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The effect of disease activity on leptin, leptin receptor and suppressor of cytokine signalling-3 expression in relapsing–remitting multiple sclerosis

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Abstract

In this study we observed higher serum leptin levels in relapsing–remitting multiple sclerosis (RRMS) patients during remission than in controls. The expression of leptin receptor (ObR) was higher in CD8+ T cells and monocytes from RRMS patients in relapse than in patients in remission and in controls. Relapsing patients showed high levels of pSTAT3 and low expression of SOCS3 and leptin administration induced an up-regulation of pSTAT3 only in monocytes from patients in relapse. Our data suggest that ObR may be involved in the development of clinical relapses in RRMS patients and suggest a rationale for potential targeting of the leptin axis during MS.

Keywords: Leptin; Transcription factors; SOCS3; STAT3; Multiple sclerosis

1. Introduction

Leptin, a cytokine-like hormone, mainly secreted by adipose tissue, regulates food intake, energy expenditure (Zhang et al., 1994) and immune response (Matarese, 2000). Leptin has a key function in the regulation of the Th1/Th2 balance (Lord et al., 1998), as it induces the secretion of proinflammatory cytokines such as TNF-α, IL-2, IL-6 and IFN-γ following the exposure to several stimuli (i.e. inflammation, infection, etc.) (Fantuzzi and Faggioni, 2000; Santos-Alvarez et al., 1999; Zarkesh-Esfahani et al., 2001). Leptin acts on immune cells by the long signalling form of its receptor (ObRb), a member of the class I cytokine receptor family, which includes receptors for IL-6, IL-11, leukaemia inhibitory factor, granulocyte-colony stimulating factor, ciliary neurotrophic factor, oncostatin M and cardiotrophin-1 (Heinrich et al., 2003). After binding to ObRb, leptin activates ObRb-associated JAK2 that induces the activation of STAT3 (Baumann et al., 1996). After thyrosine-phosphorylation, pSTAT3 translocates into the nucleus, inducing STAT3-responsive genes including SOCS3 (Niemand et al., 2003), which in turn provides inhibition of leptin signalling by binding to phosphorylated tyrosines of ObR and JAK-2 (Shuai and Liu, 2003). SOCS are a family of intracellular proteins, which have been shown to regulate the response of immune cells to cytokines in innate and adaptive immunity. SOCS3 genes are constitutively expressed in naive Th cells and regulate the balance of the Th1/Th2/Th17 immune responses, the TCR- and cytokine-induced proliferation. SOCS3 expression is induced in a variety of immune and inflammatory conditions by a wide number of cytokines, including IFN-γ, IL-2, IL-3, IL-6, and IL-10 (Starr et al., 1997). On the other hand, in vitro studies suggested that SOCS3 is able to modulate IL-6, IL10 and leptin signalling (Kubo et al., 2003). The soluble leptin receptor (sObR) represents the main leptin-binding activity in human blood. This receptor splice form can modulate steady-state leptin levels after binding free leptin in the circulation and

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consequently preventing hormone degradation and clearance (Huang et al. 2001). Therefore, high sObR levels in the blood may act as a potential reservoir of bioactive leptin (Ge et al., 2002). The involvement of leptin in autoimmune diseases has been shown in mice with a defective leptin (*ob/ob* mice). *Ob/ob* mice are resistant to the induction of several experimental models of inflammatory and autoimmune diseases, such as experimental arthritis (Busso et al., 2002), T cell-mediated hepatitis (Faggioni et al., 2000), acute and chronic intestinal inflammation (Mykoniatis et al., 2003; Siegmund et al., 2002) and experimental autoimmune encephalomyelitis (EAE) (Matarese et al. 2001; Sanna et al., 2003), an animal model of MS. Recent studies have suggested a possible role of leptin in the pathogenesis of MS (Matarese et al., 2005; Chatzantoni et al., 2004; Batocchi et al 2003). Gene-microarray analysis of active lesion in human MS lesions revealed elevated transcripts of leptin (Rogge et al., 2000; Lock et al., 2002) and myelin basic protein autoreactive T lymphocytes from relapsing–remitting MS (RRMS) patients produced leptin and up-regulated leptin receptor after activation (Matarese et al., 2005). A more recent study showed that ObR, expressed on resting mouse CD4+, CD8+, B cells, and monocytes, was up-regulated following cell activation and increased the expression of STAT3 (Papathanassoglou et al., 2006). We have previously reported that leptin produces a different effect on PBMC from RRMS patients during relapse or stable phase of disease (Frisullo et al., 2004). In particular, leptin was able to induce PBMC production of proinflammatory (TNF-α, IL-6) and anti-inflammatory cytokines (IL-10) only in PBMC from MS patients in relapse and not in the ones from patients in stable phase of disease or controls (Frisullo et al., 2004). In this study, we evaluated whether changes of expression of proteins involved in the regulation of the leptin signalling pathway may account for the different leptin sensitivity exhibited by PBMC of patients affected by RRMS in different phases of disease. We measured serum levels of leptin and sObR and the expression of ObR on lymphomonocytes from RRMS patients in stable and active phase of disease and in healthy subjects. Moreover we tested the ability of leptin to induce pSTAT3 expression and cytokine production by PBMC from RRMS patients and healthy subjects after SOCS3 induction.

