Leptin increase in multiple sclerosis associates with reduced number of $CD4^+CD25^+$ regulatory T cells

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We analyzed the serum and cerebrospinal fluid (CSF) leptin secretion and the interaction between serum leptin and $CD4^+CD25^+$ regulatory T cells (T_{Regs}) in naïve-to-therapy relapsing-remitting multiple sclerosis (RRMS) patients. Leptin production was significantly increased in both serum and CSF of RRMS patients and correlated with IFN-γ secretion in the CSF. T cell lines against human myelin basic protein (hMBP) produced immunoreactive leptin and up-regulated the expression of the leptin receptor (ObR) after activation with hMBP. Treatment with either anti-leptin or anti-leptin-receptor neutralizing antibodies inhibited *in vitro* proliferation in response to hMBP. Interestingly, in the RRMS patients, an inverse correlation between serum leptin and percentage of circulating T_{Regs} was also observed. To better analyze the finding, we enumerated T_{Regs} in leptin-deficient (*ob*/*ob*) and leptin-receptor-deficient (*db*/*db*) mice and observed the significant increase in T_{Regs}. Moreover, treatment of WT mice with soluble ObR fusion protein (ObR:Fc) increased the percentage of T_{Regs} and ameliorated the clinical course and progression of disease in proteolipid protein peptide ($PLP_{139-151}$)-induced relapsing-experimental autoimmune encephalomyelitis (R-EAE), an animal model of RRMS. These findings show an inverse relationship between leptin secretion and the frequency of T_{Regs} in RRMS and may have implications for the pathogenesis of and therapy for multiple sclerosis.

autoimmunity | tolerance | metabolism | hormones

It has recently been shown that leptin, a cytokine-like hormone mainly secreted by adipocytes, can play a significant role in the pathogenesis of several autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE), antigen-induced arthritis, and experimentally induced colitis (1–6). EAE is an animal model for the human demyelinating disease multiple sclerosis (MS) (7, 8). EAE can be induced in susceptible strains of mice through immunization with myelin antigens or by adoptive transfer of myelin-specific T helper 1 (Th-1) cells (7, 8). Genetically leptin-deficient (*ob*/*ob*) mice are resistant to the induction and progression of EAE, and WT EAE-susceptible mice show an increase in serum leptin preceding the clinical onset of the disease that correlates with inflammatory anorexia and disease susceptibility (2, 3). In addition, pathogenic Th-1 cells and macrophages in active EAE brain lesions secrete consistent amounts of leptin (3). These data account for an involvement of leptin in CNS autoimmunity, at least in the EAE model. Despite this finding, in humans, the role of leptin in the pathogenesis of MS is not yet fully elucidated. In this study, we analyzed the secretion of leptin in the cerebrospinal fluid (CSF) and serum of naïve-to-treatment relapsing–remitting MS (RRMS) patients and leptin's interaction with the $CD4^+CD25^+$ regulatory T cells (T_{Regs}). T_{Regs} are known to dampen autoreactive responses mediated by $CD4^+CD25^-$ T cells and may influence the onset and progression of autoimmunity (9). In mice, depletion of T_{Regs} is associated with autoimmunity, and defects of T_{Regs} have been described in nonobese diabetic mice and in humans with type 1 diabetes (9, 10). Also, reduced frequency of T_{Regs} and/or defective suppressor function have been observed in humans with systemic lupus erythematosus, juvenile idiopathic arthritis, autoimmune polyglandular syndrome type II, and RRMS (11–15). T_{Regs} are anergic *in vitro* but can expand *in vivo* (9). Although the mechanisms operated by T_{Regs} in suppression are not fully delineated, the forkhead transcription factor FoxP-3 seems to be required for suppression to occur (9).

Here, we report increased leptin levels in CSF and serum of naïve-to-therapy RRMS patients and an inverse correlation with T_{Regs} frequency. These findings may be relevant in better understanding the disease pathogenesis and may have therapeutic implications.

Materials and Methods

Subjects. All MS patients and controls were recruited at the Università di Napoli "Federico II." For serum and CSF leptin measurement, we included in the study 126 individuals (Table 1) with MS defined according to the criteria of McDonald *et al.* (16) and 117 age-, gender-, and body mass index (BMI)-matched controls with other noninflammatory neurologic disorders (NIND). All MS patients had RRMS and an expanded disability status scale score of $\leq$3.5 and were naïve to treatment. The inclusion criteria for RRMS patients were (*i*) onset of relapse within 4 weeks of serum/CSF collection, (*ii*) a history of at least two clinical relapses during the preceding 2 years, and (*iii*) the presence of one or more enhancing lesions on MRI at the time of entry into the study. NIND included Parkinson's disease, spinocerebellar degeneration, amyotrophic lateral sclerosis, brain tumors, cranial trauma, nonspecific headache, and hydrocephalus. We additionally included, for only serum leptin measurement and the immune-phenotypic analysis, 27 donors who were healthy age-, sex-, and BMI-matched with the RRMS and NIND populations. None of the NIND and healthy controls had a history of autoimmune disorders, infection, or endocrine disease. The study was approved by the institutional ethics committee and all individuals gave written informed consent.

