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"Leptin and autoimmunity: a key role of leptin in the control of regulatory T cell proliferation"

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“Leptin and autoimmunity: a key role of leptin in the control of regulatory T cell proliferation”

Table of Contents

LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

1) Matarese G., Carrieri P.B., La Cava A., Perna F., Sanna V., De Rosa V., Aufiero D., Fontana S., Zappacosta S. Leptin increase in multiple sclerosis associates with reduced number of CD4(+)CD25+ regulatory T cells. Proc Natl Acad Sci U S A. 102(14): 5150-5, 2005.

2) Matarese G., De Rosa V., Aufiero D., Procaccini C., Alviggi C., De Placido G., Fontana S., Zappacosta S. Adipokines, Metabolism and the Immune Response in the Regulation of Inflammation. Curr. Med. Chem. Anti-Inflamm. Anti- Allergy Ag. 4: 619-624, 2005.

3) De Rosa V., Procaccini C., La Cava A., Chieffi P., Nicoletti G.F., Fontana S., Zappacosta S., Matarese G. Leptin neutralization interferes with pathogenic T cell autoreactivity in autoimmune encephalomyelitis. J Clin Invest. 116(2): 447-455, 2006.

4) Chan J.L., Matarese G., Shetty G.K., Raciti P., Kelesidis I., Aufiero D., De Rosa V., Perna F., Fontana S., Mantzoros C.S. Differential regulation of metabolic, neuroendocrine, and immune function by leptin in humans. Proc Natl Acad Sci U S A. 103(22): 8481-6, 2006.

5) Farooqi IS, Wangensteen T, Collins S, Kimber W, Matarese G, Keogh JM, Lank E, Bottomley B, Lopez-Fernandez J, Ferraz-Amaro I, Dattani MT, Ercan O, Myhre AG, Retterstol L, Stanhope R, Edge JA, McKenzie S, Lessan N, Ghodsi M, De Rosa V, Perna F, Fontana S, Barroso I, Undlien DE, O'Rahilly S. Clinical and molecular genetic spectrum of congenital deficiency of the leptin receptor. N Engl J Med. 356(3): 237-47, 2007.

6) De Rosa V., Procaccini C., Calì G., Pirozzi G., Fontana S., Zappacosta S., La Cava A., Matarese G. A key role of leptin in the control of regulatory T cell proliferation. Immunity 26(2): 241-55, 2007.

7) Matarese G., De Rosa V., La Cava A. Regulatory CD4 T cells: sensing the environment. Trends Immunol. In press 2007.

ABSTRACT

Leptin is an adipocyte-derived hormone/cytokine that links nutritional status with neuroendocrine and immune functions. As a hormone, leptin regulates food intake and basal metabolism, and is sexually dimorphic — that is, its serum concentration is higher in females than in males with a similar body fat mass. As a cytokine, leptin can affect thymic homeostasis and the secretion of acute-phase reactants such as interleukin-1 (IL-1) and tumour-necrosis factor-α (TNF-α). Similar to other pro-inflammatory cytokines, leptin promotes T helper 1 (Th1)-cell differentiation and can modulate the onset and progression of autoimmune responses in several animal models of disease.

We report here that leptin can act as a negative signal for the proliferation of human naturally occurring Foxp3+CD4+CD25+ regulatory T (Treg) cells. Indeed, freshly isolated Treg cells produced leptin and expressed high amounts of leptin receptor (ObR). *In vitro* neutralization with leptin monoclonal antibody (mAb), during anti-CD3 and anti-CD28 stimulation, resulted in Treg cell proliferation, which was interleukin-2 (IL-2) dependent. Treg cells that proliferated in the presence of leptin mAb had increased expression of Foxp3 and remained suppressive. The phenomena appeared secondary to leptin signalling via ObR and, importantly, leptin neutralization reversed the anergic state of the Treg cells, as indicated by downmodulation of the cyclindependent kinase inhibitor p27 (p27kip1) and the phosphorylation of the extracellular-related kinases 1 (ERK1) and ERK2. Together with the finding of enhanced proliferation of Treg cells observed in leptin- and ObR-deficient mice, these results suggest a potential for therapeutic interventions in immune and autoimmune diseases.