2. Materials and methods

2.1. Patients

One hundred and two patients with RRMS (69 females and 33 males), defined by McDonalds criteria, attending the MS Outpatient Clinic of Catholic University in Rome were included in our study. The control group consisted of 68 age- and sex-matched healthy subjects (40 females and 28 males), screened for infectious conditions or other inflammatory diseases. Disability degree was assessed by Expanded Disability Status Scale. No patient had ever received immunosuppressive or immunomodulatory drugs, such as Interferon beta or Glatiramer acetate. No patients were treated with corticosteroid during 3 months before the inclusion in the study. All patients underwent clinical examination, body mass index (BMI) recording and brain and spinal cord magnetic resonance imaging (MRI) before starting the experiment. Disease activity was evaluated by clinical examination and MRI. Patients were considered in active phase of disease (relapse) when they showed an episode of new neurological disturbance lasting at least 24 h and MRI activity (≥1 Gd-DTPA enhancing lesion); in stable phase of disease (remission), when neither new neurological symptoms nor MRI activity were registered in the 6 months before and in the 2 months after the beginning of the study. Peripheral blood cells were collected from patients in relapse within 48 h from the onset of new clinical symptoms. Western blot and flow cytometric analysis was performed on PBMC of 45 RRMS patients (27 females and 13 males; mean age=36±8.6) and of 15 age- and sex-matched healthy subjects (9 females and 6 males; mean age=32±6.2). This study was approved by the ethic committee of Catholic University, and all the participants gave written informed consent before the enrolment.

2.2. Radioimmunoassay

Serum leptin levels were measured by radioimmunoassay (RIA), using a commercially -available iodine 125-labeled human leptin RIA kit (DRG International, Inc, USA). All samples were run in duplicate. The sensitivity of the leptin RIA was 0.5 ng/ml. The intra- and inter-assay coefficients of variation were 3 and 8% respectively.

2.3. Human soluble ObR quantification

sObR was assayed using a commercial ELISA kit (Chemicon International, Inc, Tamecula, CA) in serum from RRMS patients and healthy controls. The minimum detection limit of the assay was 0.4 U/ml s Data were expressed as U/ml. The intra- and inter-assay coefficients of variation were 2 and 6% respectively. All samples were assayed in duplicate.

2.4. Western Blot

PBMC were homogenized in RIPA buffer (50 mM Tris (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml Apoprotin, 1 μg/ml Leuptin, 1 mM Na_3VO_4, 1 mM NaF). After centrifugation at 10,000 rpm for 10 min, the supernatant was collected and protein concentration was measured using a colorimetric assay (Bio-Rad Laboratories, Hercules, CA). 300 micrograms (μg) of proteins were loaded in each well of a 10% polyacrylamide gel, separated by electrophoresis and blotted to nitrocellulose membrane (Schleicher & Schuell, Rellienhausen, Germany) using standard protocols. Blots were blocked in 5% non-fat dry milk and incubated with goat anti-leptin receptor antibody (1:150; Chemicon International, Temecula, CA, USA) or goat anti-SOCS3 antibody (1:100; Santa Cruz, California, USA) and goat anti-β-actin (1:100; Santa Cruz) overnight at 4 °C. After being incubated with appropriate peroxidase-conjugated secondary antibody, blots were developed using ECL Western Blotting Analysis System (Amersham Biosciences, Piscataway, USA). The relative quantity of each protein was estimated by density calculation of the corresponding band on scanned image using Totalab software.

2.5. Flow cytometry

Isolated PBMC were washed once in culture medium (Dulbecco) containing FCS and once in 0.1 M PBS and incubated with specific PC5-conjugated anti-CD4, CD8, and CD14 antibody (Beckman Coulter, Miami, FL, USA), anti-human Leptin Receptor (Chemicon International, Temecula, CA, USA) and FITC-conjugated anti-goat IgG (Caltag Laboratories, Burlingame, CA, USA). For pSTAT3 detection, PBMC were fixed with 2% paraformaldehyde for 10 min and then permeabilized using a commercially available perm/wash kit (BD Biosciences/Pharmingen). Upon permeabilization, 3×10^5 cells were resuspended in 100 μl of PBS and incubated for 30 min with the specific PE-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After incubation, cells were washed again with cold PBS and resuspended in PBS for flow cytometry (FCM) (Beckman Coulter, EPICS XL™).

For pSTAT3 detection after leptin stimulation, PBMC from RRMS patients were incubated with 250 ng/ml of leptin and tested for pSTAT3 expression in monocytes before, 30 min, 1 h and 3 h after stimulation.

2.6. Stimulation of cytokine release

PBMC were isolated from venous blood of healthy subjects and MS patients by density gradient centrifugation (2500 rpm, 30 min) over a Ficoll-Hypaque density gradient (Pharmacia, Uppsala, Sweden). PBMC were then harvested by pipetting cells from the Ficoll/plasma interface and washed twice. 5×10^6/ml cells were then transferred into 24-well plates in RPMI 1640 (EuroClone, West York, United Kingdom) containing 2 n-glutamine and 5% fetal calf serum (Hyclone Laboratories Inc, Logan, UT) and treated either with 100 U/mL IL-10 (Sigma-Aldrich Co. St Louis, USA) +100 ng/mL LPS (*Escherichia coli*, serotype 0111:B4, Sigma) or medium alone. We used a co-stimulation with LPS and IL10 in order to enhance the expression of SOCS3 and the dose of LPS and IL10 was chosen on the basis of a previous report (Cassatella et al., 1999). Three hours after incubation with IL10+LPS, corresponding to the peak of SOCS3 expression, PBMC with and without pre-treatment were washed and incubated for 72 h (5% CO_2) either with medium alone or with leptin (500 ng/ml, Sigma-Aldrich Co.). At the end of incubation, the supernatants were collected, centrifuged at 400 *g* for 10 min at 15 °C and stored at −80 °C until cytokine determination. For Western Blot analysis, PBMC were collected at baseline and 3 h after pre-treatment with IL10 and LPS or medium alone.