Leptin and IFN-γ Measurement. All serum and CSF samples were collected at 8:30 a.m. after overnight fast and stored at −80°C. For leptin measurement, a human leptin ELISA kit (R & D Systems) was used according to the manufacturer's instructions. Human IFN-γ and IL-4 were measured in the CSF, in parallel with leptin, with ELISA kits (Endogen, Cambridge, MA).

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Abbreviations: BMI, body mass index; CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalomyelitis; hMBP, human myelin basic protein; MS, multiple sclerosis; NIND, noninflammatory neurologic disorders; ObR, leptin receptor; ObR:Fc, fusion protein of ObR and Fc fragment of IgG; $PLP_{139-151}$, proteolipid protein peptide 139–151; R-EAE, relapsing EAE; RRMS, relapsing–remitting MS; Th-1, T helper 1; T_{Regs}, regulatory T cells.

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Table 1. Anthropometric parameters, leptin, and IFN-γ measurements in RRMS patients and NIND controls

Parameter measured	RRMS patients	NIND controls	*P*
No. of patients	126	117	
Sex (male/female)	58/68	52/65	
Age, yr	36.5 ± 9.5	38.2 ± 15.5	0.30
Height, m	1.65 ± 0.07	1.66 ± 0.07	0.52
Mass, kg	65.9 ± 9.6	65.4 ± 10.7	0.65
BMI, kg/m^2	24.1 ± 3.3	23.4 ± 3.2	0.12
Serum leptin, pg/ml	21,517.0 ± 15,676.0	11,727.0 ± 13,057.0	0.0001
Serum leptin/BMI	900.0 ± 650.0	488.6 ± 482.0	0.0001
CSF leptin, pg/ml	1,143.1 ± 1,389.5	205.3 ± 222.5	0.0001
CSF leptin/BMI	47.7 ± 57.3	8.2 ± 8.7	0.0001
CSF leptin/serum leptin	0.09 ± 0.18	0.03 ± 0.06	0.001
CSF leptin/CSF albumin	$5.7 \pm 6.5 \times 10^{-6}$	$1.2 \pm 2.1 \times 10^{-6}$	0.0001
CSF leptin index*	20.7 ± 53.4	7.0 ± 12.2	0.008
CSF IFN-γ, pg/ml	3.9 ± 3.1	0.45 ± 1.3	0.0001

*The CSF leptin index is a measure of *in situ* synthesis of leptin in the CNS, calculated with the following formula: (CSF leptin/CSF albumin)/(serum leptin/serum albumin).

Flow Cytometry. Immunophenotypic analysis of peripheral blood from RRMS patients and healthy controls was performed with an EPICS XL flow cytometer (Beckman Coulter) using the Beckman Coulter software program XL SYSTEM II. Triple combinations of various anti-human mAbs were used (Coulter Immunotech, Marseille, France). All samples were analyzed within 3–4 h of sampling, and staining was performed according to standard procedures as described in ref. 17.

$CD4^+CD25^+$ T_{Regs} in various mouse strains were analyzed by flow cytometry with a FACSCalibur flow cytometer (Becton Dickinson) and the Becton Dickinson software program CELLQUEST. mAbs were added to single cell suspensions of lymphocytes obtained from spleens and lymph nodes after lysis with the ACK buffer [0.15 M NH_4Cl/10 mM $KHCO_3$/0.1 mM Na_2EDTA (pH 7.4)]. The analysis and quantification of the T_{Regs} population was obtained by gating on $CD4^+$ T cells.

Human Myelin Basic Protein (hMBP) T Cell Lines. hMBP-specific short-term T cells lines were generated according to a method reported in ref. 18. The T cell lines were derived from peripheral blood lymphocytes of three naïve-to-treatment RRMS patients.

Proliferation and Suppression Assays. For *in vitro* blocking experiments, Abs against human leptin provided by Radek Sokol (BioVendor, Brno, Czech Republic) and mAb against the human leptin receptor (R & D Systems) were used at a final concentration of 10–25 μg/ml; the control was irrelevant IgG Ab (BioVendor).

The *in vitro* suppressive capacity of T_{Regs} isolated from RRMS patients and healthy controls was measured after magnetic cell sorting by using the Dynal $CD4^+CD25^+$ T_{Reg} kit (Dynal, Oslo). Briefly, $CD4^+CD25^-$ T cells (5×10^4 cells per well) were cocultured with $CD4^+CD25^+$ (5×10^4 cells per well) in a 1:1 ratio (both 98% pure) and stimulated for 3 d in the presence of anti-CD3/CD28 Dynabeads (0.1 bead per cell) (Dynal). In mice, T_{Regs} were isolated with the Regulatory T Cell Isolation kit (Miltenyi Biotec, Gladbach, Germany) and stimulated with anti-CD3 antibody (2C11 hybridoma) at 200 ng/ml final concentration and irradiated (30 Gy) T cell-depleted syngeneic splenocytes (1:1 ratio, 5×10^4 cells per well).

Immunocytochemistry. T cells cultured, or not, with hMBP were washed twice with PBS on d 5 of culture, spotted onto glass slides, and fixed with methanol for 2 min. Leptin and ObR were detected with polyclonal Abs (Santa Cruz Biotechnology) (3).