1. Background

1.1 Leptin

Living organisms require a relatively steady energy supply to sustain biological functions. Moreover, energy reserves must not only be sufficient to serve all physiological needs, but must also be wisely allocated to a wide variety of often competing physiological functions. Energy intake and energy expenditure undergo substantial daily and seasonal fluctuations, however.

Immunity requires adequate and balanced energy supply for optimal function. Although the risk of infection and death is highest when energy reserves are not sufficient, obesity, a state of energy excess, has also been associated with increased susceptibility to infection, bacteremia, and poor wound healing (Samartin and Chandra 2001).

The discovery of the adipocyte-derived hormone leptin, the levels of which reflect the amount of energy stored in the adipose tissue and are altered by conditions such as fasting and overfeeding, has proved to be fundamental to our understanding of the concept of energy availability influencing several physiological systems. More specifically, the past few years of research on leptin — the product of the obese (ob) gene — have provided important insights into the intricate network that links nutrition, metabolism and immune homeostasis (Friedman and Halaas 1998). Leptin is mainly produced by the adipose tissue in proportion to the body fat mass and, at lower levels, by tissues such as the stomach, skeletal muscle and placenta. Although an important role of leptin is to regulate body weight through the inhibition of food intake and stimulation of energy expenditure by increased thermogenesis, recent evidence has indicated that leptin is much more than a 'fat-o-stat' sensor (Matarese et al. 2002). Indeed, leptin-deficient (ob/ob) mice and leptin-receptor-deficient (db/db) mice are not only severely obese, but also have a series of marked abnormalities that are secondary to the effects of leptin on reproduction (Chehab et al. 1996), haematopoiesis (Bennet et al. 1996), angiogenesis (Sierra-Honigmann et al. 1998), insulin secretion (Friedman and Halaas 1998), metabolism of bone (Ducy et al. 2000), lipids and glucose and, last but not least, innate and adaptive immunity.

1.2 Leptin as a neuroendocrine and immune mediator

Leptin is a 16-kDa nonglycosylated protein encoded by the *obese* (*ob*) gene, which is located on human chromosome 7 and on mouse chromosome 6. In both humans and mice, mutations of the *ob* gene are associated with hyperphagia

and obesity, reduced energy expenditure, and other reproductive, neuroendocrine, and metabolic dysfunction. Serum leptin is usually higher in obese individuals and has a strong sexual dimorphism, being higher in females than males matched by age and body weight (Friedman and Halaas 1998).

Leptin is classically considered a hormone because it regulates the balance between food intake and energy expenditure, signaling to the brain the changes in stored energy. Serum leptin is directly correlated with the body fat stores, increasing with fat accumulation, and decreasing during fasting. Leptin gene expression is regulated by several factors, including other hormones. Insulin stimulates leptin secretion during feeding, while a decrease in insulin levels anticipates a fall in leptin during starvation. Glucocorticoids also operate synergistically with insulin in the secretion of leptin from cultured adipocytes, although an inverse relationship between leptin and glucocorticoids is generally observed (Friedman and Halaas 1998). Finally, leptin expression is inhibited by testosterone, increased by ovarian sex steroids, and directly influences the hypothalamic-pituitary-adrenal axis, the reproductive system, hematopoiesis, and angiogenesis.