2.7. Cytokine measurement

The production of IL-6 was measured by enzyme-linked immunosorbent assay (ELISA) using commercial kit (R&D Systems, Minneapolis, USA) following manufacturer's instructions. The intra- and inter-assay coefficients of variation were 5 and 9% respectively. The minimum detectable dose of IL-6 is less than 2 pg/ml. Data were expressed as pg/ml. All samples were assayed in duplicate.

2.8. Statistical analysis

Differences in variables among groups were tested by two-ways analysis of variance (ANOVA). Results are expressed as mean±SD. p-values ≤ 0.05 were considered statistically significant

3. Results

3.1. Patients

We included in our study 102 RRMS patients and 68 age, sex and BMI matched healthy subjects (Table 1). Fifty-six patients (38 females and 18 males) were in acute phase of the disease and 46 (31 females and 15 males) in stable phase of the disease. Clinical characteristics (age, disease duration, BMI) of the two groups of patients did not differ when they were included in the study (Table 1).

Table 1
Clinical features, BMI and serum leptin levels in RRMS patients and controls

	Male in relapse	Male in remission	Control male	p value	Female in relapse	Female in remission	Control female	p value
Number of patients	33		28		69		40	
	18	15			38	31		
Age (years)	30.2±9.8		39.8±10.9	NS	33.1±7.5		37.2±11.2	NS
	27.8±6.8	32.2±7.2		NS	33.2±7.2	34.1±8.5		NS
Disease duration (years)	5.6±4.6	6.7±5.0		NS	5.2±4.7	8.1±7.9		NS
BMI (kg/m^2)	22.6±5.1		25.1±4.6	NS	23.9±4.1		23.9±4.3	NS
	21.6±3.6	22.9±3.1		NS	22.9±3.2	24.9±3.5		NS
EDSS	2.2±0.9	1.9±0.4		NS	2.8±1.8	1.7±0.8		0.009
Gd-enhancing lesions (number)	4.4±4.1	0		0.020	4.8±5.1	0		0.001
Leptin (ng/ml)	10.4±6.4		4.2±2.9	0.05	17.2±11.7		10.2±7.9	0.005
	8.2±6.4	11.1±7.6		NS	14.8±11.7	24.7±13.9		0.041
Leptin/BMI	0.46±0.29		0.17±0.98	0.04	0.69±0.47		0.43±0.32	0.026
	0.36±0.25	0.51±0.44		NS	0.61±0.49	0.99±0.56		0.032

Clinical features of RRMS patients included in the study. All data expressed as mean±SD. EDSS=Expanded Disability Status Scale; BMI=Body Mass Index; NS=not significant. NA=Not applicable.

3.2. Serum leptin and soluble ObR levels in MS patients and healthy subjects

Serum leptin levels were higher in RRMS patients than in healthy subjects ($p=0.0165$). When we divided our two populations of patients and controls according to gender, we observed that serum leptin levels were higher in female RRMS patients and controls than in males patients and controls ($p<0.05$) (data not shown). Leptin levels were significantly higher both in female and male MS patients than in female and male controls respectively ($p=0.005$, $p=0.05$ respectively; Table 1). When we divided MS patients according to disease activity and gender, we found higher serum leptin levels in female RRMS patients in remission than in female RRMS patients in relapse and female controls ($p=0.041$ and $p=0.012$ respectively; Fig. 1A and Table 1). In male remitting RRMS patients we observed higher serum leptin levels than in male controls ($p=0.036$; Fig. 1B). These differences were maintained even when serum was normalized for BMI (Table 1). Moreover we observed a significant correlation between BMI and serum leptin level in male and female controls and in both male and female relapsing MS patients. No significant correlation was observed in MS patients in remission phase (data not shown). These results should be related to the increased levels of serum leptin levels observed only in remitting patients.

We did not find any significant difference in serum sObR levels among MS patients in relapse or in remission or healthy subjects (data not shown). Also there was no significant difference in serum sObR levels between male and female individuals.

3.3. ObR expression in T cells and monocytes from MS patients and healthy subjects

In order to evaluate ObR expression, we performed western blot and cytometric analysis on PBMC from 15 controls and 45 RRMS patients in different phases of disease (17 in relapse, 28 in remission). The two subgroups of patients did not show significant difference in baseline clinical features (data not shown). Interestingly, we found a significantly higher expression of ObR in PBMC homogenates from RRMS patients than in healthy subjects ($p<0.05$). In order to verify whether ObR expression was modified by disease activity, we performed western blotting analysis on PBMC from RRMS patients during relapse or remission phase. PBMC expression of ObR was higher in PBMC from relapsing than in ones from remitting RRMS patients and healthy subjects ($p=0.013$ and $p<0.001$ respectively; Fig. 2A, B). The expression of ObR was higher in remitting MS patients than in healthy subjects, however without reaching a statistical significance (Fig. 2A, B). Moreover, we found that, mObR was expressed on 19.37% (± 4.79) of monocytes, 5.96% (±1.09) of CD8+ T cells and 1.05% (±0.62) of CD4+ T cells from MS patients during relapse (Fig. 3A–C). Conversely, in MS patients during remission only 3.23% (±0.8) of monocytes, 1.87% (±0.86) of CD8+ T cells and 0.40% (±0.26) of CD4+ T cells were mObR positive (Fig. 3A–C). In healthy subjects, the mObR was expressed on 2.36% (±0.85) of monocytes, 2.40% (±0.76) of CD8+ T cells and on 1.20% (±0.30) of CD4+ T cells (Fig. 3A–C). There was a significantly higher number of circulating CD8+ObR+ and CD14+ObR+ cells in relapsing than in remitting MS patients ($p=0.004$ and $p=0.015$, respectively; Fig. 3B and 3C) and in healthy subjects ($p=0.003$ and $p=0.031$, respectively; Fig. 3B and 3C). No difference was observed in circulating CD4+ObR+ T cell among relapsing or remitting RRMS patients and controls (Fig. 3A). There was no significant difference in CD4+ObR+, CD8+ObR+and CD14+ ObR+cell percentage between remitting RRMS patients and controls ($p=0.062$, $p=0.502$ and $p=0.722$, respectively) (Fig. 3A–C). Representative two-parameter plots of mObR

