

No association of leukemia inhibitory factor (LIF) DNA polymorphisms  
with multiple sclerosis

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## **Effects of IFN- $\beta$ , leptin and simvastatin on LIF secretion by T lymphocytes of MS patients and healthy controls**

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**Running title: Effect of IFN- $\beta$ , simvastatin and leptin on LIF secretion**

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## **ABSTRACT**

In Multiple Sclerosis (MS), oligodendrocyte injury is believed to be caused by an aberrant immune response initiated by autoreactive T cells. Increasing evidence indicates that inflammatory responses in the central nervous system are not exclusively detrimental, but may also exert protective effects. Such protective effects are potentially mediated by the local secretion of neurotrophic factors by immune cells. We previously reported that T cells and monocytes in vitro and in inflammatory MS lesions produce leukemia inhibitory factor (LIF), a member of the neurotrophic family of neurotrophins. In the present study we report a reduced LIF production by CD4<sup>+</sup> T cells of relapsing remitting MS patient as compared to healthy controls. Furthermore, immunomodulatory agents such as leptin, IFN- $\beta$  and simvastatin were studied for their potential to alter LIF and secretion of other cytokines by T cells and monocytes of relapsing remitting MS patients and healthy controls. Low doses of simvastatin, but not IFN- $\beta$  or leptin enhanced LIF secretion by CD4<sup>+</sup> T cells of RR-MS patients. We further demonstrated that LIF did not influence viability, proliferation and cytokine secretion of T cells. Together these data provide new information on the regulation of LIF secretion by immune cells. Further insights into the complex regulation of neurotrophic factors such as LIF may prove useful for treatment of MS.

**Keywords:** multiple sclerosis, neuroprotection, leukemia inhibitory factor, immunotherapy

## 1. INTRODUCTION

Multiple sclerosis has long been viewed as a chronic inflammatory disease of the central nervous system in which the patient's own immune cells mediate injury to myelin and oligodendrocytes. The resulting demyelination causes neuronal dysfunction leading to neuronal loss (Pouly & Antel, 1999; Pouly *et al.*, 2000). From passive transfer studies in animal models, it is well established that T cells reactive to CNS antigens are able to induce disease (Ben Nun *et al.*, 1981; Pettinelli & McFarlin, 1981) and are directly involved in damaging CNS resident cells (Dandekar *et al.*, 2001; Giuliani *et al.*, 2003). However, during the last decade a number of independent observations caused a shift in the perception of the role of the immune system in CNS pathology. Schwartz and colleagues showed that injection of activated T cells specific to a CNS antigen (myelin basic protein, MBP), reduced secondary degeneration of neurons after a primary crush injury of CNS axons (Moalem *et al.*, 1999). A neuroprotective role for myelin reactive T cells was further illustrated in several rodent models of stroke, optic nerve injury and spinal cord trauma (Yoles *et al.*, 2001; Barouch & Schwartz, 2002; Moalem *et al.*, 1999; Hammarberg *et al.*, 2000; Frenkel *et al.*, 2005). Also in humans there are indications for a protective role of CNS specific T cells. These T cells expand upon non-specific CNS insults such as viral infections (Miller *et al.*, 1997), stroke (Wang *et al.*, 1992) and peripheral nerve trauma (Olsson *et al.*, 1992). Together, these observations have led to the concept of protective autoimmunity. This paradigm states that autoimmunity is beneficial and is only disease promoting when it is dysregulated (Moalem *et al.*, 1999; Schwartz & Kipnis, 2002).

The mechanisms underlying the neuroprotective effect of T cells have not yet been established. Withdrawal of growth factors and deprivation of neurotrophic support is a well supported mechanism leading to death of neurons and glial cells in CNS injuries and neurodegenerative diseases (Semkova & Kriegstein, 1999; Novikov *et al.*, 1995). Therefore, it has been suggested that myelin reactive T cells may exert their protective effect, at least in part, by local production of various factors such as anti-inflammatory cytokines and neurotrophins. Autoreactive T cells have been shown to secrete BDNF, NGF, NT-3 and NT-4/5 *in vitro* (Moalem *et al.*, 2000). BDNF was also found to be produced by T cells and macrophages in inflammatory brain lesions (Kerschensteiner *et al.*, 1999a). Hammerberg and colleagues demonstrated that neurodamaging effects of T cell derived cytokines such as TNF- $\alpha$  and IFN- $\gamma$  can be halted by the production of neurotrophic factors by these same cells

(Hammarberg *et al.*, 2000). We have recently reported that autoreactive T cells secrete leukemia inhibitory factor (LIF), a family member of the neuropoietic factors. We also showed the presence of LIF producing T cells in both active and chronic active lesions (Vanderlocht *et al.*, 2006). Butzkueven and coworkers further demonstrated that LIF can ameliorate experimental autoimmune encephalomyelitis (EAE), an animal model for MS by promoting survival of oligodendrocytes *in vivo* (Butzkueven *et al.*, 2002). In line with these results we showed that LIF dose dependently protects oligodendrocytes against TNF- $\alpha$  induced apoptosis *in vitro* (Vanderlocht *et al.*, 2006) as was reported previously for the neurokine ciliary neurotrophic factor (CNTF) (D'Souza *et al.*, 1996).

Due to the impermeability of the blood brain barrier, the delivery of sufficient amounts of neurotrophins to CNS lesions has hampered the therapeutic application of neurotrophic factors (Sagot *et al.*, 1997). The identification of agents that stimulate the local production of neurotrophic factors such as neurokines and neurotrophins of the NGF/NT family by T cells may lead to new tools for MS therapy. In this study we demonstrate that LIF does not affect the viability, proliferation and cytokine secretion of T cells. We further observed a reduced LIF secretion by peripheral blood CD4<sup>+</sup> T cells of relapsing remitting MS patients as compared to secondary progressive MS patients and healthy individuals. We tested whether immunomodulatory agents with therapeutic potential for MS, such as IFN- $\beta$  and simvastatin are able to enhance LIF secretion by CD4<sup>+</sup> T cells of MS patients. We further evaluated the effect of leptin on LIF secretion by immune cells. Leptin, initially identified as a hormone regulating appetite and body weight (Friedman & Halaas, 1998), is implicated in the induction and progression of EAE (Matarese *et al.*, 2001b; Matarese *et al.*, 2001a) and the regulation of immune and anti-inflammatory responses (Loffreda *et al.*, 1998; Lord *et al.*, 1998). Leptin stimulates LIF secretion by endometrial cells and a detailed analysis of gene transcripts in brain lesions from MS patients showed elevated leptin transcripts (Lock *et al.*, 2002b; Steinman & Zamvil, 2003). Therefore, we tested whether leptin is able to stimulate LIF secretion by immune cells *in vitro*.

## **2. MATERIALS AND METHODS**

### **Patients and healthy controls**

Heparinized venous blood samples were collected from 12 women and 9 men with clinically definite MS. Thirteen patients had a relapsing remitting disease course, the remaining eight showed a progressive disease course. Four subjects were received medication at the time of blood sampling. Two RR-MS patients received IFN- $\beta$ , 1 RR-MS patient received glatiramer acetate (GA) and 1 secondary progressive MS patient received methotrexate (MTX). Table 1 provides an overview of the characteristics of these MS patients. In addition blood was drawn from 16 healthy control subjects with a mean age of 29. Informed consent was obtained from all subjects volunteering for this study.