Mice. Female *ob/ob* (C57BL6/J-*ob/ob*), WT controls (C57BL6/J-WT), female leptin-receptor-deficient (*db/db*) mice (C57BL-Ks-*db/db*), C57BL-Ks-*db/+* controls (*db/+*), and SJL/J mice (all 6–8 weeks old) were obtained from Harlan Italy (Corezzana, Italy). Experiments were performed following the guidelines of the Istituto Superiore di Sanità, Rome.

EAE Induction and Treatment with the Fusion Protein of ObR and the Fc Fragment of IgG (ObR:Fc). The peptide used for EAE induction in SJL/J female mice was the proteolipid protein peptide $(PLP)_{139–151}$ (HSLGKWLGHPDKF). The peptide was synthesized by INBIOS (Pozzuoli, Italy), purity was assessed by HPLC (>97% pure), and amino acid composition was verified by mass spectrometry. For EAE induction, mice were immunized s.c. in the flank with 100 μl of complete Freund's adjuvant (Difco) emulsified with 100 μg of $PLP_{139–151}$ peptide on d 0 and with 200 ng of pertussis toxin (Sigma) i.p. on d 0 and d 1. Mice were scored for clinical symptoms and weighed daily according to a system described in refs. 2 and 3. Brains and spinal cords were dissected 15–20 d after immunization and fixed in 10% formalin. Paraffin-embedded sections of 5 μm thickness were stained with hematoxylin/eosin, and sections from 4–10 segments per mouse were examined blindly for the number of inflammatory foci by using a scoring system described in ref. 3.

The chimeric fusion protein ObR:Fc (R & D Systems) in 200 μl of PBS was injected i.p. at a dose of 100 μg per mouse per day for three consecutive days. Thus, treatment with ObR:Fc of SJL/J mice was performed on d −1, d 0, and d +1 both before and after $PLP_{139–151}$ immunization. The same amount of control IgG was injected i.p. in the control SJL/J mice.

Real-Time Quantitative PCR. mRNA was extracted from purified $CD4^+CD25^+$ cells (98% pure by FACS analysis) by using the MicroFastTrack 2.0 kit followed by cDNA synthesis with the SuperScript System (Invitrogen). Expression levels of the transcription factor FoxP3 were analyzed by real-time quantitative PCR (TaqMan gene expression assay) by using an ABI PRISM 7700 thermal cycler (Applied Biosystems). TaqMan primers and probes for FoxP3 and for the housekeeping gene GAPDH were purchased as premade kits (Applied Biosystems). For relative quantitation of gene expression to the endogenous control, the comparative C_T method was used in accordance with the manufacturer's guidelines. Results are expressed as the percentage of FoxP3 increase compared with $CD4^+CD25^-$ effector T cells.

Statistical Analysis. Nonparametric analyses were performed by using the Mann–Whitney *U* test for unrelated two-group analyses. The ANOVA test was used to assess differences between groups.