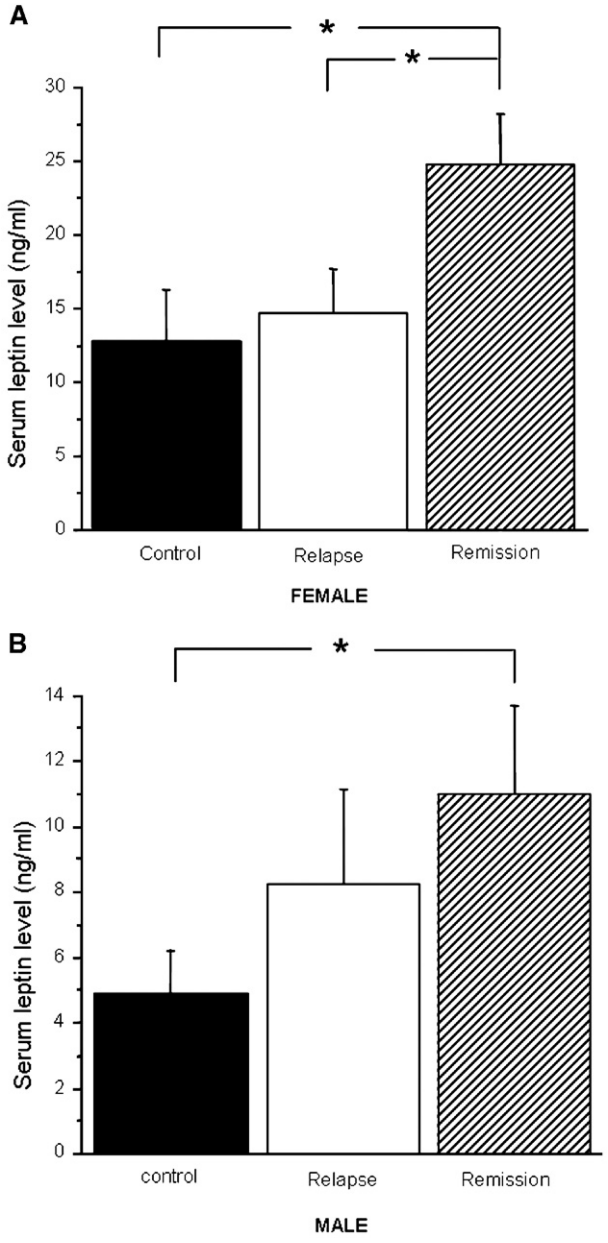

Fig. 1. Serum leptin values in healthy subjects and in RRMS patients according to gender and disease activity. (A) Serum leptin levels in 102 RRMS patients in different phases of disease and in 68 healthy subjects. (B) Serum leptin levels in 69 female RRMS patients and in 40 female controls. (C) Serum leptin levels in 33 male RRMS patients and in 28 male controls. Serum leptin levels in female (D) and male (E) controls, relapsing and remitting RRMS patients. Asterisks indicate statistical significances among groups: *$p<0.05$. The data are shown as histograms representing means±S.E.M.

