNS and represents a very reliable and reproducible model for therapy studies, relapsing and remitting forms of EAE such as those observed in Swiss-Jackson Laboratories (SJL) mice and Dark Agouti (DA) rats may better represent the corresponding RR form of MS, with the latter strictly mimicking histopathology of multiple sclerosis, and myelin oligodendrocyte protein (MOG)-induced EAE that with its chronic progressive course may resemble the SP/PP forms of MS.

Even though caution must be exercised when translating pathogenic concepts and pharmacological data from rodent EAE to human MS, it is generally accepted that rodent EAE models represent important in vivo tools to gain insight into MS-like immunopathogenic mechanisms and the modes of action of present and future therapies (Steinman L and Zamvil SS, 2006).

There are major differences between EAE and MS, since EAE is induced by active sensitization with brain tissue antigens and makes use of strong immune adjuvant eliciting a pathophysiological response, and since EAE is mainly studied in inbred animals (Gold R et al., 2006).

Nonetheless, most of our current knowledge regarding principal mechanisms of brain inflammation has been gathered from studies on EAE and drugs of proven efficacy in MS such as glucocorticoids, mitoxantrone and glatiramer acetate- The murine equivalent monoclonal antibody of natalizumab, IFN-beta and cyclophosphamide are at least effective in one of these 4 rodent models (Mix E et al., 2008; Mangano K et al., 2009; Donia M et al., 2010). However, given the heterogeneity of MS and EAE as well, it is obvious that there is no single definitive EAE model, but rather a combination of different approaches that could eventually help us to develop new and more effective therapeutic approaches (Gold R et al., 2006). Thus, EAE represents an excellent tool for studying basic mechanisms of brain inflammation and immune-mediated CNS tissue injury and to obtain proof of principle studies on whether a certain therapeutic strategy has the potential to block immunopathogenic pathways in MS patient subpopulations.

The present study aims for a better elucidation of the involvement of MIF in modulating the encephalitogenic immune responses underlying MS and to the development of anti-MIF based therapeutic strategies.

Materials and methods

Microarray analysis

Gene Ontology analysis on MIF knockout cells

A global transcriptomic profiling of MIF knockout cells was obtained from the publicly available dataset GSE10241. Briefly, HEK293 cells were transfected with either MIF siRNA (100pmol/ml) or control RNA using Lipofectamine^{TM}2000 reagent and incubated for 24 hours, before isolation of total RNA. Transcriptional analysis was carried out using the Illumina human-6 v2.0 expression beadchip platoform. Details on the experimental design can be retrieved form the relative publication (Liu et al., 2008). Gene Ontology analysis for the category "Biological Process" was performed separately on the significant up- and downregulated genes using the web-based utility Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov).

Analysis of CD4 T cells from preclinical models of MS

In order to evaluate the transcriptional levels of genes of interest in encephalitoenic T cells from preclinical models of MS, the GSE57098 and GSE38645 datasets were selected. GSE57098 included data from CD4+ 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. The Agilent-026655 Whole Mouse Genome Microarray 4x44K v2 platform was used for the generation of the transcriptional profile of the samples. For the GSE38645 dataset, ex vitro myelin specific T cells (blasts and resting state, day 2 and 7 after antigen challenge respectively) or isolated from the spleen (3 days p.t.) derived from adoptive transfer EAE Lewis rats. Details on the experimental design can be retrieved form the relative publications (Hoppmann et al., 2015; Odoardi et al., 2012)

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. Data on genes of interest were evaluated from unstimulated cells and following 24h incubation with anti-CD3/CD28 antibodies. Data from 14 patients and 14 control subjects are included. The Agilent-039494 SurePrint G3 Human GE v2 8x60K Microarray platoform was used (Hellberg et al., 2016).

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 obtained from PBMCs using the Affymetrix Human Genome U133A 2.0 Array (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 frequency of acute relapses.

Dataset selection for the analysis of oligodendrocyte damage and remyelination processes

Expression datasets were obtained from the NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/). GSE60847, GSE38010 and GSE48872 were chosen for the analysis. 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). GSE48872 included gene expression profiles from adult OPCs 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 previously treated for 5 weeks with cuprizone (0.2%). Adult oligodendrocytes (OLs) were obtained from brains of 2-month-old mice (Moyon et al., 2015). Total RNA from Acute plaques, Chronic-Active plaque and Chronic plaque from MS brains and white matter from healthy controls was used for GSE38010 and the Affymetrix Human Genome U133 Plus 2.0 Array was used for assessment of transcriptional differences (Han et al., 2012).

Ex vivo and in vitro analysis

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 Animal Plant of the Department of Biomedical Sciences and Biotechnology, General Pathology Section, Catania, Italy. They were kept under standard laboratory conditions (non-specific for pathogens) with free access to food (Harlan Global Diet 2018) and water and were allowed to adapt to their environment for at least a week before starting the study. Automatically controlled ambient conditions had been set to maintain the temperature at 20 - 24 ° C with a relative humidity (RH) of 30-70%, 10-30 variations of air per hour and a natural light cycle: dark. The protection of animals used in the experiment complies with Directive 86/609 / EEC, implemented by D.Lgs. 26/2014.

Induction of EAE induced by MOG in C57BL / 6 mice.

MOG35-55 was synthesized by Genemed Synthesis Inc (San Francisco CA). The mice were immunized with 200 ug of MOG emulsified in CFA with 1 mg of Mycobacterium tuberculosis H37RA (Difco, Detroit, MI, USA). Each mouse received subcutaneous injections of 200 ul of emulsion subdivided between two sites that drain into axillary lymph nodes. Pertussis toxin (Calbiochem, Nottingham, UK) was used as co-adjuvant and was administered i.p. at the dose of 200 ng / mouse on days 0 and 2 after immunization. Mice were observed daily by measuring their body weights and clinical signs of EAE. Clinical classification was performed by an obscure observer of the treatment: 0 = no sign of illness; 0.5 = Partial paralysis of the tail; 1 = paralysis of the tail; 1.5 = tail paralysis + indirect unilateral frontal paralysis; 2 = tail paralysis + back weakness or partial paralysis back; 2.5 = tail paralysis + partial paralysis of the back (lowered pelvis); 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.