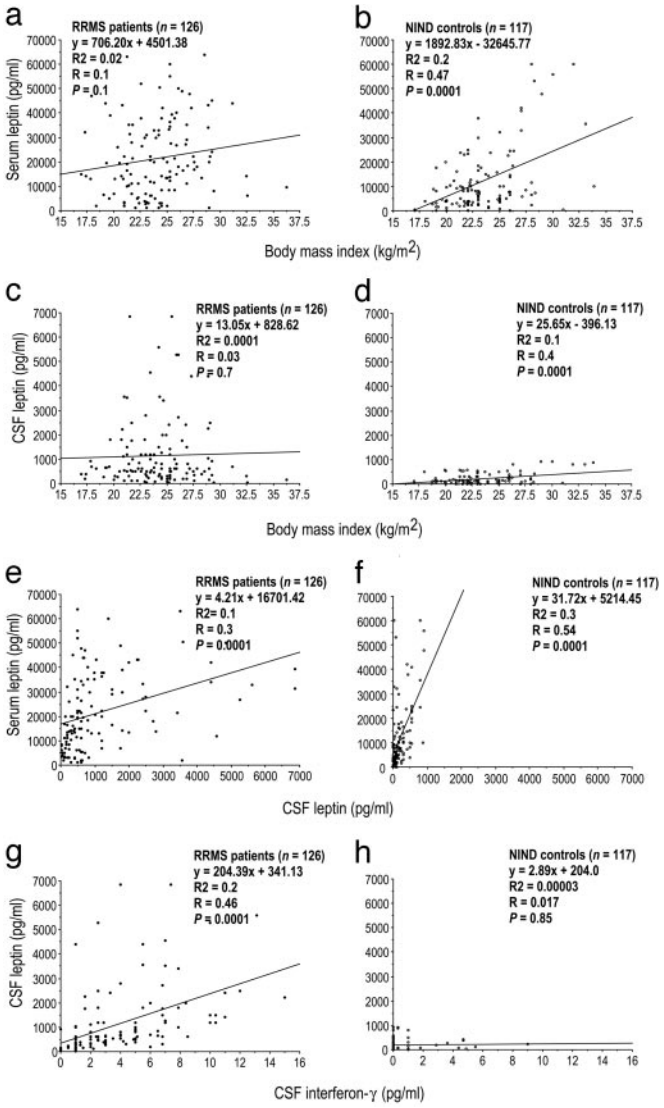

Fig. 1. Naïve-to-therapy RRMS patients show an increased secretion of leptin in serum and CSF that correlates with IFN-γ production in CSF. Statistical analyses of these data are summarized in Table 1. (*a* and *b*) Simple regression analysis between serum leptin and BMI in RRMS patients ($n = 126$) and NIND controls ($n = 117$). The correlation was lost in RRMS patients, whereas correlation was maintained in NIND controls. (*c* and *d*) The correlation between CSF leptin and BMI was lost in RRMS patients whereas correlation was very strong in NIND controls. (*e* and *f*) Significant correlation between serum and CSF leptin in both RRMS patients and NIND controls; the correlation was stronger in patients than in controls. (*g* and *h*) Simple regression analysis between the CSF leptin and the IFN-γ levels in CSF in both RRMS patients and NIND controls. Only in RRMS patients was there a statistically significant positive correlation between the CSF leptin and the IFN-γ levels in CSF. In NIND controls, the IFN-γ average levels were very low (see Table 1), and no correlation was observed with CSF leptin.

Simple regression analysis and the Pearson's correlation coefficients were adopted to study the relationship between different variables. The program used was STATVIEW (Abacus Concepts, Cary, NC). Results are expressed as mean ± SD; $P < 0.05$ was considered statistically significant.

Results

Increased Serum and CSF Leptin in Naïve-to-Treatment RRMS Patients Correlates with IFN-γ Production in CSF.

We found that leptin was increased in both serum and CSF of naïve-to-therapy RRMS patients (Table 1 and Fig. 1 *a–d*). These differences were maintained even when serum and CSF leptin were normalized for BMI (Table 1 and Fig. 1 *a–d*). In addition, as expected, serum and CSF leptin secretion positively correlated with BMI in NIND controls (Fig. 1 *b* and *d*). This correlation was lost in RRMS patients (Fig. 1 *a* and *c*). Conversely, the correlation between serum leptin and CSF leptin was maintained in both RRMS patients and NIND controls; however, this correlation was stronger in NIND controls than in RRMS patients (Fig. 1 *e* and *f*). We also compared the CSF-leptin/serum-leptin ratio and observed a statistically significant increase of this value in RRMS (Table 1). This evidence was further supported by the lack of increase of albumin in the CSF of RRMS patients, a marker of blood–brain-barrier (BBB) damage. In addition, we calculated the CSF-leptin/CSF-albumin ratio as a further indicator of BBB integrity and the CSF leptin index, calculated as the (CSF leptin/CSF albumin)/(serum leptin/serum albumin), to evaluate the *in situ* production of leptin by CNS. As shown in Table 1, the CSF-leptin/CSF-albumin ratio and the CSF leptin index were higher in RRMS patients (Table 1), suggesting the production of leptin by CNS in RRMS.

Finally, we measured the amount of IFN-γ and IL-4 in CSF and observed a significant increase in IFN-γ (Table 1) and a positive correlation with CSF leptin secretion in RRMS patients only (Fig. 1 *g* and *h*). IL-4 did not show any significant increase in CSF, and the concentration of IL-4 was always below the detection limit of the assay in both RRMS and NIND controls (data not shown).

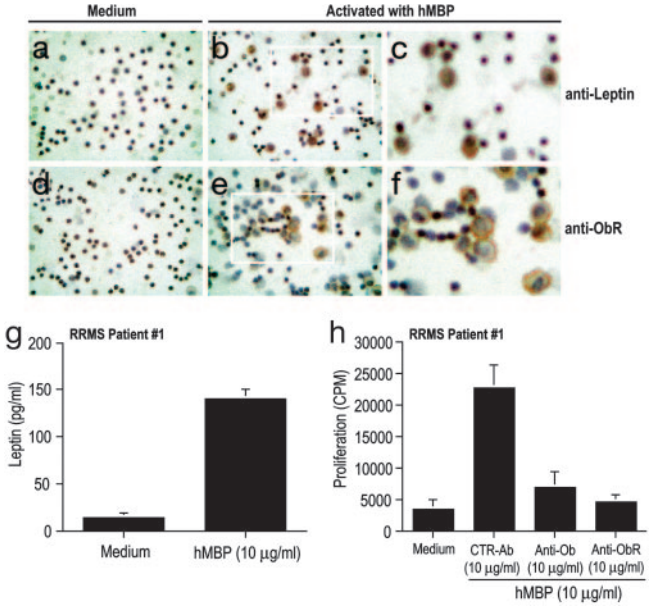

Fig. 2. T cell lines against hMBP derived from naïve-to-treatment RRMS patients produce immunoreactive leptin, up-regulate the ObR, and are inhibited in their proliferation by anti-leptin or anti-leptin-receptor blocking antibodies. (*a–c*) Expression of leptin in T cells from a naïve-to-treatment RRMS patient in the presence of medium alone (*a*) or after activation with hMBP (*b* and *c*). Leptin was detectable only after activation in the cytoplasm of T cells. (*d–f*) Expression of ObR on T cells in the presence of medium only (*d*) or after activation with hMBP (*e* and *f*). The ObR was expressed at very low levels before activation and was significantly up-regulated on the cell membrane after activation with hMBP (*e* and *f*). All photos show immunoperoxidase staining with diaminobenzidine chromogen (brown) and hematoxylin counterstaining (violet). The open squares in *b* and *e* represent the zone of higher magnification shown in *c* and *f*, respectively. (Magnification: *a*, *b*, *d*, and *e*, ×100; *c* and *f*, ×400.) (*g*) Anti-hMBP short-term T cell lines secrete immunoreactive leptin. (*h*) The anti-hMBP proliferative response of T cells is inhibited by the addition to cell cultures of either of the anti-Ob or anti-ObR antibodies. The data shown are from one representative experiment of three.