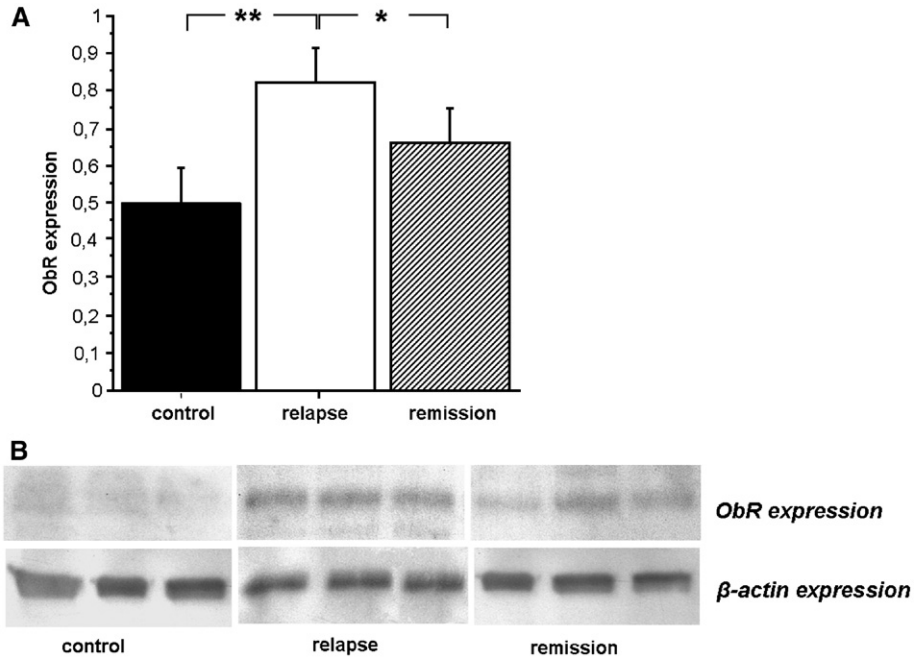

Fig. 2. ObR expressions in PBMC from healthy subjects and RRMS patients in different phases of disease. (A) Western Blot for long isoforms of ObR in homogenates of PBMC from 15 controls, 17 relapsing and 28 remitting RRMS patients. The relative quantity of each protein was estimated by density calculation of the corresponding band on scanned image using Totalab software. ObR expression values were obtained as ratios between ObR density and each actin density. Results are presented as means ± S.D. Asterisks indicate statistical significances among groups: *$p<0.05$; **$p<0.001$. The data are shown as histograms representing means ± S.E.M. (B) Representative blots of three different patients during relapse and remission and three different controls. The same membranes were reprobed with anti-actin antibodies as a control for loading.

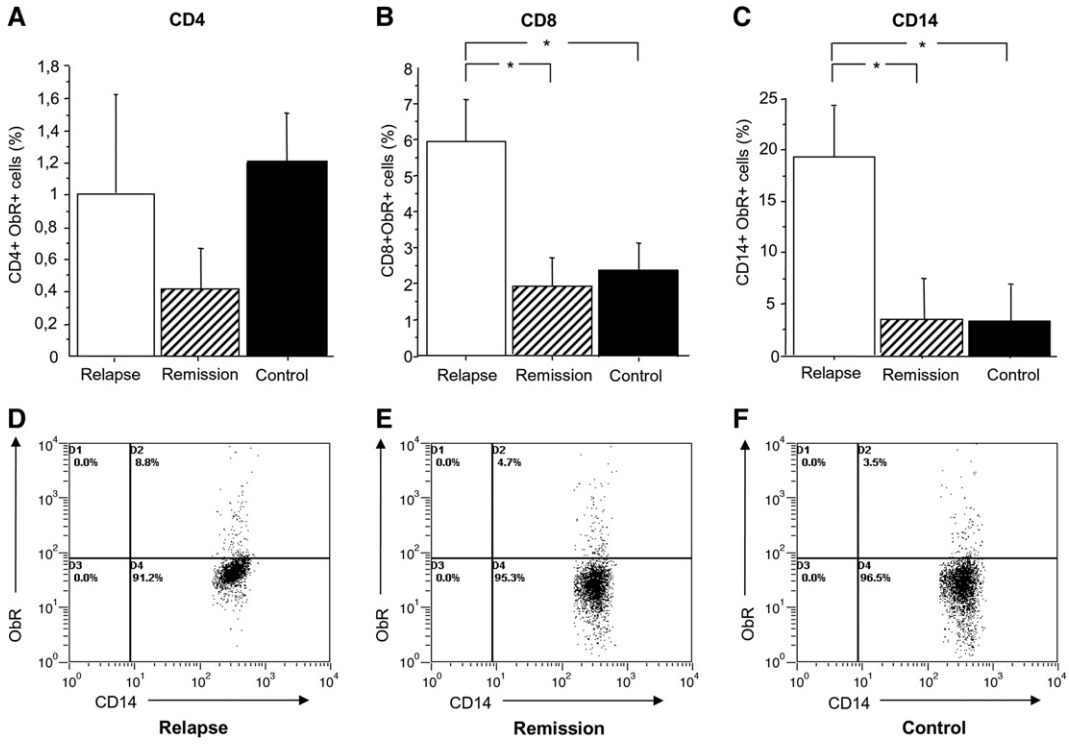

Fig. 3. Expression of mObR in CD4+, CD8+ and CD14+ cells from RRMS patients in different phases of disease. (A–C) Comparison of percentage of mObR+CD4+, mObR+CD8+ and mObR+CD14+ cells from controls, relapsing and remitting RRMS patients. Asterisks indicate statistical significances among groups: *$p<0.05$. (D–F) Representative two-parameter plots of mObR expression gated on CD14+in one RRMS patient in relapse (D), one in remission (E) and one healthy subject (F). Quadrants were set using appropriate isotype controls for each intra- and extracellular antibody.