Ex vivo restimulation of splenocytes with MOG35-55

At the 14th day after EAE induction, spleens and lymph nodes were harvested from C57BL/6 mice. Cell suspensions were prepared by grinding the organs with the plunger of a 5 ml disposable syringe 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.

Cells were pelleted and washed twice with PBS. Cells were then resuspended at 2 × 106 cells/mL in complete medium and re-restimulated with 40 µg/mL of MOG or Concanavalin A (ConA; 10 µg/ml, Sigma Aldrich St. Louis MO, USA).

Determination of TNF-alpha in cell culture supernatant s

Splenocytes were plated in triplicate in 24-well microtiter plates at a concentration of $2*10^6$ cells/well, in the same medium, stimulated with MOG35-55 (40 µg/ml) and incubated for 48h 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 concentrations by sandwich ELISA, using a commercially available kit (R&D systems, Minneapolis, MN, USA).

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.

RESULTS

Effects of MIF Knocking out on cellular processes

In order to ascertain the effects of MIF inhibition on cellular functions, gene ontology analysis for the most enriched Biological Processes (BPs) was performed on the significantly downregulated and upregulated genes upon MIF siRNA treatment of HEK293 cells. The most represented BPs by the downregulated genes resulted to be: protein folding (GO:0006457); negative regulation of hippo signaling (GO:0035331); negative regulation of myeloid leukocyte differentiation (GO:0002762) and maintenance of centrosome location (GO:0051661) (Figure 1). Gene that resulted upregulated upon MIF inhibition were instead involved in extracellular matrix organization (GO:0030198); response to interleukin-6 (GO:0070741); semaphorin-plexin signaling pathway (GO:0071526); blood vessel remodeling (GO:0001974); luteinizing hormone secretion (GO:0032275) and positive regulation of neuron differentiation (GO:0045666) (Figure 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 ($p<0.01$) (Figure 2). No significant differences were instead observed in the levels of DDT (Figure 2). 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) (Figure 2). Similarly, the two co-receptors CXCR2 and CXCR4 were markedly upregulated in the CNS-infiltrated cells, entailing a strong statistical significance ($p<0.01$ for both genes) (Figure 2).

Evaluation of MIF and its receptors in encephalitogenic cells in different activation states

Next, we wanted to determine the expression levels of MIF, the homologue DDT and receptors in myelin-specific T cells from a rat model of adoptive-transfer EAE, in three different activation states: resting state; upon ex vivo activation and ex vivo sorted from the spleen (migratory phenotype). A significant higher level of MIF expression was observed in both resting and activated T cells as compared to spleen-derived encephalitogenic T cells ($p<0.001$ and $p<0.05$, respectively) (Figure 3). No variations among the different activation states were observed as regards DDT transcriptional levels (Figure 3). Resting T cells expressed lower levels of CD74 as compared to the activated T cells ($p<0.01$) and the migratory spleen-sorted cells ($p<0.05$) (Figure 3). On the other hand, CD44 levels were found to be significantly lower than those in resting ($p<0.001$) and migratory cells ($p<0.001$) ($p<0.001$). Significantly higher levels of CXCR2 ($p<0.05$) and CXCR4 ($p<0.001$) were observed in the migratory cells as compared to both resting and activated cells (Figure 3).

Analysis of circulating CD4 T cells in Multiple Sclerosis 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 can be observed between the two groups of people, with the only exception for CD44 (Figure 4). Indeed, basal CD44 levels resulted to be significantly higher in the CD4 T cells from MS patients as compared to those from the healthy donors ($p<0.05$) (Figure 4).

In addition, it was observed a marked upregulation of MIF and CD74 upon anti-CD3/CD28 activation of the cells from both groups of people, entailing a strong statistical significance ($p<0.01$ and $p<0.001$, for MIF and CD74, respectively) (Figure 4). On the other hand, CD44 levels slightly decreased following T cell activation, reaching the statistical significance only in the MS group ($p<0.05$) (Figure 4). No modulation could be observed for DDT, CXCR2 and CXCR4 (Figure 4).

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

In the current analysis, we wanted to determine whether the different expression levels of MIF and its receptors in PBMCs from MS patients could either promote or confer protection from acute relapses. Data from 62 non-treated patients with definite MS were included. 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 (Figure 5). However, a trend of protection from relapses (p=0.079) was observed in the patients expressing lower levels of DDT and higher levels of CD44 (p=0.1029) (Figure 5).

Effects of ISO-1 on MOG-specific encephalitogenic cells

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. ISO-1 treatment was associated to dose-dependent reduction MOG-specific proliferation, reaching a ~40% reduction at the concentration of 100 nM, as compared to vehicle-treated MOG-stimulated splenocytes (Figure 6).

MOG-restimulation of splenocytes form EAE-affected mice was associated to a significant upregulation of TNF-alpha production, as detected by ELISA. ISO-1 treatment dose-dependently inhibited TNF-alpha secretion, entailing a strong statistical at all the three tested concentrations (25 nM, p=0.0496; 50 nM, p=0.0111; 100 nM, p=0.0071) (Figure 5).

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 spinal cords of EAE-affected mice, no significant modulation in MIF and DDT expression was observed, as compared to healthy control mice (Figure 6). A significant increase in CD74 ($p<0.01$) and 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 (Figure 6).

Along the same lines, CD74 and CD44 were found to be 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 (Figure 7).

Higher levels of CD74, CD44 and CXCR4 could also be observed in the Active plaques from brain of MS patients, with no substantial modulation of either MIF or DDT genes. On the other hand, a downregulation of CD74, CD44 and CXCR4 was observed in both the Chronic and Chronic-Active plaques as compared to normal control brains (Figure 8). Due to the low number of replicates, the statistical significance was not reached in any of the comparisons

Conclusions

Multiple sclerosis (MS) is one of the most common chronic inflammatory diseases of the central nervous system leading to demyelination and neurodegeneration. MS is a variable condition and the symptoms depend on which areas of the central nervous system have been affected. There is no set pattern to MS and every patients has a different set of symptoms, which vary from time to time and can change in severity and duration, even in the same person. Increasing body of data supports the hypothesis that MIF is located at an upstream position in the events leading to possible dysregulated immunoinflammatory responses leading to autoimmune reactions and may therefore represent a new potential target for the treatment of autoimmune diseases. Indeed, it has been reported in independent studies that MIF plays a key role in the pathogenesis of both organ-specific and systemic autoimmune diseases (Stosic-Grujicic et al., 2009).