RRMS Patient-Derived T Cell Lines Activated with hMBP Produce Immunoreactive Leptin and Up-Regulate the ObR. To investigate whether leptin could be secreted by hMBP-activated autoreactive T cells present in the CNS, we generated short-term T cell lines from RRMS patients and stained them with leptin- and ObR-specific antibodies. As shown in Fig. 2 *a–f*, hMBP-activated T cells from three naïve-to-therapy RRMS patients produced consistent amounts of leptin and up-regulated the ObR. The production of leptin was also confirmed with the measurement of immunoreactive leptin in the culture medium by a human-leptin-specific ELISA (Fig. 2*g*).

Neutralization of Leptin or Its Receptor Inhibits T Cell Activation of hMBP-Specific T Cell Lines Derived from RRMS Patients. We measured the proliferative response against hMBP on T cells from three naïve-to-treatment RRMS patients and added either an anti-leptin or an anti-leptin-receptor blocking antibody to the culture medium (Fig. 2*h*). We observed a significant reduction in the proliferative response of all three patients tested, ranging from 45% to 60% inhibition of proliferation (Fig. 2*h*).

Inverse Correlation Between Serum Leptin and Circulating T_{Regs} in Naïve-to-Treatment RRMS Patients. The analysis of the immune phenotype was also performed on the peripheral blood of 31 individuals from the naïve-to-therapy RRMS patient population, selected on the basis of increase in serum leptin concentration (a serum leptin increase to ≥2.5-fold higher than the mean serum leptin observed in NIND and healthy controls). We compared these phenotypes with the immune phenotype of 27 healthy controls matched for age, sex, and BMI. The relative percentage and the absolute cell count per mm^3 of the $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, $CD3^-CD16^+CD56^+$, and $CD4^+CD25^+$ T_{Regs} subpopulations were performed (see Table 3, which is published as supporting information on the PNAS web site). Interestingly, naïve-to-therapy RRMS patients, selected on the basis of their serum leptin increase, showed a significant reduction in the percentage and absolute number of T_{Regs} in the peripheral blood (Fig. 3*a* and Table 3), whereas no difference was observed in the frequency of the other cell subpopulations (Table 3). T_{Regs} measurement in healthy controls was in agreement with that found in other studies (14). Regression analysis between serum leptin and the percentage of T_{Regs} showed an inverse correlation in RRMS patients (Fig. 3*b*) but not in the controls (Fig. 3*c*). *In vitro* analysis of T_{Regs}-mediated suppression in RRMS patients indicated a reduced ability to suppress T cell proliferation as compared with healthy controls (Fig. 3*d*), as reported in ref. 15. Moreover, the addition of leptin (100 ng/ml) to human T_{Regs} alone, or during coculture with $CD4^+CD25^-$ effectors, did not alter significantly either proliferation or the suppressive capacity of T_{Regs} (see Fig. 5 *a* and *b*, which is published as supporting information on the PNAS web site).

***ob*/*ob* and *db*/*db* Mice Have Increased T_{Regs}.** To analyze in more detail the effect of leptin on the generation of T_{Regs} in the periphery, we measured the effect of chronic leptin deficiency on the number of T_{Regs} in *ob*/*ob* mice. These mice showed an increased frequency of T_{Regs} in lymphoid organs when compared with normal WT mice (10.4 ± 3.7% vs. 4.7 ± 1.7%, respectively; $P < 0.02$). In addition, we counted T_{Regs} in the lymphoid organs of *db*/*db* mice and, again, observed an increased percentage of T_{Regs} when compared with *db*/+ heterozygote controls (13.9 ± 1.9 vs. 7.9 ± 0.9, respectively; $P < 0.01$). Finally, the suppressive capacity and phenotype of T_{Regs} from *db*/*db* mice were evaluated. No significant differences were observed in terms of either suppressive capacity or hyporesponsiveness of T_{Regs} (see Fig. 6 *a–c*, which is published as supporting information on the PNAS web site). In addition, expression levels of FoxP3 in T_{Regs} of *ob*/*ob* and *db*/*db* mice were comparable to those in normal control mice (Fig. 6*d*).