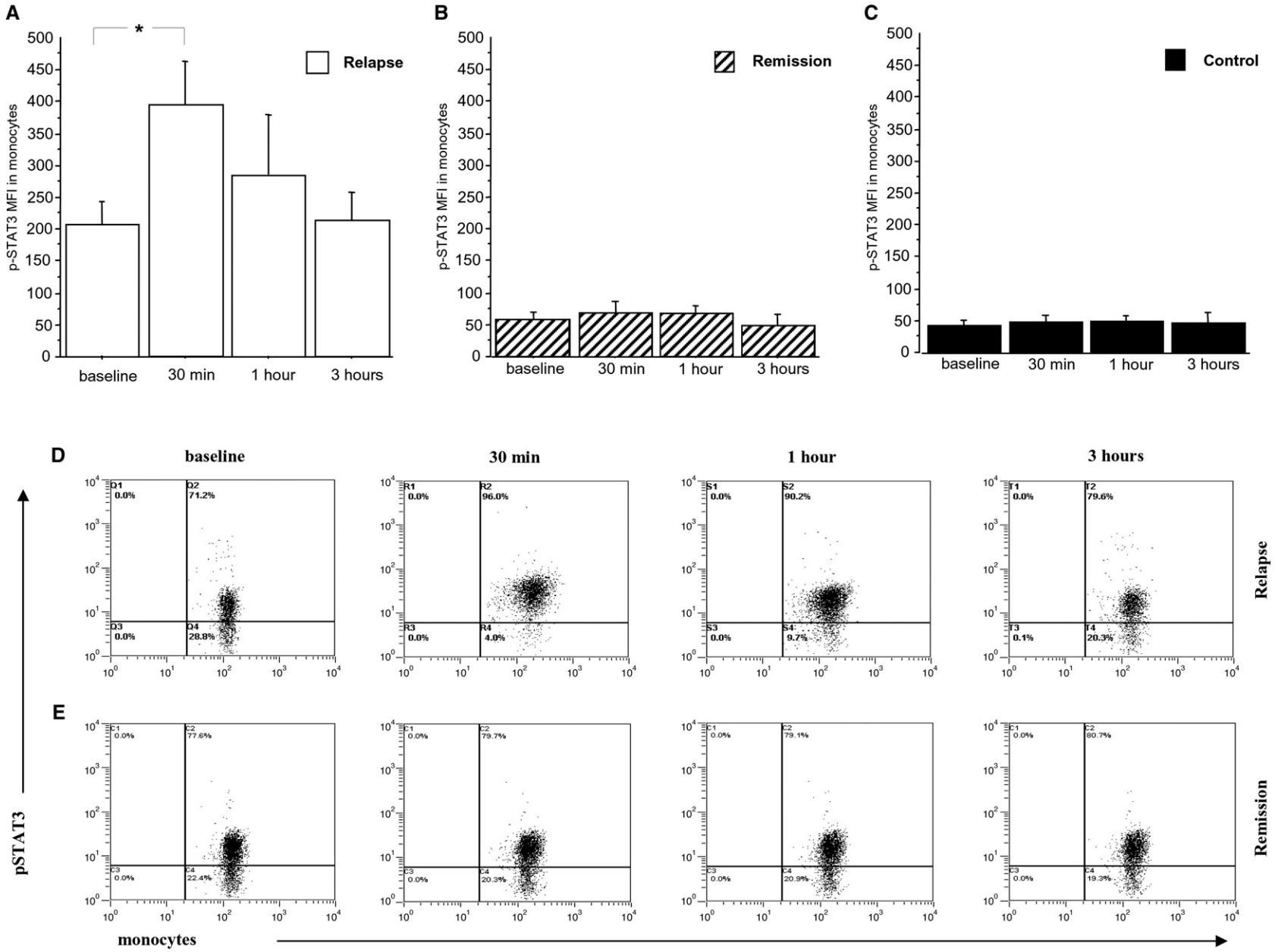

Fig. 4. p-STAT3 expression of PBMC from relapsing and remitting RRMS patients after incubation with leptin. PBMC from RRMS patients were incubated with 250 ng/ml of leptin and tested for p-STAT3 expression in monocytes before, 30 min, 1 h and 3 h after stimulation. Time course of mean p-STAT3 expression in monocytes from RRMS patients in relapse (A) and remission (B) and from healthy subjects (C) after leptin incubation. Asterisks indicate statistical significances among groups: $*p<0.05$. (D–E) Representative two-parameter plots of p-STAT3 and CD14 expressions gated on CD14+cells from two different RRMS patients in different phases of disease, one in relapse (D) and one in remission(E). Quadrants were set using appropriate isotype controls for each intra- and extracellular antibody.

expression gated on CD14+ in one RRMS patient in relapse (D), one in remission (E) and one healthy subject (F) are shown in Fig. 3D–F.

3.4. p-STAT3 expression in T cells and monocytes from MS patients and healthy subjects after leptin stimulation

In order to assess the sensitivity to leptin we evaluated by cytofluorimetric analysis the changes of pSTAT3 expression in monocytes from RRMS patients in different phases of disease activity after incubation of mononuclear cells with recombinant leptin. The baseline levels of pSTAT3 was significantly higher in monocytes from relapsing than remitting MS patients ($p<0.01$) and controls ($p<0.01$), while there was no difference between MS patients in remission and controls (Fig. 4A–C). The percentage of pSTAT3+ monocytes and the pSTAT3 mean florescence intensity on monocytes from relapsing MS patients increased 30 min after leptin treatment and declined to baseline approximately after 3 h (Fig. 4A, D). After leptin incubation we did not observe significant changes in pSTAT3+ cells or mean pSTAT3 expression in monocytes from RRMS patients in stable phase of disease (Fig. 4B, E) or controls (Fig. 4C).

3.5. IL6 production of PBMC from MS patients and healthy subjects after SOCS3 induction

To address whether leptin-induced IL-6 production can be modulated by SOCS3 expression, we incubated PBMC from RRMS patients in relapse and in remission and from controls with either IL10+LPS or medium alone. Basal levels of SOCS3 expression in PBMC were higher in MS patients in remission than in the relapsing ones ($p<0.001$) and controls ($p<0.001$) (Fig. 5A–C). After 3 h, we observed an increased expression of SOCS3 in PBMC treated with IL10/LPS both from MS patients in different phase of disease and from healthy subjects (Fig. 5A–C). There was no significant difference in basal IL-6 production by PBMC from RRMS patients in different phase of disease and controls (Fig. 5D–F). After leptin stimulation we observed a stronger increase of IL-6 production by PBMC from relapsing MS patients than remitting patients ($p=0<0.05$) and controls ($p<0.05$). (Fig. 5D–F). Therefore after addition of high dose of leptin, IL-6 production was higher in supernatants of PBMC incubated with medium alone than in those incubated with IL10+ LPS ($p<0.001$) (Fig. 5D–F). These data confirm that an increased expression of SOCS3 can inhibit, directly or by the modulation of