Macrophage migration inhibitory factor (MIF) is a cytokine with pleiotropic actions associated to clinical worsening and relapses in MS patients. However, the mechanism through which MIF promotes MS progression remains debated. In a previous study, it has been shown that MIF plays a role in regulating CNS effector mechanisms necessary for the development of a mouse model of MS, the experimental autoimmune encephalomyelitis (EAE) (Cox et al., 2013). Indeed, despite the ability to generate pathogenic myelin-specific immune responses peripherally, MIF-deficient mice have been

shown to undergo reduced EAE severity, with a greater percentage of resting microglia and fewer infiltrating inflammatory macrophages. In addition, intraspinal stereotaxic microinjection of MIF resulted in an upregulation of inflammatory mediators in microglia, which was sufficient to restore EAE-mediated inflammatory pathology in MIF-deficient mice (Cox et al., 2013). As far as the EAE model is concerned, we must emphasize the contribution to the development, validation and testing of MS drugs and, even more so, to the understanding of MS pathogenesis.

Drugs targeting the MIF pathway are currently under extensively studied for their possible use as cancer chemotherapeutics and as immunosuppressive agents, although only a limited number of clinical trials are ongoing to evaluate their effectiveness in the context of immunological disorders.

In the present work, we have first performed an in silico study to evaluate the involvement of MIF, DDT, CD74, CD44, CXCR2 and CXCR4 in MS and oligodendrocytes function. Subsequently, we have tested ex vivo the potential immunomodulatory action of a MIF inhibitor on MOG-induced EAE. In order to provide consistent results, we have generated data from both preclinical models of MS, and MS patients.

We show here, that MIF and its receptor CD74 are significantly upregulated upon T cell activation, as it can be observed in both the MOG-induced EAE model and in circulating CD4 T cells from MS patients. In addition, we show that higher levels of CD74 in PBMCs may predispose to earlier clinical relapses and that, a trend of protection from relapses was observed in the patients expressing lower levels of DDT and higher levels of CD44.

We have also determined the transcriptomic levels of MIF receptors in MS-associated CNS lesions. In spinal cords of EAE-affected mice was observed a significant increase in CD74 and CD44 and CXCR4 compared to control samples and a similar trend of increase was observed for CXCR2. Along the same lines, CD74 and CD44 were found to be significantly increased in adult oligodendrocyte precursors from cuprizone-challenged animals with respect to control adult oligodendrocyte precursors.

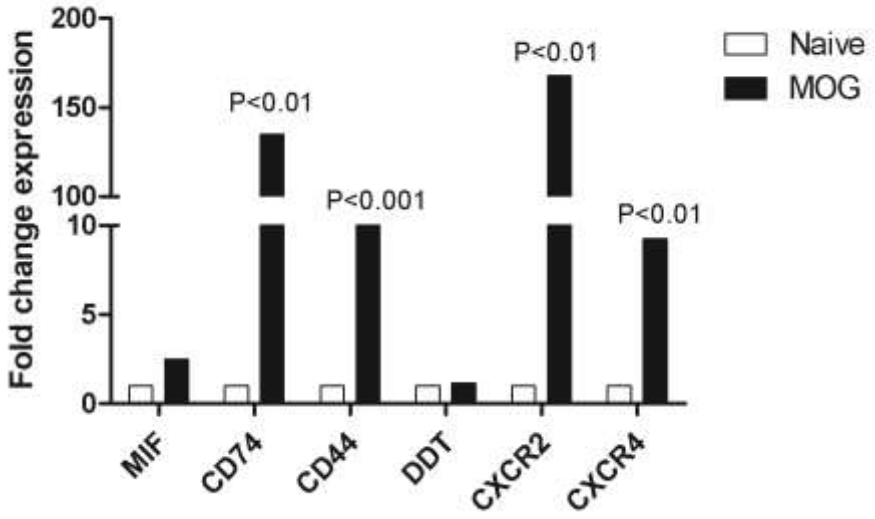
We also observed higher levels of CD74, CD44 and CXCR4 in the active plaques from brain of MS patients. When we considered chronic and chronic-active plaques compared to normal control brain, a downregulation of CD74, CD44 and CXCR4 was observed

Overall, our data indicate that there is a significant involvement of MIF in MS ethiopathogenesis and that treatment with ISO-1 may be a useful therapeutic approach in the clinical setting. In particular, we show that ISO-1 is able to reduce antigen-specific proliferation and TNF-alpha secretion from

encephalitogenic T cells. This is particularly relevant as anti-MIF therapy is under clinical development for solid tumors (NCT01765790) and lupus nephritis (NCT01541670). Also, anti-CD74 antibodies are available and tested in different conditions, such as B Cell Malignancies (NCT00504972), Refractory Chronic Lymphocytic Leukemia (NCT00868478) and Non-Hodgkin Lymphoma (NCT00989586). Inhibitors of CXCR4, such as BMS-936564 and BL-8040, are also listed in the ClinicalTrials.gov website for their testing in Multiple Myeloma and Chronic Myelogenous Leukemia.

As described above, MIF exerts various pro-inflammatory actions and is involved in the pathogenesis of inflammatory and autoimmune diseases. Among the therapeutic therapies used to counter these pathologies, the most promising therapy is the anti-cytokine immunopharmacological strategy, neutralization of MIF with either anti-MIF antibody, or the chemically derived inhibitors of MIF enzymatic activity.