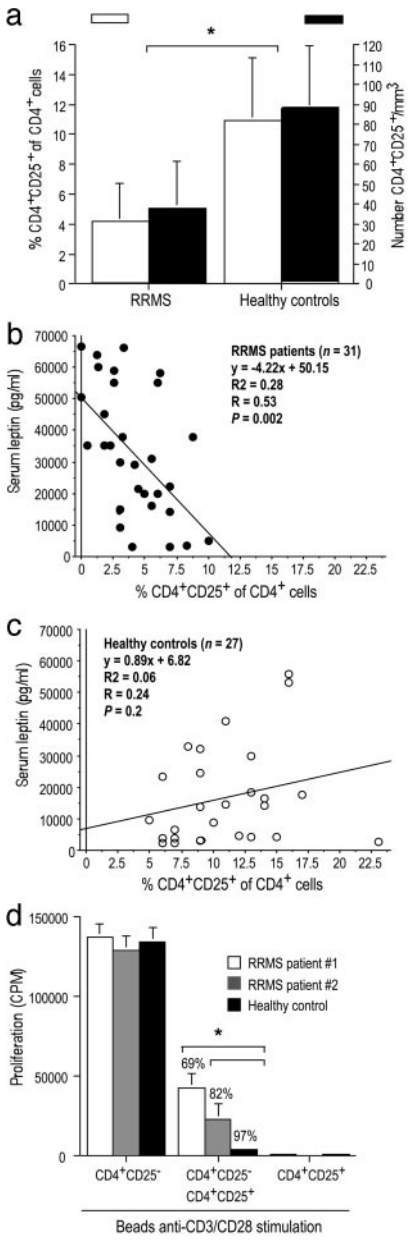

Fig. 3. Inverse correlation between serum leptin and circulating T_{Regs} in RRMS patients. (*a*) The immune phenotype of circulating lymphocytes in RRMS patients selected on the basis of their increase in serum leptin (RRMS patients with a serum leptin increase to ≥2.5-fold higher than the mean of serum leptin observed in NIND and healthy controls) revealed a significant reduction in the percentage and the absolute number of circulating $T_{Regs.}$ (*, $P = 0.0001$ and *, $P = 0.0001$, respectively). (*b* and *c*) A statistically significant inverse correlation was observed between serum leptin and circulating T_{Regs} in RRMS patients (*b*), whereas no correlation was observed in healthy controls (*c*). (*d*) Functional analysis of $CD4^+CD25^+$ T_{Regs} of two RRMS patients selected on the basis of an increase in serum leptin. The proliferative response was inhibited upon addition of $CD4^+CD25^+$ cells to the $CD4^+CD25^-$ responder population at a 1:1 ratio in normal controls (black bars). $CD4^+CD25^+$ cells from two naïve-to-therapy patients with RRMS exhibited significantly less suppressor activity (white and gray bars). *, $P = 0.03$. $CD4^+CD25^+$ cells alone were unresponsive upon stimulation as reported in ref. 9. The numbers above the bars represent the percent of inhibition of proliferation in the experiment. The data shown are from one representative experiment of five.

ObR:Fc Soluble Chimera Increases the Number of T_{Regs} and Ameliorates Clinical Course and Progression of Relapsing EAE (R-EAE). Treatment of normal R-EAE-susceptible SJL/J mice with anti-leptin

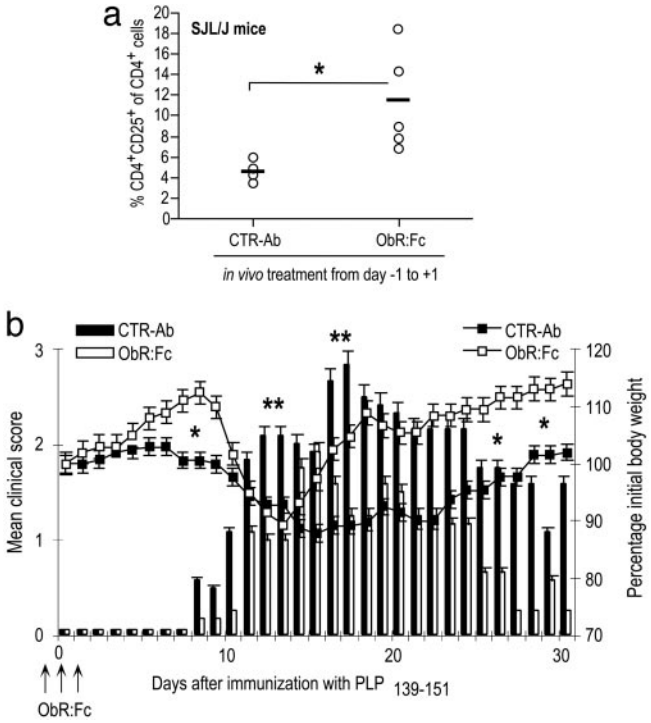

Fig. 4. Neutralization of leptin with ObR:Fc increases the number of T_{Regs} and ameliorates the clinical course of R-EAE. (*a*) Treatment of R-EAE-susceptible SJL/J female mice with ObR:Fc induced a significant increase in the circulating T_{Regs}. *, $P = 0.01$. (*b*) Mean clinical score (bars) and body weight (curves) of SJL/J female mice pretreated with the ObR:Fc (white bars and squares) or the CTR-Ab (black bars and squares) on d −1, d 0, and d +1 and immunized with the $PLP_{139–151}$ on d 0. Statistical analyses of these data are summarized in Table 2. The data shown are from one representative experiment of two ($n = 6$ mice per group). *, $P = 0.01$; **, $P = 0.002$.