### **Reagents**

Human recombinant leptin, LIF and IFN- $\beta$  were obtained from R&D systems (Minneapolis, MN), Peprotech (London, UK) and PBL biomedical laboratories (Piscataway, NJ, USA) respectively. Simvastatin was purchased from Calbiochem (Bad Soden, Germany) and lipopolysaccharide (LPS) and phytohemagglutinine (PHA) from Sigma Aldrich (St Louis, MO, USA). All antibodies except anti-CD190 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and the swine-anti-rabbit Ab (Dako Diagnostics, Glostrup, Danmark) were purchased from BD biosciences (Erembodegem, Belgium). Cells were cultured in RPMI 1640 (Life Technologies, Invitrogen), supplemented with 10% fetal calf serum (FCS) (Hyclone Europe, Erembodegem, Belgium), 0.1 mM non essential amino acids, 1 mM sodium pyruvate and the antibiotics penicillin (50 U/ml) and streptomycin (50  $\mu$ g/ml).

### **LIF secretion by CD4<sup>+</sup> T cells**

Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation using Ficoll Hypaque (Pharmacia, Sweden). PBMC ( $2-3 \times 10^8$ ) were incubated for 30 min. with anti-CD4-FITC labeled antibodies (BD, Erembodegem, Belgium) at 4°C. CD4<sup>+</sup> T cells were isolated directly from PBMC by means of high speed FACS-sorting (FACSaria™ BD, Erembodegem, Belgium). Purity of FACS-sorted cell fractions was routinely 98%. CD4<sup>+</sup> T cells were subsequently cultured at  $2 \times 10^4$  cells/well with  $1 \times 10^5$  irradiated autologous

PBMC (as feeder cells) in the presence of 2 µg/ml of anti-CD3 Ab (house made Ab clone 2G3). After 3 days, cell free supernatants were collected for LIF and cytokine measurements.

#### **Treatment of immune subsets with LIF, IFN-β, leptin and simvastatin**

Monocytes and CD4<sup>+</sup> T cell fractions were isolated from PBMC by negative selection using antibody-coated immunomagnetic beads according to manufacturer's instructions (DynaL Biotech, Oslo, Norway). The purity of the collected cell fractions was >95% as assessed by flow cytometry. PBMC were plated in 96-well microtiter plates at a density of 10<sup>5</sup> cells/well, whereas monocytes were plated at a density of 5 x 10<sup>4</sup> cells/well. CD4<sup>+</sup> T cells were cultured at a density of 5 x 10<sup>4</sup> cells/well with 5 x 10<sup>4</sup> irradiated feeder cells. Different cell fractions were treated for 3 days with the indicated concentration of the immunomodulatory drugs in presence or absence of a specific stimulus (100 ng/ml LPS for monocytes and 2 µg/ml anti-CD3 (house made Ab 2G3 clone) for T cells).

#### **Proliferation assay**

Cell free supernatants were collected after 3 days of culture and <sup>3</sup>H-thymidine (1 µCi, Amersham, Buckinghamshire, UK) was added. After 16 hr cells were harvested and <sup>3</sup>H-thymidine incorporation was measured with a beta counter (Wallac, Turku, Finland). The proliferation of drug-treated, anti-CD3 stimulated conditions (mean cpm of 6 wells) was expressed as percentage of the proliferative response in untreated anti-CD3 stimulated control wells (100 %).

#### **MTT assay**

Viability of monocytes was established using the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described (Mosmann T., 1983). After 3 days of treatment cell free supernatant was collected and 100 µl of MTT solution (0.5 mg/ml) was added to the cultures. Cells were incubated for 4 hours at 37° C and 5% CO<sub>2</sub>, where after the supernatant was removed and 175 µl DMSO/glycine was added to each well to dissolve the produced formazan crystals. The optic density (OD) at 540 nm was measured using a microplate reader. The OD is a measure of the produced formazan and correlates with the metabolic activity of the cells. The mean absorption (4 wells) of drug-treated, LPS stimulated

monocytes was expressed as percentage of the viability (mean absorption) in wells containing non-treated, LPS stimulated monocytes (100%).

### **Quantification of cytokines by ELISA**

Cell free supernatants of T cell cultures were collected after 3 days of stimulation with the indicated factors. IFN- $\gamma$ , TNF- $\alpha$  and IL-10 levels in the supernatants were measured using a sandwich ELISA based on commercially available Ab pairs (CytoSets, biosource Europe, Nivelles, Belgium) as described earlier (Hermans *et al.*, 1997). LIF production was assessed using a commercially available sandwich ELISA for human LIF according to the manufacturer's instructions (Bender MedSystems, Vienna, Austria). Net cytokine production was calculated by subtracting background levels (no stimulation) from the cytokine levels measured in the stimulated cultures. The relative cytokine secretion in drug-treated, anti-CD3 (or LPS) stimulated conditions was calculated by dividing the net cytokine secretion in this condition by the net cytokine secretion of non-treated, anti-CD3 (or LPS) stimulated condition (100%).

### **Flow cytometric analysis**

Expression of LIF receptor subunits, gp130 and CD190 (LIFR- $\beta$ ) was assayed by flowcytometric analysis. PBMC were suspended in FACS buffer (phosphate buffered saline with 2% FCS) pre-treated for 15 minutes with human serum and anti-Fc $\gamma$  RIII blocking antibody (StemCell Technologies, Paris, France) and stained for 30 minutes at 4° C with phycoerythrin (PE) conjugated anti-gp130. For the LIFR- $\beta$  staining, PBMC were first permeabilized using the cytofix/cytoperm plus<sup>TM</sup> (PharMingen, San Diego, CA, USA), stained with a primary rabbit anti-LIFR- $\beta$  (30 min 4° C) and subsequently with a secondary FITC labelled swine-anti-rabbit (30 min 4° C). After washing, cells were resuspended in FACS buffer and counterstained with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or Peridinin-chlorophyll-protein complex (PerCP) conjugated antibodies specific for CD3, CD4, CD8, CD25, CD56, CD14, CD19. Finally, cells were washed twice in FACS buffer and analyzed on a FACSCalibur cytometer (BD Biosciences). Data acquisition ( $2 \times 10^4$  events for each sample) and analysis were performed using Cell Quest software.

To examine toxic effects of LIF, we performed an annexin-V-FITC/7-AAD staining on  $10^5$  PBMC as previously described (Lecoeur *et al.*, 2002), which we combined with a CD3-PE

staining. The annexin-V/7-AAD staining of CD3 positive events in the lymphocyte gate allows distinguishing viable, apoptotic and necrotic T cells.

### **Statistical analysis**

For comparisons of healthy controls with MS patients Student-t tests were used. Differences were considered significant when  $p < 0.05$ . Differences between compound treated conditions compared to a control value (100 %) were assessed using the Wilcoxon signed rank test calculated with Graphpad prism software (Graphpad Software, San Diego, CA, USA).

### **3. RESULTS**

#### **Reduced LIF production by CD4<sup>+</sup> T cells of RR-MS patients**

We compared the LIF production by CD4<sup>+</sup> T cells of MS patients versus controls to study possible quantitative differences. To this end, flow sorted CD4<sup>+</sup> T cells of 9 healthy controls (HC), 10 RR-MS patients (MS 1-10) and 8 SP-MS patients (MS 14-21) were stimulated with soluble anti-CD3 antibody (2 µg/ml) in the presence of irradiated autologous PBMC. LIF secretion was measured in the supernatant three days after stimulation (Figure 1). The optimal time point for LIF secretion was determined in pilot experiments (Figure 2A and 2B). Interestingly, the mean LIF secretion by CD4<sup>+</sup> T cells was significantly lower in RR-MS patients ( $78 \pm 23$  pg/ml) as compared to healthy controls ( $422 \pm 108$  pg/ml;  $p < 0.01$ ) (Figure 1A). This reduced LIF production was not observed for SP-MS patients ( $307 \pm 160$  pg/ml). The mean proliferation of anti-CD3 stimulated CD4<sup>+</sup> T cells was not significantly different when comparing the different study groups, indicating that the observed difference is not due to a reduced proliferation of CD4<sup>+</sup> T cells of RR-MS patients (Figure 1B). The observed difference in LIF secretion remained significant when RR-MS patients that received medication (MS 6-8) were left out. In addition, the level of IFN- $\gamma$  secreted by CD4<sup>+</sup> T cells was not significantly different between the study groups (data not shown). Taken together these data indicate that CD4<sup>+</sup> T cells of relapsing remitting MS patients but not secondary progressive patients display a reduced LIF secretion as compared to controls.