In addition, it is known that glucocorticoid activity is closely related to MIF and inhibition of MIF could therefore lead to the enhancement of anti-inflammatory actions of glucocorticoids, with consequent reduction of inflammation. Direct cytokine neutralization has generally been shown to be more effective than inhibition of downstream effects of the cytokine in question. The importance of the preclinical studies presented here is to identify immunopathogenic pathways that can be pharmacologically targeted in the absence of visible side effects or toxicities. As the efficacy and safety of MIF inhibitors increase, these drugs may find therapeutic applications in the treatment of immunoinflammatory disorders. MIF neutralization showed beneficial effects almost exclusively in the treatment of inflammatory conditions in animal models but despite the application being limited to this kind of experiments, the data obtained provide a good starting point for the future application in treating such disorders in humans. However, more potent and specific inhibitors targeting MIF or its downstream effects are needed for the development of novel pharmaceutical therapies for MIF-associated diseases, such as MS.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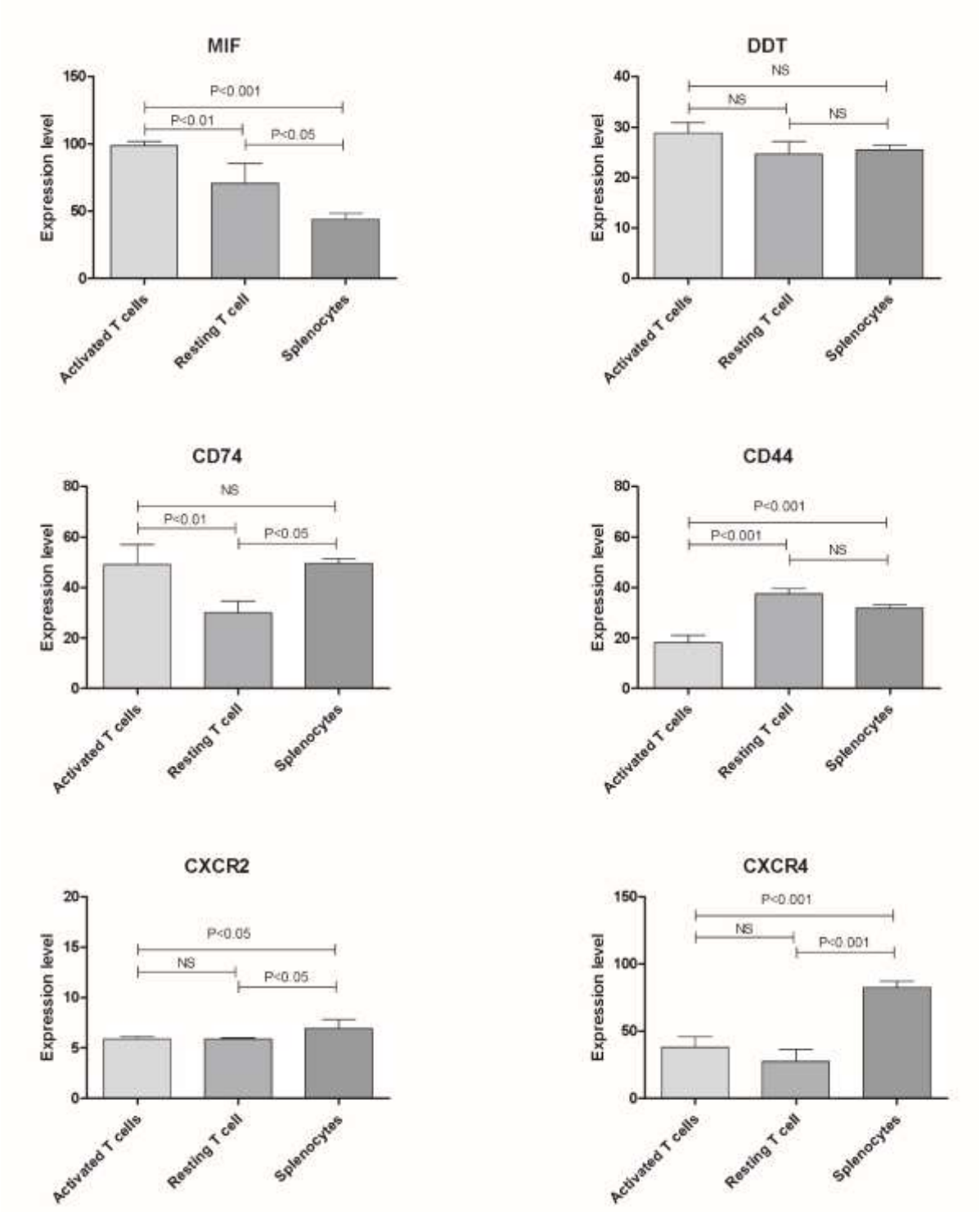
200
150
100
10
5
0
P<0.01
P<0.001
P<0.01
P<0.01
Naive
MOG
MIF
CD74
CD44
DDT
CXCR2
CXCR4

Figure 1.