blocking ObR:Fc soluble chimera induced an increase of the percentage of T_{Regs} in the periphery (Fig. 4*a*). To test whether this treatment could also modify the induction/progression of R-EAE, we pretreated SJL/J female mice with ObR:Fc chimera before immunization with the encephalitogenic peptide $PLP_{139–151}$. The treatment was performed from d −1 to d +1 with i.p. injection of 100 μg per day per mouse of ObR:Fc chimera dissolved in PBS (Fig. 4*b*). ObR:Fc-treated mice showed a reduced peak clinical score, an improvement in disease relapses and progression, and a reduction in the percentage of body weight loss (Fig. 4*b* and Table 2). Moreover, a significant increase in body weight before/during the early phases of disease in ObR:Fc-treated mice (on d 9, the percentage of initial body weight in ObR:Fc-treated mice was 112.4 ± 0.9% vs. 100.6 ± 0.7% in control (CTR)-Ab-treated mice, $P = 0.01$) was observed, compared with a classical reduction in body weight preceding the onset of clinical symptoms in CTR-Ab-treated mice. In addition, ObR:Fc-treated mice showed very rapid reduction of body weight after d 10 and a rapid recovery after d 13 of disease to a weight that was significantly higher than that of CTR-Ab-treated mice (Fig. 4*b* and Table 2). On the contrary, control mice showed a more stable body weight loss that was maintained over the disease course (Fig. 4*b* and Table 2). Finally, CNS inflammatory lesions were also significantly reduced in ObR:Fc-treated mice (Table 2). A significant increase in T_{Regs} was observed on d 15 of the disease course in mice pretreated with ObR:Fc (Table 2).

Discussion

In this report, we analyze the secretion of leptin in serum and CSF of naïve-to-treatment RRMS patients in correlation with the secretion of IFN-γ in CSF and the percentage of circulating T_{Regs}. The data presented here provide evidence that a significant increase of leptin secretion occurs in the acute phase of MS and that this event positively correlates with the CSF production of IFN-γ. Increased secretion is present in both the serum and CSF of RRMS patients and determines the loss of correlation between leptin and BMI (Fig. 1 *a* and *c*). Moreover, the increase of leptin in the CSF is higher than that in the serum (a 5.6-fold increase in CSF leptin vs. a 1.8-fold increase in serum leptin, $P = 0.0001$, Table 1), possibly secondary to *in situ* synthesis of leptin in the CNS and/or an increased transport across the blood–brain barrier, upon enhanced systemic production. Indeed, the CSF-leptin/serum-leptin ratio, the CSF-leptin/serum-albumin ratio, and the CSF leptin index all significantly increase in RRMS patients when compared with NIND controls (Table 1).

Recently, gene-microarray analysis of Th-1 lymphocytes and active MS lesions in humans revealed elevated transcripts of many genes of the neuroimmunoendocrine axis, including leptin (19, 20). Leptin's transcript was also abundant in the gene-expression profile of human Th-1 clones, demonstrating that the leptin gene is induced in and associated with polarization toward Th-1 responses, commonly involved in T cell-mediated autoimmune diseases such as MS (19, 20). We previously reported *in situ* leptin secretion by inflammatory T cells and macrophages in active EAE lesions (3). Here, we show that autoreactive hMBP-specific T cells from RRMS patients can produce immunoreactive leptin and up-regulate the leptin receptor after activation (Fig. 2 *a–f*), possibly explaining, in part, the increased *in situ* CSF leptin levels in RRMS patients. Interestingly, both anti-leptin and anti-leptin-receptor blocking antibodies reduced the proliferative responses of hMBP-specific T cell lines (Fig. 2 *h*, *j*, and *l*), underscoring the possibilities of leptin-based intervention on this autocrine loop.

Many questions need to be answered about whether and how T_{Regs} can regulate autoimmunity in humans. In animal models of autoimmune diseases, the role of T_{Regs} has been demonstrated (21). More recently, a reduced function and/or generation of T_{Regs} in human autoimmune diseases such as systemic lupus erythematosus, type 1 diabetes, autoimmune polyglandular syndrome type II,

Table 2. Effect of pretreatment with soluble ObR:Fc chimera on neurological impairment and percentage of $CD4^+CD25^+$ during active R-EAE induction with the $PLP_{139-151}$ encephalitogenic peptide in SJL/J female mice

Group of mice	Antigen	Incidence, no./total (%)	Day of onset (range)	Peak clinical score	Average CDI*	Percentage of initial body weight at disease peak	No. of inflammatory foci	Percentage of $CD4^+CD25^+$ after treatment
SJL/J CTR-Ab (d −1 to d+1)	$PLP_{139–151}$	6/6 (100.0)	8.1 ± 0.4 (8–9)	2.8 ± 0.7	42.7 ± 7.9	89.4 ± 0.5	30.8 ± 1.8	4.5 ± 0.7
SJL/J ObR:Fc (d −1 to d+1)	$PLP_{139–151}$	6/6 (100.0)	10.6 ± 2.0 (8–13)	1.9 ± 0.7†	21.8 ± 5.3‡	108.2 ± 0.7‡	15.0 ± 1.5‡	11.3 ± 4.3†

The data shown are from one representative of two independent experiments shown in Fig. 4*b*. CTR-Ab, control Ab.
*Cumulative disease index, sum of daily scores determined for each mouse of that group and averaged.
†$P = 0.01$.
‡$P = 0.002$.