#### **Effect of IFN- $\beta$ on LIF production and cell proliferation of T cells and monocytes**

We subsequently studied whether some selected drugs could affect LIF production by T cells. To this end, we studied the production of LIF and other cytokines in anti-CD3 stimulated PBMC that were treated with the selected drug. PBMC were chosen since it is expected that upon anti-CD3 stimulation both CD4<sup>+</sup> and CD8<sup>+</sup> T cells will proliferate. Therefore, the cytokine content in anti-CD3 stimulated PBMC mainly reflects cytokines produced by T cells. Since monocytes may constitute another important source of LIF producing cells, we also compared the effects on LPS activated monocytes. First we determined whether there are kinetic differences in LIF secretion between monocytes and T cells. As shown in figure 2.A and 2B the optimal time point for measuring LIF secretion for both T cells and monocytes was three days in culture. We subsequently compared the LIF production of anti-CD3 stimulated PBMC from 5 HC and LPS stimulated monocytes from 8 HC. As shown in Figure

2, LPS stimulated monocytes ( $25 \pm 3$  pg/ml) secrete significantly more LIF than unstimulated monocytes ( $12 \pm 2$  pg/ml), but this production is significantly lower than that of anti-CD3 stimulated ( $204 \pm 41$ ) or resting ( $25 \pm 6$ ) PBMC. IFN- $\beta$  is a cytokine that is used for the treatment of RR-MS. To examine the effect of IFN- $\beta$  on LIF secretion by T cells, we isolated PBMC from 5 healthy controls (HC) and treated the cells for 3 days with IFN- $\beta$  (1, 10, 100 and 1000 U/ml) in presence or absence of anti-CD3. Figure 3A shows the proliferation and cytokine secretion of anti-CD3 stimulated PBMC treated with a dose of 1000 U/ml of IFN- $\beta$  relative to untreated, anti-CD3 stimulated PBMC. Treatment with IFN- $\beta$  dose-dependently reduced anti-CD3-induced T cell proliferation, with a maximal reduction of  $66 \pm 6$  % at a dose of 1000 U/ml. IFN- $\beta$  treatment further resulted in a dose-dependent decrease in the secretion of LIF, TNF- $\alpha$  and IFN- $\gamma$  but enhanced the secretion of IL-10 (Figure 3A). The secretion of IL-10 was also slightly but significantly enhanced after IFN- $\beta$  treatment in resting PBMC (from 18 pg/ml to 33 pg/ml), whereas the secretion of TNF- $\alpha$ , IFN- $\gamma$  and LIF were not significantly altered. (Mean cytokine production of untreated resting PBMC of all experiments:  $24 \pm 30$  pg/ml IFN- $\gamma$ ;  $119 \pm 65$  pg/ml TNF- $\alpha$ ;  $9 \pm 8$  pg/ml IL-10 and  $25 \pm 14$  pg/ml LIF).

We also isolated monocytes from 4 HC and treated the cells with IFN- $\beta$  in presence or absence of LPS. Viability and cytokine secretion of LPS stimulated plus IFN- $\beta$  (1000 U/ml) treated monocytes were plotted relatively to the LPS treated condition (Figure 3B). Treatment with IFN- $\beta$  resulted in a small but significant decrease in the viability/activation and LIF production of monocytes. Additionally, we noted a small but significant increase in TNF- $\alpha$  and a marked increase in IL-10 secretion after IFN- $\beta$  treatment. IFN- $\gamma$  was not detectable in the supernatants of monocytes. No significant differences in viability and cytokine secretion were observed when resting monocytes were treated with IFN- $\beta$  (Mean cytokine production of untreated resting Monocytes of all experiments:  $40 \pm 41$  pg/ml TNF- $\alpha$ ;  $1 \pm 2$  pg/ml IL-10 and  $11 \pm 6$  pg/ml LIF).

To evaluate whether LIF secretion by T cells from MS patients can be stimulated by IFN- $\beta$ , anti-CD3 stimulated PBMC from 5 untreated RR-MS patients (MS 9-13) were treated with IFN- $\beta$  as indicated above. As observed in healthy controls (Figure 3A), LIF secretion by anti-CD3 stimulated PBMC of MS patients was dose-dependently reduced in response to IFN- $\beta$

(Figure 3C). Interestingly, this LIF repression in cultures treated with 1000 U/ml IFN- $\beta$  was significantly more pronounced in healthy controls ( $92 \pm 4\%$ ) as compared to MS patients ( $39 \pm 19\%$ ) (Figure 3C). All other cytokine profiles and the proliferative response were not significantly different between PBMC of healthy controls and MS patients (data not shown).

Together these data indicate that treatment with IFN- $\beta$  induces a repression of type 1 cytokines (TNF- $\alpha$  and IFN- $\gamma$ ) and a stimulation of IL-10 (type 2 cytokine). LIF secretion by T cells and monocytes is repressed rather than stimulated by IFN- $\beta$ . This repression is significantly more pronounced in healthy controls as compared to MS patients.

### **Effect of leptin on LIF production and cell proliferation of T cells and monocytes**

Because leptin increases LIF secretion in endometrial cells (Gonzalez *et al.*, 2004) and because leptin transcripts are increased in MS lesions (Lock *et al.*, 2002a; Steinman & Zamvil, 2003), we tested whether leptin stimulates LIF secretion by T cells and monocytes. To this end, PBMC and monocytes from healthy volunteers and PBMC of 5 untreated RR-MS patients (MS 9-13) were treated with human recombinant leptin (0.1, 1, 10 and 100nM) in presence and absence of their specific stimulus. Proliferation and cytokine secretion of anti-CD3 stimulated PBMC (5 HC) treated with 100 nM leptin are plotted as indicated above (Figure 4A). Although the anti-CD3 induced proliferative response is dose-dependently reduced by leptin (reduction of  $44 \pm 8\%$  at 100 nM), we observed a marked increase of IFN- $\gamma$ , TNF- $\alpha$  and IL-10 in the supernatants of anti-CD3 stimulated PBMC treated with 100 nM leptin (Figure 4A). Notably, LIF secretion was significantly decreased following leptin treatment. We also observed a dose-dependent increase of IFN- $\gamma$ , TNF- $\alpha$ , IL-10 (corresponding in absolute figures with an increase ( $\Delta$ ) of respectively 1043 pg/ml, 513 pg/ml and 61 pg/ml) but not of LIF, when unstimulated PBMC were treated with leptin. Similar effects on proliferation and cytokine secretion were observed when both resting and anti-CD3 stimulated PBMC from MS patients were treated with leptin. Figure 4.C shows the dose-dependent repression of LIF secretion by leptin in anti-CD3 stimulated PBMC from 5 RR-MS patients.

Treatment of LPS stimulated monocytes with 100 nM leptin did not affect viability but induced a significant increase in the secretion of TNF- $\alpha$ , IL-10 and LIF (Figure 4B).

Although the increase in LIF ( $68 \pm 31$  %) content was observable in all donors, this corresponds with a minor absolute increase of only 10 pg/ml. When non-stimulated monocytes were treated with leptin, we noted a dose dependent activation ( $\Delta$  0.061 OD MTT assay) and a marked increase in the secretion of TNF- $\alpha$ , IL-10 and LIF, corresponding with an increase ( $\Delta$ ) of 1456 pg/ml, 83 pg/ml and 18 pg/ml respectively.