MIF
Expression level
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100
50
0
P<0.001
P<0.01
P<0.05
Activated T cells
Resting T cell
Splenocytes
DDT
Expression level
40
30
20
10
0
NS
NS
NS
Activated T cells
Resting T cell
Splenocytes
CD74
Expression level
80
60
40
20
0
NS
P<0.01
P<0.05
Activated T cells
Resting T cell
Splenocytes
CD44
Expression level
80
60
40
20
0
P<0.001
P<0.001
NS
Activated T cells
Resting T cell
Splenocytes
CXCR2
Expression level
20
15
10
5
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P<0.05
NS
P<0.05
Activated T cells
Resting T cell
Splenocytes
CXCR4
Expression level
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P<0.001
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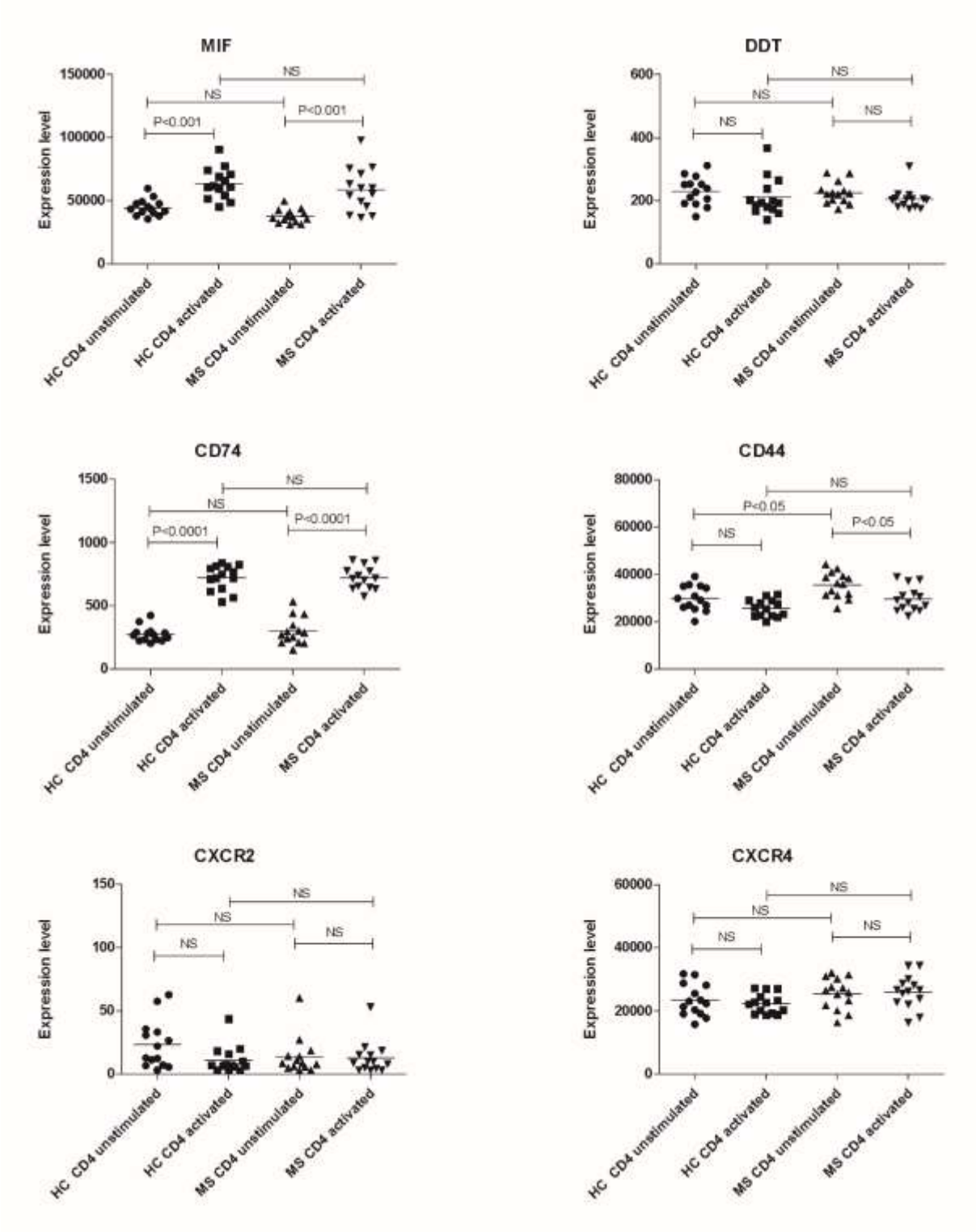
P<0.001
Activated T cells
Resting T cell
Splenocytes

Figure 2.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Expression level
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50000
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NS
NS
P<0.001
P<0.001
HC CD4 unstimulated
HC CD4 activated
MS CD4 unstimulated
MS CD4 activated
DDT
Expression level
600
400
200
0
NS
NS
NS
HC CD4 unstimulated
HC CD4 activated
MS CD4 unstimulated
MS CD4 activated
CD74
Expression level
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1000
500
0
NS
NS
P<0.0001
P<0.0001
HC CD4 unstimulated
HC CD4 activated
MS CD4 unstimulated
MS CD4 activated
CD44
Expression level
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60000
40000
20000
0
NS
P<0.05
NS
P<0.05
HC CD4 unstimulated
HC CD4 activated
MS CD4 unstimulated
MS CD4 activated
CXCR2
Expression level
150
100
50
0
NS
NS
NS
NS
HC CD4 unstimulated
HC CD4 activated
MS CD4 unstimulated
MS CD4 activated
CXCR4
Expression level
60000
40000
20000
0
NS
NS
NS
NS
HC CD4 unstimulated
HC CD4 activated
MS CD4 unstimulated
MS CD4 activated