Many studies have linked the immune and neuroendocrine systems. Physiological responses to stress usually involve finely integrated interactions between the autonomic nervous system and the hypothalamo-pituitary-adrenal (HPA) axis and the immune system and metabolism (Turnbull and Rivier 1999, Addad et al. 2002). For example, peripheral inflammation stimulates the central release of corticotrophin-releasing hormone (CRH), which in turn regulates the stress response through the production of adrenocorticotrophic hormone (ACTH) — a hormone that promotes the synthesis and release of glucocorticoids from the adrenal glands. The glucocorticoids — hormones that get their name from their ability to raise levels of blood glucose — have potent anti-inflammatory effects and dampen humoral and cell-mediated immune responses. Interestingly, mediators that are common to the neuroendocrine and immune systems, such as the cytokines interleukin-1 (IL-1), IL-6 and tumour-necrosis factor (TNF), can all modulate inflammation through the HPA axis (Turnbull and Rivier 1999, Addad et al. 2002). Indeed, these peripherally derived cytokines can cross the blood–brain barrier and act on the hypothalamus and pituitary gland to regulate the secretion of ACTH in response to inflammation. These cytokines also mediate a negative feedback on their own peripheral pro-inflammatory activity and are counter-regulated by endogenous glucocorticoids produced by the HPA axis.

Leptin is one of the mediators that are common to the neuroendocrine and immune systems (Zhang et al. 1994). In

immune system, leptin, together with c-reactive protein (CRP), IL-1 and IL-6, can act as an early acute-phase reactant, produced at high levels during inflammation, sepsis and fever, and it can be induced by other inflammatory mediators such as TNF and IL-1 (Friedman and Halaas 1998, Landman et al. 2003, Orbak et al. 2003). However, although these findings have been demonstrated in several systems, other studies have not found increased leptin in inflammatory conditions in humans, including acute experimental endotoxaemia, newborn sepsis, HIV infection and during anti-inflammatory therapy (Bornstein et al. 1998, Koc et al. 2003, Yarasheski et al. 1997).

The neuroendocrine role of leptin is most evident in conditions such as fasting — during which the production of leptin by adipose tissue is markedly reduced — or in relation to the effects of sex hormones on its production (testosterone reduces the secretion of leptin, whereas oestrogens increase its production). The link between leptin and sex hormones is also indicated by the marked gender dimorphism, manifested by a higher serum concentration in females than in males with similar body fat mass (Friedman and Halaas 1998).

The fact that leptin has effects on both neuroendocrine and immune systems should not come as a surprise, given the functional connection and anatomical contiguity between adipocytes and lymphoid cells (Matarese et al. 2002). Morphologically, aggregations of lymphoid tissue, including the lymph nodes, omentum, thymus and bone marrow, are associated with adipose tissue (Matarese et al. 2002). Fat deposits do not simply have a structural, metabolic and heat-insulating function, but provide a microenvironment that helps the immune system to sustain immune responses. In particular, lymphoid and adipose tissue interact locally through common mediators known as adipokines — adipocyte-derived molecules that bridge metabolism and immune homeostasis (these molecules include leptin, adiponectin, chemokines and other pro-inflammatory cytokines).

1.3 Leptin signaling in immune cells

Leptin, as previously mentioned, is mainly secreted by the adipose tissue, which is also present within both primary and secondary lymphoid organs and has a significant metabolic and immunomodulatory role (Laharrague et al. 1998). Leptin's three-dimensional structure is similar to that of a cytokine consisting of a four α helix bundle motif which is common to the IL-6, IL-12, IL-15 family of cytokines (Zhang et al. 1997) (indeed leptin belongs to the family of long-chain helical cytokines). Leptin receptor (ObR), is also a member of the class I cytokine receptor superfamily and has at least six