Taken together, these data indicate that leptin induces both Th1 cytokines (IFN- $\gamma$  and TNF- $\alpha$ ) and the Th2 cytokine IL-10 in activated T cell and monocyte cultures. Leptin induces a small but significant increase in the LIF secretion of monocytes, but represses LIF secretion in T cells of both MS patients and healthy controls.

#### **Effect of Simvastatin on LIF production and cell proliferation of T cells and monocytes**

Statins are currently approved cholesterol lowering agents. Since the recent identification of potent anti-inflammatory and immunomodulatory effects, statins are also considered promising candidates for treatment in MS. Therefore, we tested whether these molecules are able to increase the secretion of LIF and we studied the effects of simvastatin (50 nM, 250 nM, 1  $\mu$ M and 10  $\mu$ M) on PBMC of 5 HC and 5 RR-MS patients (MS 9-13) and monocytes from 4 HC.

Figure 5A shows the effects of 10 $\mu$ M simvastatin on anti-CD3 stimulated PBMC of 5 HC. Treatment with 10  $\mu$ M simvastatin induced a significant increase in the secretion of IFN- $\gamma$  and a significant decrease of IL-10 and LIF production by anti-CD3 stimulated T cells. The secretion of TNF- $\alpha$  remained unaltered. No significant changes in cytokine secretion were observed when unstimulated PBMC were treated with simvastatin. Simvastatin treatment did not affect viability and cytokine secretion of resting and LPS stimulated monocytes (Figure 5B). We did not observe differences in proliferative response and cytokine secretion in MS patients compared to healthy controls (data not shown). Interestingly, when treated with a low dose of simvastatin we observed a small increase in LIF secretion by anti-CD3 stimulated PBMC of MS patients ( $\Delta$ conc=30 pg/ml at 1  $\mu$ M, Fig 5C) but this was not seen in HC (data not shown).

To further study whether this small dose-dependent increase on PBMC level is caused by a subpopulation of T cells, we studied the effect of simvastatin on purified CD4<sup>+</sup> and CD8<sup>+</sup> T

cell fractions. As observed in Figure 1, CD4<sup>+</sup> T cells from healthy controls display a higher LIF secretion as compared to RR-MS patients. CD4<sup>+</sup> and CD8<sup>+</sup> T cell fractions were isolated from 4 RR-MS patients and 4 HC by means of magnetic bead selection and cultured with different doses of simvastatin in the presence or absence of anti-CD3 and irradiated feeder cells.

LIF secretion of simvastatin treated, anti-CD3 stimulated conditions is plotted as percentage of untreated, anti-CD3 stimulated conditions (Figure 6). Simvastatin treatment of CD4<sup>+</sup> T cells resulted in a significant increase in LIF secretion by anti-CD3 stimulated CD4<sup>+</sup> T cells of MS patients, but not healthy controls (Fig. 6A). The maximal stimulation ( $43 \pm 15\%$  corresponding with an increase of 41 pg/ml) was observed with a simvastatin dose of 250 nM ( $p < 0.05$ ). A similar trend ( $p > 0.05$ ) was observed with CD8<sup>+</sup> T cells of MS patients (increase of  $25 \pm 16\%$  corresponding with 10 pg/ml) (Figure 6B).

Taken together, these data show that treatment with a low dose of simvastatin (250 nM) results in an increase in LIF secretion by CD4<sup>+</sup> and CD8<sup>+</sup> T cells of MS patients but not in healthy individuals. For both study groups, we also observed an increase in IFN- $\gamma$  secretion and a decrease in IL-10 by anti-CD3 stimulated PBMC following simvastatin treatment.

### **Immunomodulatory effects of LIF on T cells**

We then studied the possibility that LIF may affect the cytokine production and proliferation of T cells. To study immunomodulatory effects of LIF on T cells, PBMC were isolated from 6 healthy controls and treated with LIF (1, 15, 50 and 100 ng/ml) in presence or absence of anti-CD3. After 3 days, viability of T cells was checked using a CD3/annexinV/7-AAD staining, supernatants were collected to measure cytokine secretion and a classical <sup>3</sup>H thymidine incorporation assay was performed. LIF treatment did not significantly affect viability, proliferation and cytokine secretion (IL-10, TNF- $\alpha$  and IFN- $\gamma$ ) of both non-stimulated and anti-CD3 stimulated PBMC (data not shown). We further examined the distribution of gp130 and CD190 (LIFR- $\beta$ ) on peripheral mononuclear cells by means of flow cytometry. The results, summarized in table 2, represent the mean of 3 independent experiments with PBMC of 3 healthy volunteers. Gp130 expression was observable on 40-65% of all PBMC and within the PBMC fraction gp130 expression was mainly attributable to monocytes (90-95%).

In addition, 65 % of T cells (65%) and a small percentage of B cells (4.5%) expressed gp130. Notably, within the T cell population a larger proportion of CD4<sup>+</sup> T cells (70%) as compared to CD8<sup>+</sup> T cells (41%) expressed gp130. When PBMC were stimulated for 3 days with phytohemagglutinin (PHA), only 18% of resting T cells and 6% activated cells (CD3<sup>+</sup>CD25<sup>+</sup>) expressed gp130<sup>+</sup> indicating that gp130 expression is decreased upon activation (data not shown). LIFR-β expression within the PBMC fraction was restricted to 88% of monocytes and 55% of NK cells. Only a small percentage of T cells (3.5%) and B cells (0.7%) stained positive for the LIF binding receptor subunit (LIFR-β).

Taken together, these data show that the vast majority of T cells do not express the receptor requirements necessary for LIF signalling, which may explain why LIF does not affect the proliferation and cytokine secretion of T cells.

#### 4. DISCUSSION

We demonstrate for the first time that peripheral CD4<sup>+</sup> T cells of relapsing remitting MS patients display a reduced LIF secretion. This is of particular relevance since peripheral CD4<sup>+</sup> T cells are among immune cells the most important producers of neurotrophic factors such as BDNF and LIF *in vitro* (Kerschensteiner *et al.*, 1999b; Vanderlocht *et al.*, 2006). The reduced LIF secretion was observed in CD4<sup>+</sup> T cells of RR-MS patients but not in SP-MS. Other cytokine and chemokine profiles were also found to differ between RR-MS and SP-MS (Sorensen & Sellebjerg, 2001; Barth *et al.*, 2002). These differences in cytokine and chemokine secretion may relate to the heterogeneity of underlying immunopathogenic mechanisms in these disease subtypes. The cause of the reduced LIF secretion by peripheral CD4<sup>+</sup> T cells of RR-MS patients remains elusive. We previously showed the lack of association between polymorphisms in the LIF gene and MS prevalence (Vanderlocht *et al.*, 2006), indicating that genetic factors also not contribute to the observed difference in LIF secretion.

It remains unclear what the consequences of a reduced LIF secretion by peripheral blood CD4<sup>+</sup> T cells in RR-MS could be. Since LIF can pass the blood brain barrier (BBB) through a saturatable transport system (Pan *et al.*, 2000) and since it was previously shown that LIF potentiates neuronal and oligodendrocyte survival *in vitro* and *in vivo* (Mayer *et al.*, 1994; Barres *et al.*, 1993; Hughes *et al.*, 1993; Azari *et al.*, 2001; Martinou *et al.*, 1992), it is conceivable that trophic support in the CNS of RR-MS patients is reduced. These findings are supported by convincing *in vivo* evidence that LIF receptor signalling ameliorates EAE severity by directly promoting oligodendrocyte survival (Butzkueven *et al.*, 2002). In addition, LIF was shown to increase the expression of NT-3 (Blesch *et al.*, 1999) and IGF-1 (Kerr & Patterson, 2005) in different models of CNS injury. As a consequence, trophic support of the inflamed CNS of RR-MS patients could be more severely impaired than expected from the reduced LIF secretion alone. Further studies are necessary to clarify this issue.