Figure 3

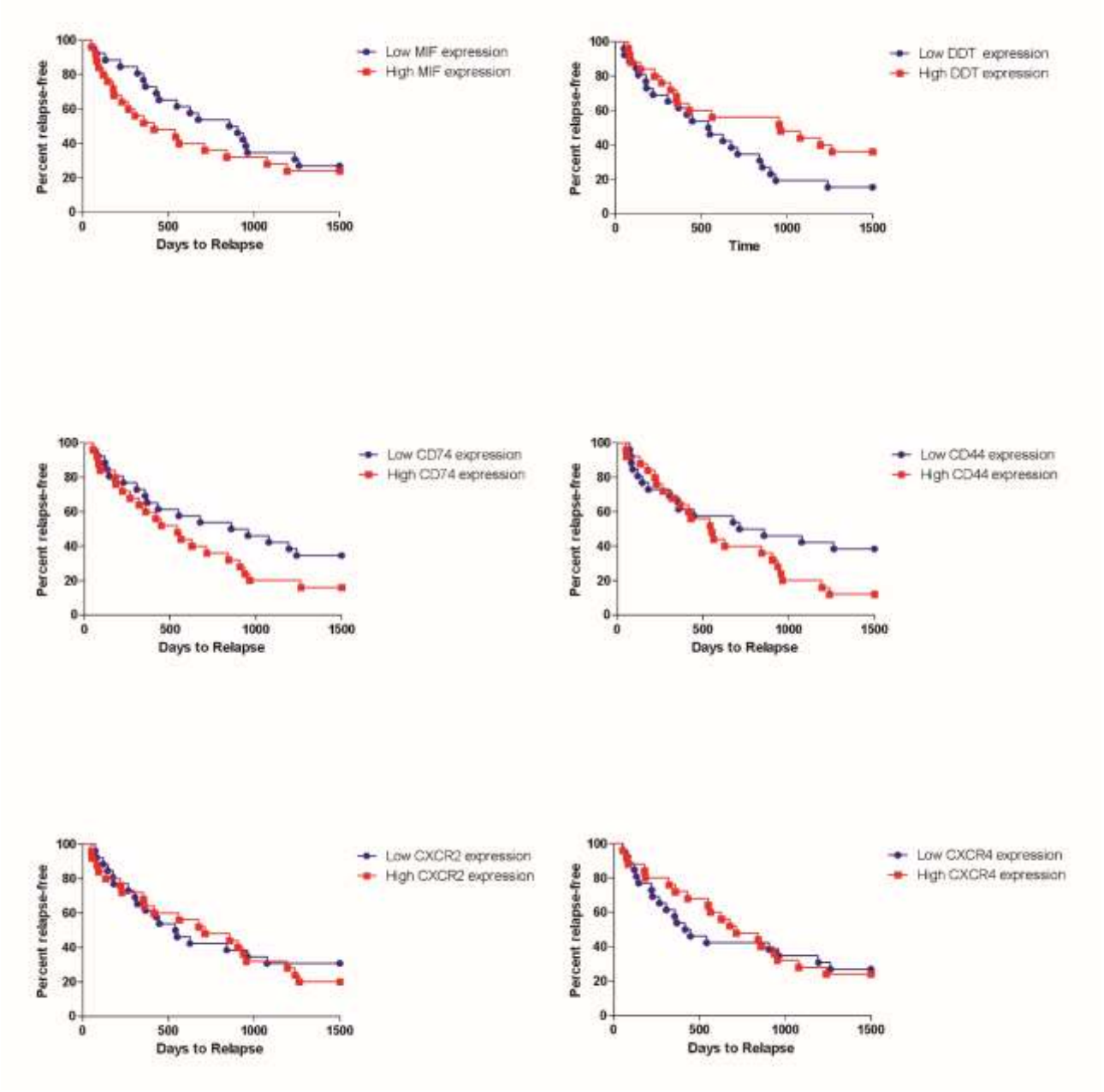
Percent relapse-free
Days to Relapse
Low MIF expression
High MIF expression
Time
Low DDT expression
High DDT expression
Low CD74 expression
High CD74 expression
Low CD44 expression
High CD44 expression
Low CXCR2 expression
High CXCR2 expression
Low CXCR4 expression
High CXCR4 expression

Figure 4

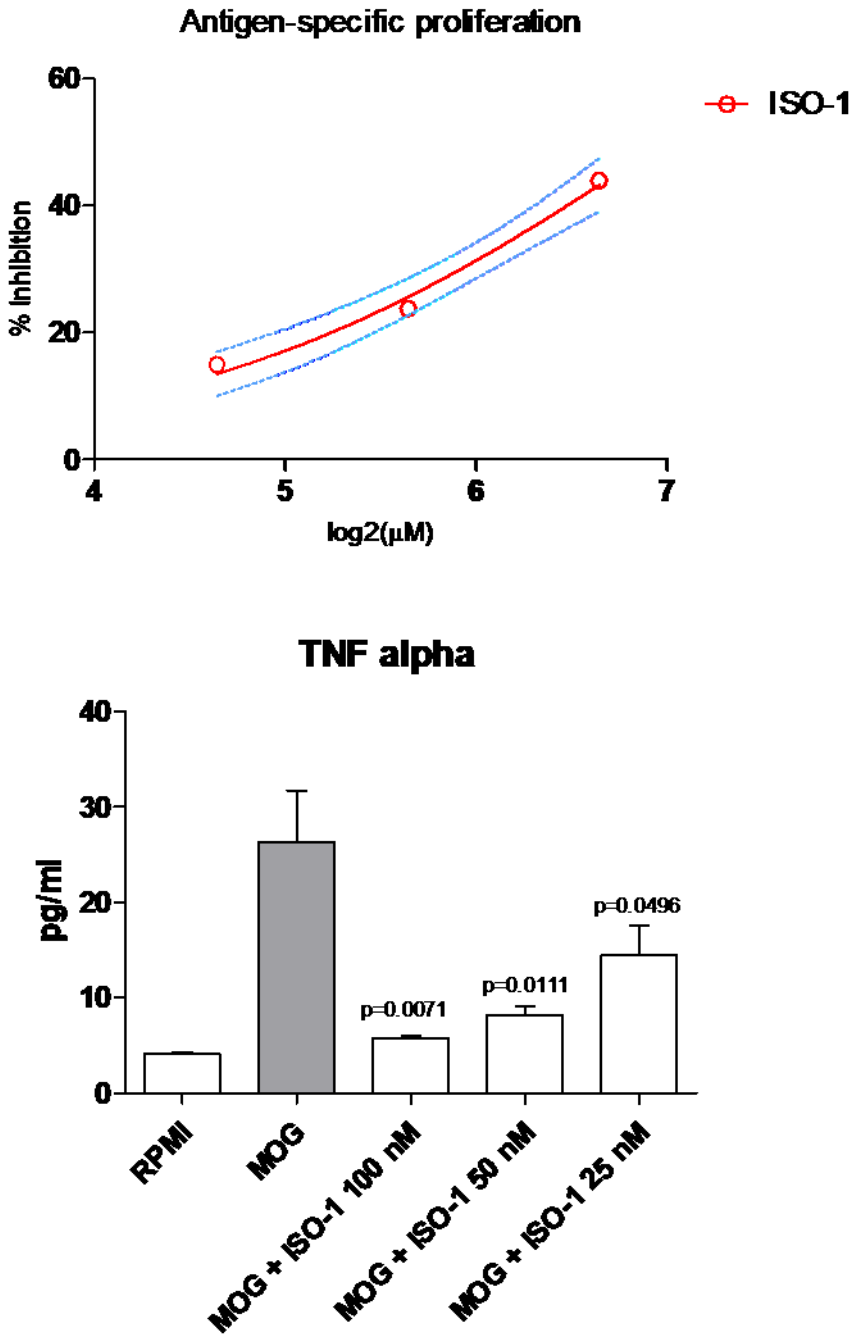
Antigen-specific proliferation
ISO-1
% Inhibition
60
40
20
0
4
5
6
7
log2(µM)
TNF alpha
pg/ml
40
30
20
10
0
p=0.0071
p=0.0111
p=0.0496
RPMI
MOG
MOG + ISO-1 100 nM
MOG + ISO-1 50 nM
MOG + ISO-1 25 nM