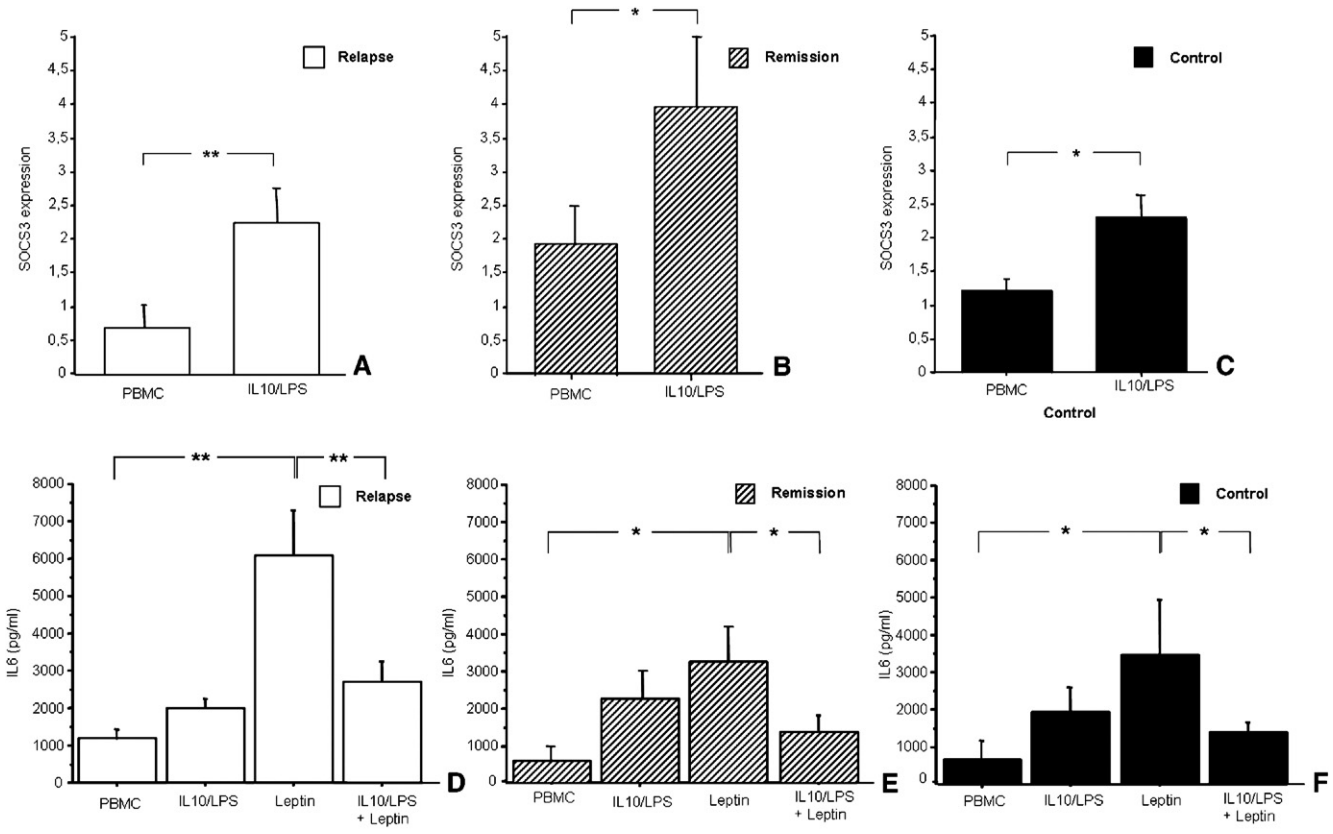

Fig. 5. SOCS3 expression and leptin-induced IL-6 production by PBMC pre-incubated with IL10 and LPS or medium alone. PBMC were cultured either with 100 U/mL IL10 and 100 ng/ml LPS or with medium alone for 3 h. Then PBMC were washed and stimulated with a high dose of leptin (500 ng/mL) or incubated with medium alone. After 3 h PBMC were lysated and separated by 10% SDS-PAGE and analyzed by immunoblotting with an anti-SOCS3 antibody and then with an anti-β-actin antibody. The supernatants were used to determine IL-6 levels by ELISA. (A–C) The histograms show the changes in SOCS3 expression after treatment of PBMC from relapsing (A) and remitting (B) RRMS patients and controls (C), with 100 U/mL IL-10+100 ng/mL LPS or with medium alone. (D–F) IL-6 levels in supernatants from PBMC from MS patients during relapse (D) and remission (E) and healthy subjects (F) pre-incubated with medium alone, IL10/LPS, Leptin or IL10/LPS+ Leptin. Asterisks indicate statistical significances among groups: *$p<0.05$; **$p<0.001$.

other cytokines, the effect of leptin on IL6 production both in MS patients and controls.