Other neurotrophic factors, like NGF and recently neurturin have been shown to possess immunomodulatory effects (Thorpe & Perez-Polo, 1987; Otten *et al.*, 1989; Mazurek *et al.*, 1986; Vargas-Leal *et al.*, 2005). We investigated whether LIF is also able to influence the proliferation and cytokine patterns of T cells. Our data indicate that a functional LIF receptor is expressed on a small proportion of T cells which most likely explains the lack of effects on

T cells reported in this study. This is consistent with the lack of any measurable effect of LIF administration on immune responses in EAE (Butzkueven *et al.*, 2002). In contrast to T cells, the majority of monocytes express both LIFR- $\beta$  and gp130. Therefore LIF may exert immunomodulatory effects on monocytes. Indeed, LIF has already been described to induce chemotaxis of mouse peritoneal macrophages *in vitro* and infiltration of macrophages in CNS lesions is delayed in LIF knock-out mice (Sugiura *et al.*, 2000). Together, findings indicate that a reduced secretion of LIF by CD4<sup>+</sup> T cells is unlikely to affect T cell responses in MS, but may modulate macrophage function.

Increasing the local neurotrophic support has been proposed as an attractive therapeutic strategy for MS. However, so far the successful application of neurotrophic factors for the treatment of CNS diseases was hampered due to difficulties in delivering sufficient amounts to the site of inflammation (Sagot *et al.*, 1997). One attractive strategy to circumvent this delivery problem would be to increase the production of neurotrophic factors by T cells and thereby exploit their homing to the CNS at times of inflammation. We further analysed the effects of three immunomodulatory drugs on LIF secretion by immune cells to explore the possibility that some of these molecules may increase LIF production. IFN- $\beta$  is an approved drug for the treatment of relapsing remitting MS. In contrast to Byskosh and Reder who reported a transient increase in LIF mRNA content of PBMC of MS patients one week after the start of IFN- $\beta$  treatment (Byskosh & Reder, 1996), we observed a dose dependent reduction of LIF secretion by T cells and monocytes *in vitro*. It should however be noted that the former study investigated LIF expression on the mRNA level on PBMC directly *ex vivo* (i.e. without activation *in vitro*) and no long term differences were observed. Although doses of IFN- $\beta$  required to see effects on immune parameters *in vitro* are generally higher than steady state plasma levels (40-80 U/ml in patients receiving 8x10<sup>6</sup> U subcutaneously three times a week (Chiang *et al.*, 1993; Khan *et al.*, 1996), we already observed a significant decrease in LIF at a dose of 100 U/ml. At a dose of 1000 U/ml, this reduction was significantly less pronounced in PBMC from untreated RR-MS patients, further indicating that regulation of LIF secretion is altered in MS.

Statins, formerly known as hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors are clinically approved cholesterol lowering drugs. Statins are promising candidates for future treatment in MS, as they were shown to possess potent anti-inflammatory effects (Neuhaus *et al.*, 2005). The stimulation of LIF secretion by CD4<sup>+</sup> and CD8<sup>+</sup> T cells of MS

patients but not healthy controls that was observed following low dose simvastatin treatment further points to a possible therapeutic application of simvastatin in MS. The optimal dose for the LIF stimulating effect is 250 nM. It should be noted that this dose is 6-fold higher than the steady state plasma concentration of statins after a 40 mg oral intake. However this does not mean a priori that these effects are irrelevant because the optimal effects on cytokine secretion were observed at a dose of 10  $\mu$ M (250 fold steady state concentration) (Neuhaus *et al.*, 2002). These different effects at low doses do not match the dose-dependent effects of statins on T cell responses as reported by Youssef and colleagues (Youssef *et al.*, 2002). However, these authors did not measure LIF and studied the T cell responses of rodent T cells. In addition to the stimulation of LIF secretion, we also noted a marked increase of IFN- $\gamma$  and decrease in IL-10 as previously reported by Neuhaus *et al.* (2002). Taken together with the reported enhancement of proteolytic activity of matrix metalloproteinase-2 by simvastatin (Kieseier *et al.*, 2004) these data also suggest pro-inflammatory actions of simvastatin. Large double-blinded clinical studies are currently underway to further analyse the therapeutic potential of statins in MS.

Furthermore, we noted a small increase in LIF secretion of monocytes, but not T cells, after leptin treatment, indicating the opposite regulation of LIF by leptin in T cells and monocytes. The increased LIF secretion by monocytes following leptin treatment may indicate that the elevated leptin transcripts in MS lesions could upregulate the local secretion of LIF by infiltrating monocytes. It should however be noted that the LIF stimulating effect of leptin is rather small and it remains to be studied whether this effect also has *in vivo* relevance.

Although our results show that none of the used immunomodulatory agents completely restore the defective production of LIF by CD4<sup>+</sup> T cells, this study illustrates the unique regulation of LIF under influence of different stimuli. Previously, Piccini and coworkers showed higher LIF concentrations in the supernatants of Th2 compared to Th1 and Th0 T cell clones. Furthermore, they showed that the development of LIF secreting T cells was downregulated by Th1 inducers (IL-12, IFN- $\alpha$  and IFN- $\gamma$ ) and upregulated by IL-4 (Piccinni *et al.*, 1998). The discrepancy in secretion profile between LIF and all other tested cytokines after leptin treatment illustrates the distinct regulation of LIF secretion.

This study provides the first evidence that the secretion of the neurotrophic factor LIF is reduced in peripheral blood CD4<sup>+</sup> T cells of relapsing remitting MS patients. Because LIF can

pass the BBB freely, a reduced peripheral LIF secretion may have consequences in the CNS. Our results further show that unlike other neurotrophic factors such as NGF, LIF does not affect T cell responses, but it remains to be established whether a reduced LIF secretion contributes to MS pathology by decreasing trophic support to the injured CNS. Although immunomodulatory agents such as IFN- $\beta$ , leptin and simvastatin do not substantially increase LIF secretion, simvastatin treatment may provide a means to specifically increase LIF secretion by T cells of MS patients. Further detailed knowledge of the regulation of LIF secretion and the identification of agents that restore the reduced LIF secretion by CD4<sup>+</sup> T cells of RR-MS patients may provide additional tools for MS therapy.

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**Figure 1: Comparison of LIF secretion by CD4<sup>+</sup> T cells of relapsing remitting MS patients, secondary progressive MS patients and healthy controls.** CD4<sup>+</sup> T cells of 9 healthy controls, 10 RR-MS patients and 8 SP-MS patients, isolated directly *ex vivo* by means of FACS sorting, were cultured for 3 days in presence of anti-CD3 (2µg/ml). The mean LIF content (±STDEV) (A) and the mean proliferative response (cpm ± STDEV) are depicted (B). \*\*p< 0.01

**Figure 2: LIF secretion by PBMC and purified monocyte cultures.** A. LIF secretion kinetics of PBMC isolated from 2 healthy controls and cultured in the presence of 2µg anti-CD3. B. LIF secretion kinetics of monocytes isolated from 2 healthy controls and cultured in the presence of 100 ng/ml LPS. C. PBMC were isolated from 18 HC and plated at a density of 10<sup>5</sup> cells/well in the presence (+) or absence (-) of anti-CD3. Monocytes were isolated from 12 HC and plated at a cell density of 5x10<sup>4</sup> cells/well in presence (+) or absence (-) of LPS. After 3 days in culture, cell free supernatants were collected and LIF content was measured with ELISA. The mean LIF content (± STDEV) is depicted.