Figure 5

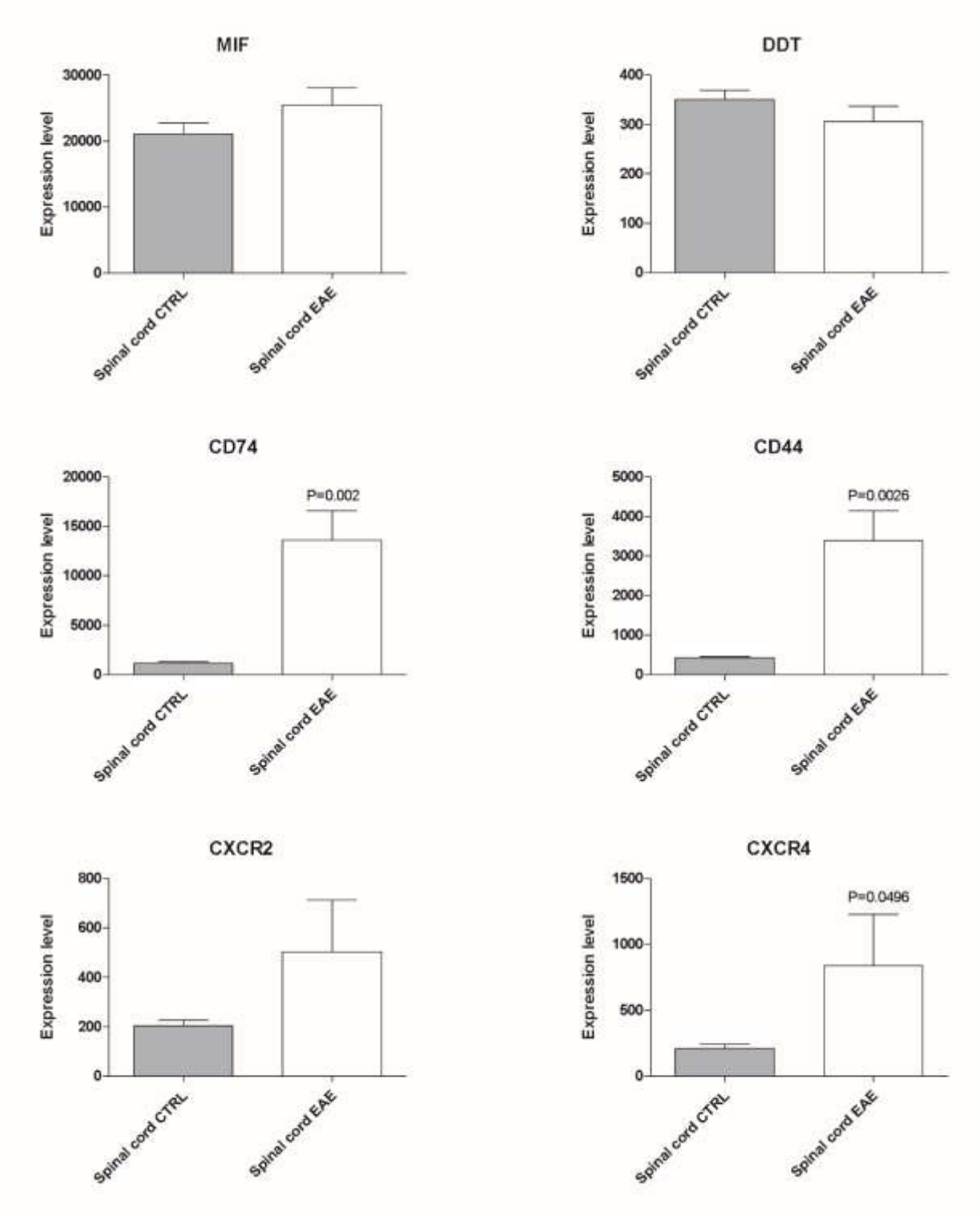
MIF
Expression level
30000
20000
10000
0
Spinal cord CTRL
Spinal cord EAE
DDT
Expression level
400
300
200
100
0
Spinal cord CTRL
Spinal cord EAE
CD74
Expression level
20000
15000
10000
5000
0
P=0.002
Spinal cord CTRL
Spinal cord EAE
CD44
Expression level
5000
4000
3000
2000
1000
0
P=0.0026
Spinal cord CTRL
Spinal cord EAE
CXCR2
Expression level
800
600
400
200
0
Spinal cord CTRL
Spinal cord EAE
CXCR4
Expression level
1500
1000
500
0
P=0.0496
Spinal cord CTRL
Spinal cord EAE