4. Discussion

In a previous study we found a higher PBMC sensitivity to leptin in RRMS patients in relapse than in patients in remission and in healthy subjects (Frisullo et al., 2004). To understand the molecular basis for this difference, we analyzed serum leptin, sObR levels as well as the expression of ObR, and the STAT3/SOCS3 signaling pathway and correlated them with disease activity of RRMS patients. Firstly, we observed that serum leptin levels were higher in RRMS patients than in sex, age and BMI matched controls. These data are in agreement with a recent report showing that leptin is increased both in the serum and the cerebrospinal fluid of RRMS patients naïve to treatment (Matarese et al., 2005). Two previous studies (Chatzantoni et al., 2004; Batocchi et al., 2003) reported comparable levels of serum leptin between RRMS patients and healthy subjects. This discrepancy may be due to different inclusion criteria (Batocchi et al., 2003) and to the small number of patients in remission tested (Chatzantoni et al., 2004). When we divided RRMS patients according to gender, both female and male patients showed higher serum leptin levels than controls. When considering disease activity, both male and female patients in remission showed higher serum leptin levels than male and female controls, respectively. These data are in agreement with our previous observation that serum leptin levels increased several weeks before relapse in RRMS patients during treatment with INF-beta and declined in the clinically active phase of disease (Batocchi et al., 2003). Also in EAE leptin surge precedes the onset of the disease and correlates with the development of pathogenic T cell response (Sanna et al., 2003). Since some authors (Liu et al., 1997) showed that sObR can inhibit binding to the mOBR functionally inactivating leptin, we evaluated serum sObR in RRMS patients and controls. In agreement with other authors (Chatzantoni et al., 2004), we did not find any difference in sObR levels between healthy subjects and RRMS patients in different phases of disease.

An up-regulation of ObR expression was found on mice T cells and monocytes after activation with anti-CD3 or endotoxin (Papathanassoglou et al., 2006) and on T cells of mice with chronic intestinal inflammation in which a selective deficiency of ObR led to delayed development of intestinal injury (Siegmund et al., 2004). ObR expression was higher on PBMC of HIV-infected subjects (Rottapel, 2001) and on T cells clones from RRMS patients after activation with human myelin basic protein (Matarese et al., 2005). The ObR expression on immune cells in patients with inflammatory diseases suggests a possible involvement of ObR in the mechanism of inflammation (Matarese et al., 2005; Sanchez-Margalet et al., 2002). In our study, at biochemical level, we observed an up-regulation of ObR in mononuclear cells from RRMS patients in relapse, but not in RRMS patients in remission and controls. By evaluating circulating ObR+ cells we found the highest ObR expression on monocytes of both RRMS patients in different phase of disease and controls. Relapsing RRMS patients had more CD14+ and CD8+ObR+ cells than patients in remission and controls. These data suggest that ObR could play a role in the pathogenesis of MS by up-regulating the immune response in the acute phase of the disease. Moreover, the high expression of mObR and the low levels of serum leptin during MS relapse suggest that therapy targeting ObR rather than leptin might be more effective in the treatment of MS. It is worth mentioning that a recent study has identified a monoclonal antibody directed against ObR, able to inhibit leptin signalling that acts as a leptin antagonist in vitro (Fateli et al., 2006).

It has been hypothesized that autoimmune diseases may result from a dysregulation of cytokine network when the right cytokine signal is not initiated and/or stopped at the appropriate time (Rottapel, 2001). SOCS3 is a member of a family of endogenous inhibitors that regulate, in part, the longevity of cytokine signaling transduced by the JAK/STAT pathway. The importance of SOCS3 in leptin action in vivo has recently been evaluated in mice with heterozygous SOCS3 deficiency showing both enhanced weight loss and increased hypothalamic leptin receptor signalling in response to exogenous leptin administration (Howard et al., 2004). To verify whether the higher leptin sensitivity of mononuclear cells of relapsing MS patients could be ascribed to a dysregulation of ObR/STAT3/SOCS3 signaling pathway, we performed an in vitro stimulation assay and observed that basal pSTAT3 levels was higher in monocytes from relapsing RRMS patients than remitting ones and controls. Moreover the addition of high leptin dose induced an up-regulation of pSTAT3 expression only in patients in relapse even if we can not exclude the possibility of a overall higher level also of total STAT3 in these patients. These data are in agreement with our previous study where we observed an up-regulation of pSTAT3 in CD4+, CD8+ T cells and in monocytes from relapsing RRMS patients (Frisullo et al., 2006). Considering that STAT3 is activated by the binding of a variety of pro-inflammatory cytokines, including leptin, IL-6, and IL-23 (Yang et al., 2007) to their receptors, the higher pSTAT3 expression observed in relapsing MS patients suggests that the engagement of an up-regulated leptin receptor in the acute phase of disease could enhance the inflammatory process. On the other hand we observed lower basal levels of SOCS3 in PBMC from relapsing MS patients than remitting ones and controls. The addition of leptin increased IL-6 production only in PBMC from relapsing MS patients, while SOCS3 up-regulation, induced by IL10+LPS, inhibited IL-6 production in all three groups. These data suggest that the SOCS3-mediated negative feed-back mechanism of regulation of leptin is apparently preserved in RRMS patients. The low levels of SOCS3 in PBMC from relapsing MS patients can be interpreted on the light of a recent in vitro study, in which it was observed that antigen stimulation of Th cells induced a dramatic but transient decline in SOCS3 expression after 48 h (Yu et al., 2004). Thus, in relapsing MS patients, the binding of TCR to putative antigens could induce a transitory decrease of SOCS3 expression enhancing the cytokine sensitivity of the activated T cells and the production of proinflammatory cytokines. A recent study (Stark and Cross, 2006) which suggests that cytokine autoregulatory mechanisms involving SOCS may play a role in determining the course of EAE seems to corroborate our data.

In conclusion, our study indicates that membrane ObR and SOCS3 expression modulates leptin sensitivity in RRMS patients. Although additional studies are needed, our data further suggest that the ObR/STAT3/SOCS3 pathway may play a critical role in the pathogenesis of MS.

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