**Figure 3: Effects of IFN-β on the proliferation and cytokine release profile of anti-CD3 stimulated PBMC and LPS treated monocytes.** Anti-CD3 stimulated PBMC isolated from 5 HC were treated with 1000 U/ml IFN-β. After 3 days of treatment, proliferation (<sup>3</sup>H) was assayed and cytokines were measured in the cell free supernatants. A. Proliferation and cytokine secretion were plotted relative to IFN-β untreated, anti-CD3 stimulated PBMC. The corresponding absolute values given as reference value (100%) are as follows: proliferation 119659 cpm; 2024 pg/ml IFN-γ; 1435 pg/ml TNF-α; 52 pg/ml IL-10 and 226 pg/ml LIF. Each box represents the interquartile range (IQR) with the median (line) and outer limits (bars). B. LPS stimulated monocytes, isolated by means of negative selection from 4 HC, were treated with 1000 U/ml IFN-β. After 3 days of treatment viability was assayed and the cytokine content in the cell free supernatants was determined. Viability and cytokine secretion were plotted relative to untreated, LPS stimulated monocytes. Reference values: optic density (OD<sub>540</sub>) 0.200; 1347 pg/ml TNF-α; 117 pg/ml IL-10 and 23 pg/ml LIF. C. Dose-dependent repression of LIF secretion by PBMC 5 MS patients by IFN-β. (Reference value: 106 pg/ml LIF)

**Figure 4: Effects of leptin on the proliferation and cytokine release profile of anti-CD3 stimulated PBMC and LPS treated monocytes.** A. Proliferation and cytokine secretion of anti-CD3 stimulated, leptin (100 nM) treated normal PBMC plotted relative to anti-CD3 stimulated, but leptin-untreated PBMC. The corresponding absolute values given as reference value (100%) are as follows: proliferation 112653 cpm; 2233 pg/ml IFN- $\gamma$ ; 1983 pg/ml TNF- $\alpha$ ; 59 pg/ml IL-10 and 243 pg/ml LIF. B. Effect of leptin (100 nM) on viability and cytokine secretion of LPS stimulated monocytes from healthy controls. Viability and cytokine secretion were plotted relative to untreated, LPS stimulated monocytes. Control values: optic density (OD<sub>540</sub>) 0.214; 1443 pg/ml TNF- $\alpha$ ; 126 pg/ml IL-10 and 33 pg/ml LIF. C. Dose-dependent effects of leptin on LIF secretion by PBMC 5 untreated MS patients. (Control value 203 pg/ml LIF)

**Figure 5: Effects of simvastatin on the proliferation and cytokine release profile of anti-CD3 stimulated PBMC and LPS treated monocytes.** A. Proliferation and cytokine secretion of anti-CD3 stimulated, simvastatin (10  $\mu$ M) treated normal PBMC plotted relative to anti-CD3 stimulated, simvastatin-untreated PBMC. The corresponding absolute values given as reference value (100%) are as follows: proliferation 74813 cpm; 1366 pg/ml IFN- $\gamma$ ; 1020 pg/ml TNF- $\alpha$ ; 27 pg/ml IL-10 and 145 pg/ml LIF. B. Effect of simvastatin (10  $\mu$ M) on viability and cytokine secretion of LPS stimulated monocytes from healthy controls. Viability and cytokine secretion were plotted relative to LPS stimulated untreated monocytes. Reference values: optic density (OD<sub>540</sub>) 0.223; 1634 pg/ml TNF- $\alpha$ ; 161 pg/ml IL-10 and 21 pg/ml LIF. C. Dose dependent effects of simvastatin on LIF secretion by PBMC 5 untreated MS patients. (Reference value: 132 pg/ml LIF)

**Figure 6: Effects of simvastatin on the LIF secretion by CD4<sup>+</sup> and CD8<sup>+</sup> T cells from healthy controls and MS patients.** CD4<sup>+</sup> and CD8<sup>+</sup> T cell fractions were isolated by means of magnetic bead selection and treated with different doses of simvastatin in the presence or absence of anti-CD3 and irradiated feeder cells. A. Effect of simvastatin on the relative LIF secretion of CD4<sup>+</sup> T cells (as percentage of untreated anti-CD3 stimulated CD4<sup>+</sup> T cells) of healthy controls (n=4) and MS patients (n=4). B. Effect of simvastatin on the relative LIF secretion of CD8<sup>+</sup> T cells of healthy controls and MS patients. \*p<0.05

**Table 1:** Characteristics of MS patients

**Table 2:** Expression of LIF receptorsubunits gp130 and LIFR- $\beta$  by immune cells.

**Figure 1**  
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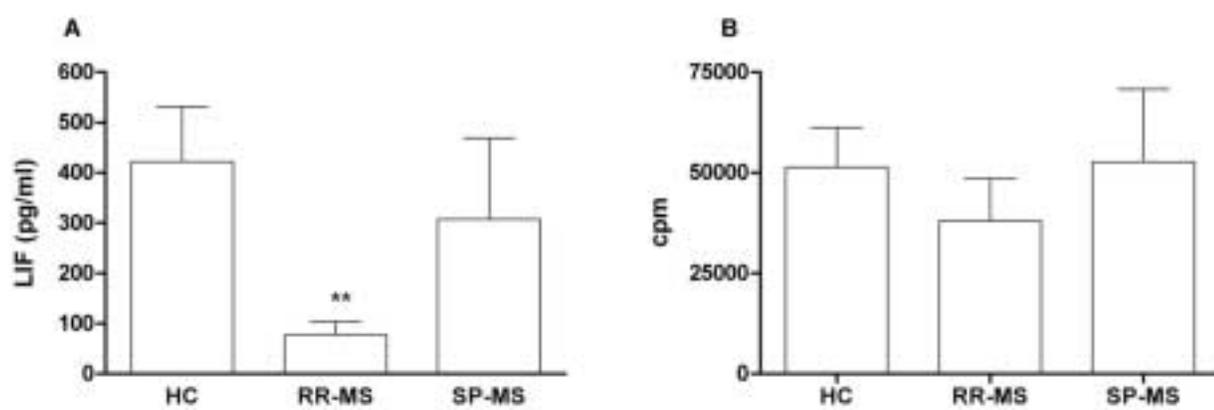


Figure 2

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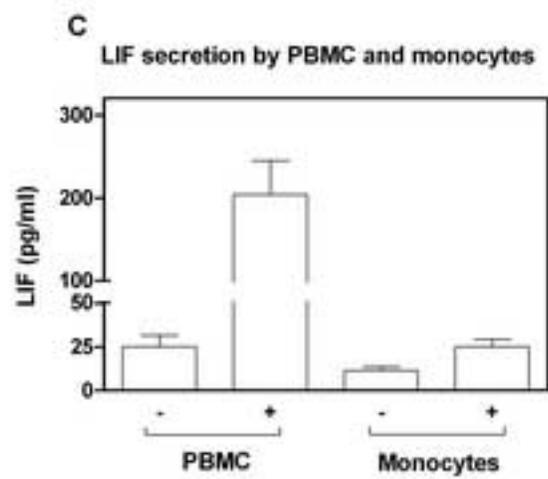
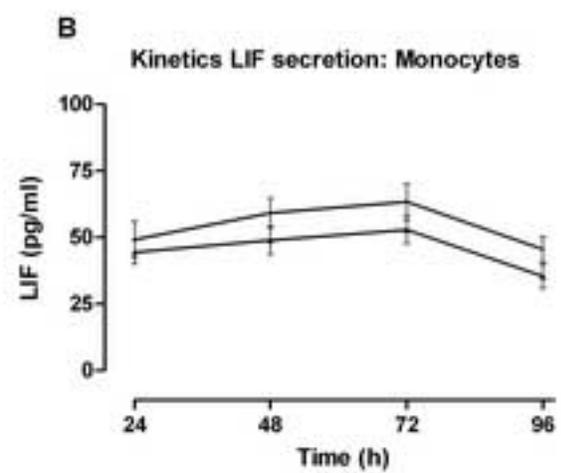
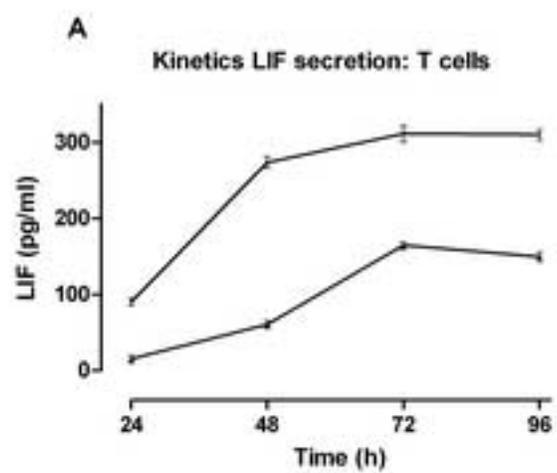