Figure 6

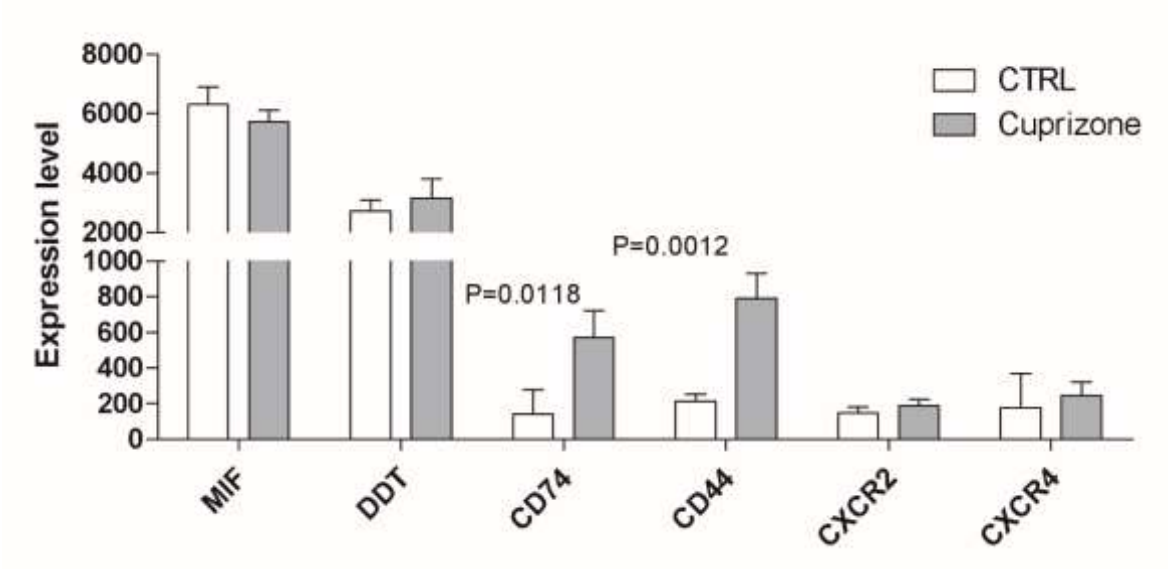
Expression level
8000
6000
4000
2000
1000
800
600
400
200
0
CTRL
Cuprizone
P=0.0118
P=0.0012
MIF
DDT
CD74
CD44
CXCR2
CXCR4

Figure 7

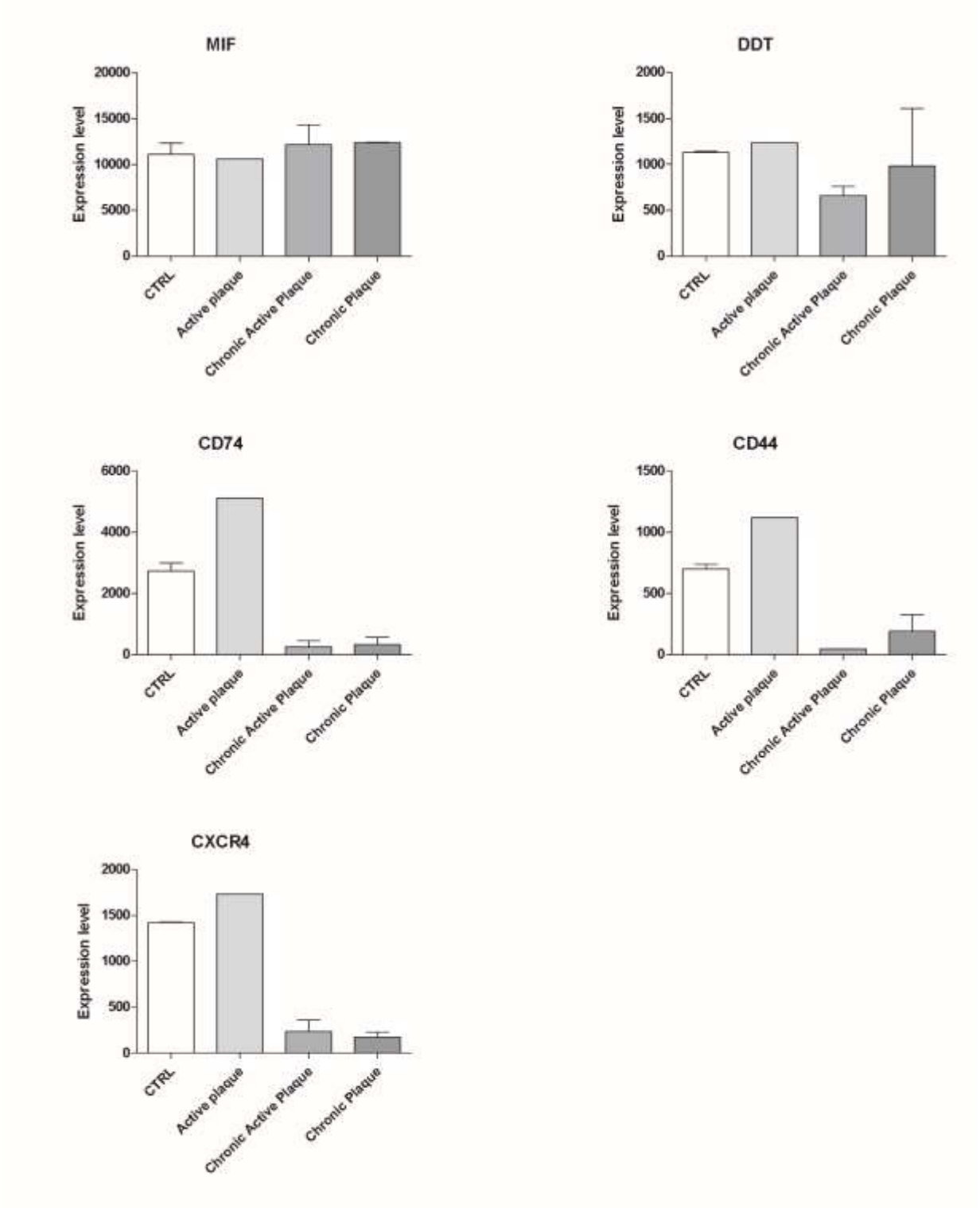
MIF
Expression level
20000
15000
10000
5000
0
CTRL
Active plaque
Chronic Active Plaque
Chronic Plaque
DDT
Expression level
2000
1500
1000
500
0
CTRL
Active plaque
Chronic Active Plaque
Chronic Plaque
CD74
Expression level
6000
4000
2000
0
CTRL
Active plaque
Chronic Active Plaque
Chronic Plaque
CD44
Expression level
1500
1000
500
0
CTRL
Active plaque
Chronic Active Plaque
Chronic Plaque
CXCR4
Expression level
2000
1500
1000
500
0
CTRL
Active plaque
Chronic Active Plaque
Chronic Plaque

Figure 8

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