juvenile idiopathic arthritis, and MS has been described in refs. 10–15. Recently, this reduction has been shown, in RRMS, to be a functional defect of T_{Regs} rather than a reduced number of T_{Regs} in the periphery (15). To address whether leptin secretion could have an effect on T_{Regs} in RRMS patients, we measured the T_{Regs} frequency in the peripheral blood of naïve-to-treatment RRMS patients selected on the basis of an increase in serum leptin to ≥2.5-fold higher than levels measured in NIND and healthy controls. Here, we show that the average percentage and the absolute number of T_{Regs} in these RRMS patients were significantly lower than those of healthy controls (Fig. 3*a* and Table 3). No significant differences in $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, and $CD3^-CD16^+CD56^+$ cells were observed in either study group (Table 3). In addition, our functional data confirmed that, in our experimental conditions, RRMS patients showed a functional T_{Regs} defect, confirming findings previously reported in ref. 15 (Fig. 3*d*). Administration of exogenous leptin to human T_{Regs} or to suppression assays did not alter hyporesponsiveness and suppressive capacity (Fig. 5 *a* and *b*), suggesting that *in vitro* leptin is not responsible for impaired T_{Regs} function. Simple regression analysis showed an inverse correlation between systemic leptin concentrations and T_{Regs} in the naïve-to-treatment RRMS population (Fig. 3 *b* and *c*). These data demonstrate an inverse relationship between leptin and T_{Regs} in MS and may account for a reduced generation of T_{Regs}, at least early in the disease, in naïve-to-treatment patients. Indeed, we hypothesize that, after therapy, these phenomena may be masked and overcome by therapy-induced effects. In fact, in the case of chronic leptin deficiency, such as in *ob/ob* mice, we found an increased number of circulating T_{Regs}, and similar results were observed in *db/db* mice. This finding was also confirmed by experiments showing a higher recovery and percentage of T_{Regs} from R-EAE-susceptible SJL/J female mice treated with leptin-blocking ObR:Fc (Fig. 4*a*). Also, this pretreatment subsequently ameliorated R-EAE onset and progression (Fig. 4*b* and Table 2). The fact that T_{Regs} from *db/db* mice had a similar suppressive capacity and phenotype compared with T_{Regs} from normal controls (Fig. 6 *a–d*) suggests that leptin does not affect *in vitro* suppressive function but, rather, *in vivo* expansion/proliferation of T_{Regs}. Further studies need to address this point. Recent reports have shown increased secretion of serum leptin before relapses in RRMS patients during treatment with IFN-β and the capacity of leptin to enhance *in vitro* secretion of TNF-α, IL-6, and IL-10 by peripheral blood mononuclear cells of RRMS patients in the acute phase of the disease but not in patients in the stable phase (22, 23). In view of the above considerations, we suggest that, in MS, leptin may be part of a wider scenario in which several proinflammatory soluble factors may act in concert in driving the pathogenic (autoreactive) Th-1 responses targeting neuroantigens (24). Recently, Hafler *et al.* (15) reported a decrease in the effector function and cloning frequency of T_{Regs} from the peripheral blood of patients with MS. We show here that, in naïve-to-therapy MS patients, not only the function but also the number of T_{Regs} is affected, and, more importantly, the finding inversely correlates with the concentration of serum leptin. It appears therefore that, early in the disease, the effects on T_{Regs} in MS may be different from the effect observed after therapy has been initiated. Regarding the correlation with leptin, it is worth mentioning that strains of mice prone to the spontaneous development of autoimmune diseases, such as nonobese-diabetic (NOD) and IL-2-deficient ($IL\text{-}2^{-/-}$) mice, show reduced frequency of T_{Regs} in the periphery (9) associated with abnormal leptin responses due to increased serum leptin concentrations (disproportionate to fat mass) (25, 26). NOD mice have higher basal serum leptin levels than normal age-, sex-, and fat-matched controls (25). $IL\text{-}2^{-/-}$ mice are prone to spontaneous development of inflammatory bowel disease and other autoimmune disorders (26). Whereas in normal mice, serum leptin decreases with fat-mass loss, in $IL\text{-}2^{-/-}$ mice there is a paradoxical rise in serum leptin compared with control mice, even after starvation, which reduces serum leptin (26). These data support the hypothesis that a disproportionate response in leptin secretion can correlate with a reduction in the periphery of the T_{Regs} compartment in these two models.

Because of the influence of leptin on food intake and metabolism, the findings reported here underscore the role of molecules at the interface between metabolism and immunity in the control of not only inflammation but also autoimmune reactivity (24, 27). Recently, molecules with orexigenic activity, such as ghrelin and neuropeptide Y (NPY), have been shown to mediate not only effects opposite to those of leptin on the hypothalamic control of food intake but also on peripheral immune responses (28, 29). Indeed, ghrelin blocks the leptin-induced secretion of proinflammatory cytokines by human T cells (28), and NPY ameliorates the clinical course and progression of EAE (29). Given these considerations, we may envisage a situation in which the influences exerted by several metabolic regulators, including leptin, might broadly influence vital functions not limited to caloric tuning but, rather, affecting immune responses and the interaction of the individual with the environment. Although additional studies are needed, our data provide direct evidence of a negative association between leptin secretion and T_{Regs} in the early stages of an autoimmune disease characterized by Th-1 autoreactivity, such as MS.

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