Figure 3

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## IFN- $\beta$ (1000 U/ml)

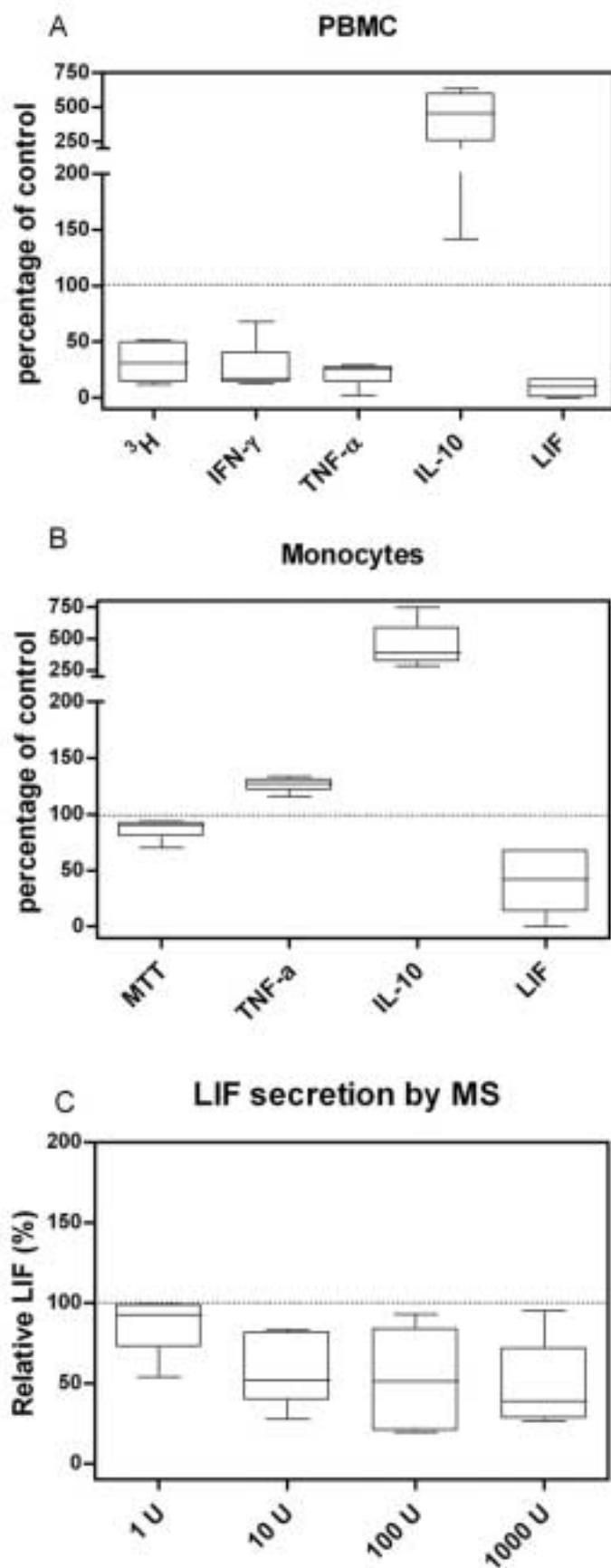
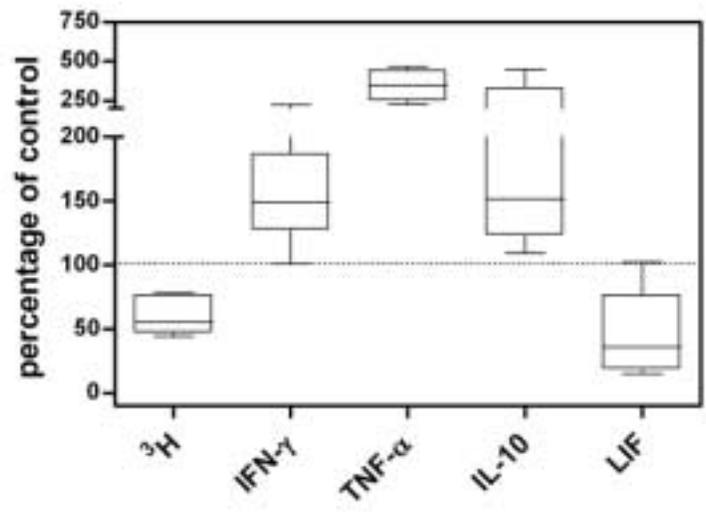


Figure 4

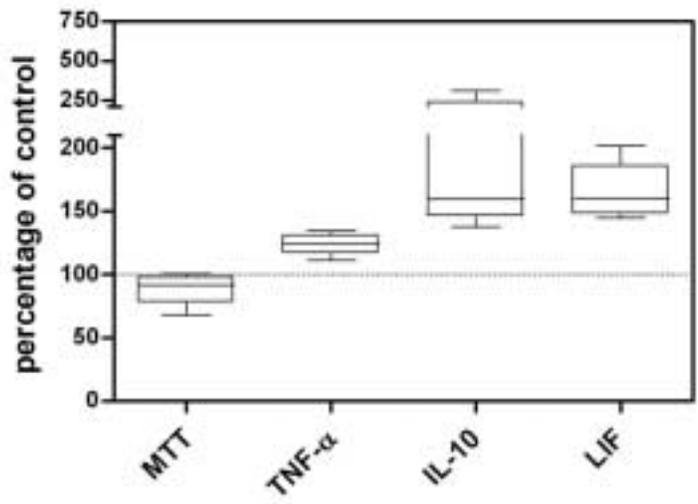
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### Leptin (100 nM)

PBMC



Monocytes



PBMC of MS patients

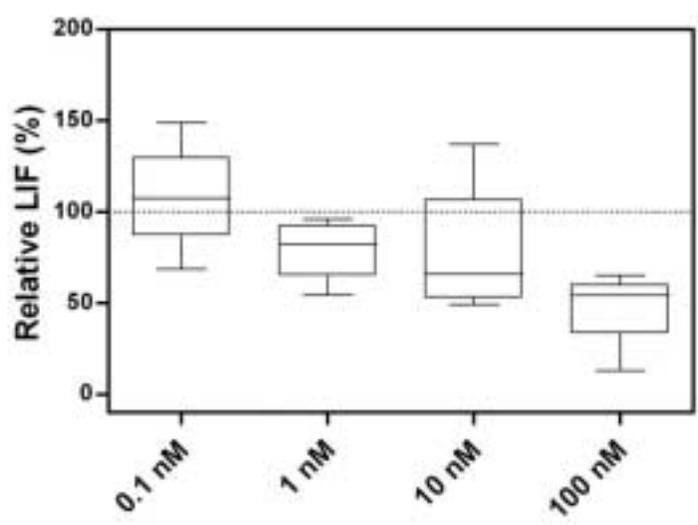


Figure 5

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## Simvastatin (10 $\mu$ M)

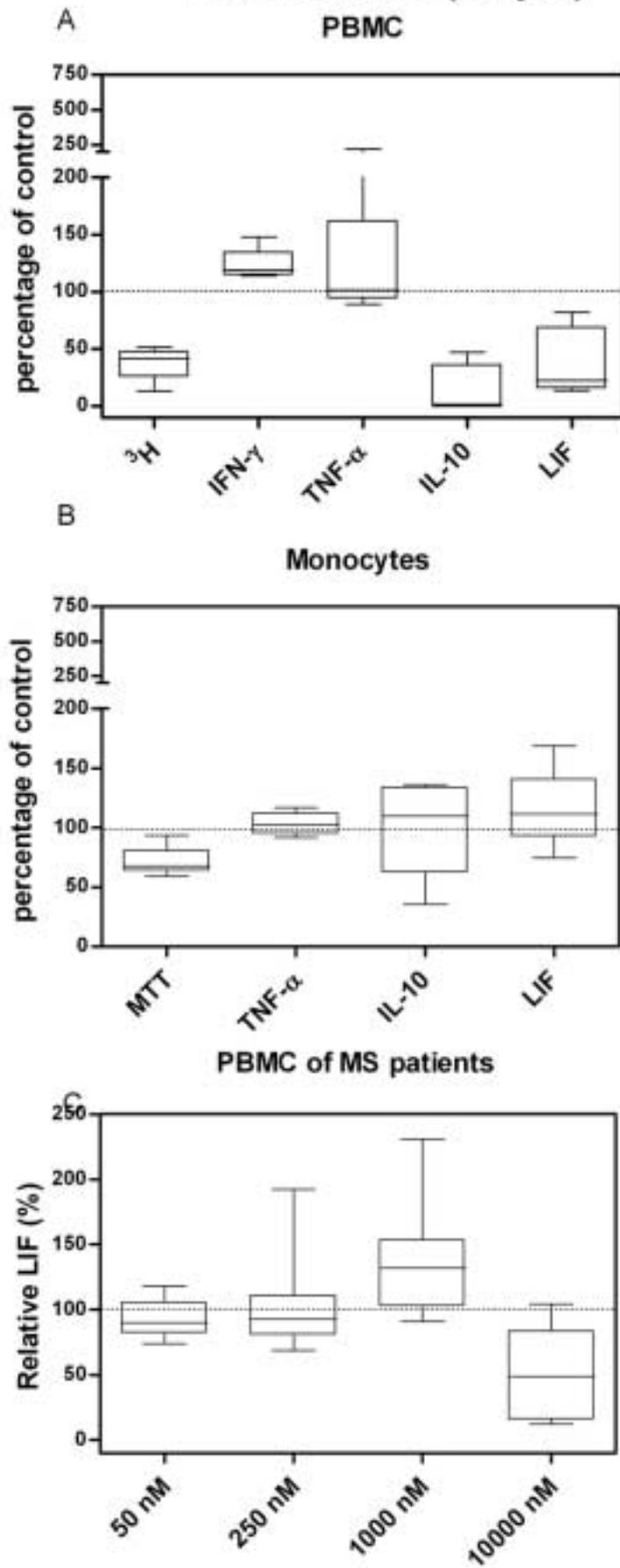
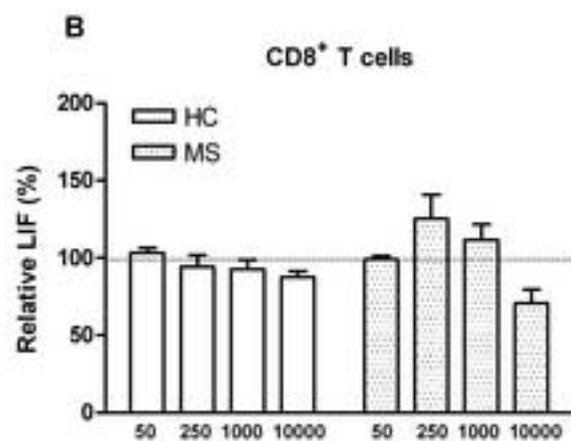
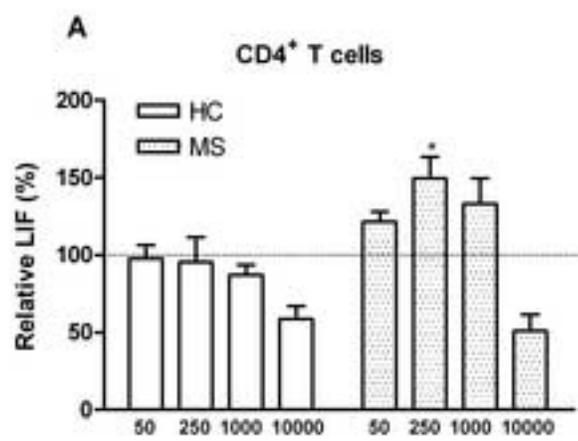


Figure 6  
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**Table 1:** Characteristics of MS patients

| RR-MS | Patient ID | Age | Sex | DD | EDSS | Med          |
|-------|------------|-----|-----|----|------|--------------|
|       | MS 1       | 31  | M   | 8  | 1    | /            |
|       | MS 2       | 24  | F   | 4  | 4    | /            |
|       | MS 3       | 57  | F   | 21 | 0    | /            |
|       | MS 4       | 57  | F   | 25 | 2    | /            |
|       | MS 5       | 44  | F   | 6  | 3,5  | /            |
|       | MS 6       | 54  | F   | 10 | 3    | IFN- $\beta$ |
|       | MS 7       | 30  | M   | 8  | 1    | GA           |
|       | MS 8       | 28  | M   | 3  | 5    | IFN- $\beta$ |
|       | MS 9       | 24  | F   | 4  | 6,5  | /            |
|       | MS 10      | 33  | M   | 6  | 1,5  | /            |
|       | MS 11      | 33  | M   | 3  | 2    | /            |
|       | MS 12      | 49  | F   | 4  | 2    | /            |
|       | MS 13      | 49  | F   | 7  | 3,5  | /            |

| SP-MS | ID    | Age | Sex | DD | EDSS | Med |
|-------|-------|-----|-----|----|------|-----|
|       | MS 14 | 61  | M   | 8  | 5,5  | MTX |
|       | MS 15 | 69  | F   | 18 | 4    | /   |
|       | MS 16 | 47  | M   | 13 | 6    | /   |
|       | MS 17 | 59  | F   | 14 | 5    | /   |
|       | MS 18 | 55  | F   | 25 | 9    | /   |
|       | MS 19 | 57  | M   | 32 | 8,5  | /   |
|       | MS 20 | 52  | F   | 19 | 8,5  | /   |
|       | MS 21 | 38  | M   | 15 | 5    | /   |

*Patient ID: patient identification, RR-MS: relapse-remitting MS, SP-MS: secondary progressive MS, DD: Disease duration (years), Med: medication a time of blood sampling, MTX: metotrexate, IFN- $\beta$ : Interferon beta, GA: glatiramer acetate, EDSS: Expanded Disability Status Scale*

**Table 2:** Expression of gp130 and LIFR- $\beta$  receptor subunits by immune cells

|                                | gp130 | CD190 (LIFR- $\beta$ ) |
|--------------------------------|-------|------------------------|
| PBMC                           | 53%   | 22%                    |
| T cells (CD3 <sup>+</sup> )    | 64%   | 3.5%                   |
| CD4 <sup>+</sup> T cells       | 70%   | 3.25%                  |
| CD8 <sup>+</sup> T cells       | 41%   | 4%                     |
| Monocytes (CD14 <sup>+</sup> ) | 90%   | 88%                    |
| NK cells (CD56 <sup>+</sup> )  | 0%    | 55%                    |
| B cells (CD19 <sup>+</sup> )   | 4.5%  | 0.7%                   |

*\*Mean percentage (n=3) of positive staining within a given immune cell subset*