isoforms as a result of alternative splicing with cytoplasmatic domains of different length, known as OBRa, OBRb, OBRc, OBRd, OBRe and OBRf (Tartaglia et al. 1995, Tartaglia et al. 1997). These receptors are membrane-spanning glycoproteins with fibronectin type III domains in the extracellular region and with a shared 200-amino-acid module containing four conserved cysteine residues and two membrane proximal cytokine-like binding motifs, Trp-Ser-Xaa-Trp-Ser. The short forms of the leptin receptor are expressed by several non-immune tissues and seem to mediate the transport and degradation of leptin. The long form of OBR, known as OBRb, is expressed by the hypothalamus in areas that are responsible for the secretion of neuropeptides and neurotransmitters that regulate appetite, body weight (Friedman and Halaas 1998, Tartaglia et al. 1995, Tartaglia et al. 1997) and bone mass. Interestingly, OBRb is also expressed by endothelial cells, pancreatic β cells, the ovary, $CD34^+$ haematopoietic bone-marrow precursors, monocytes/macrophages, T and B cells (Friedman and Halaas 1998, Park et al. 2001, Sierra-Honigmann et al. 1998, Ducy et al. 2000, Lord et al. 1998, Sancez-Margalet et al. 2003, Tartaglia et al. 1995, Tartaglia et al. 1997).

After binding leptin, OBRb-associated Janus-family tyrosine kinase 2 (JAK2) becomes activated by auto- or cross-phosphorylation and tyrosine phosphorylates the cytoplasmic domain of the receptor. Four of the phosphorylated tyrosine residues function as docking sites for cytoplasmic adaptors such as signal transducer and activator of transcription (STAT) factors, particularly STAT3 (in some cases, also STAT1 and STAT5) (Banks et al. 2000, Bjorbaek et al. 2001, Sweeney et al. 2002). The membrane distal tyrosine (position 1138) functions as a docking site for STAT3, which is a substrate of JAK2. After subsequent dimerization, STAT3 translocates to the nucleus and induces the expression of suppressor of cytokine signalling 3 (SOCS3) and other genes. SOCS3 takes part in a feedback loop that inhibits leptin signalling by binding to phosphorylated tyrosines. SRC homology 2 (SH2) domain-containing phosphatase 2 (SHP2) is recruited to Tyr985 and Tyr974, and activates extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) pathways through the adaptor protein growth factor receptor-bound protein 2 (GRB2), ultimately inducing the expression of FOS and JUN. After leptin binding, JAK2 can induce phosphorylation of the insulin receptor substrate 1/2 (IRS1/2) proteins that are responsible for the activation of phosphatidylinositol 3-kinase (PI3K) (Tartaglia et al. 1995, Tartaglia et al. 1997, Banks et al. 2000, Bjorbaek et al. 2001, Sweeney et al. 2002, Martin-Romero and Sanchez-Margalet 2001, Van den Brink et al. 2000). Moreover, Src associated in mitosis protein (Sam68),

an RNA-binding protein, regulator of RNA metabolism and effector of the PI3K is currently thought to function as an adaptor protein by binding to activated STAT-3 and to the p85 subunit of PI3K. Phosphotyrosine phosphatase 1B (PTP1B), which is localized on the surface of the endoplasmic reticulum, is involved in negative regulation of OBRb signalling through the dephosphorylation of JAK2 after internalization of the OBRb complex.

1.4 Leptin in innate and adaptive immunity

Mice lacking leptin or its functional receptor have a number of defects in both cell-mediated and humoral immunity (Chandra et al. 1980, Mandel et al. 1978). Similarly, humans with congenital leptin deficiency have a much higher incidence of infection-related death during childhood (Ozata et al. 1999), whereas recombinant human leptin (rmetHuLeptin) administration in two children with congenital leptin deficiency normalized absolute numbers of naive CD4CD45RA T cells and nearly restored the proliferation response and the cytokine release profile from their lymphocytes (Farooqui et al. 2002). A number of studies in mice have shown that the effect of leptin on the immune system is both direct and indirect, i.e., via modulation of central or peripheral pathways. Leptin has a well-established role in all cells involved in innate immunity, which “inflexibly” senses either specific pathogen-associated molecular patterns, formally not expressed by host tissues, or endogenous molecules released from “stressed” cells. In particular, leptin seems to promote activation of and phagocytosis by monocytes/macrophages and their secretion of leukotriene B4 (LTB4), cyclooxygenase 2 (COX2), nitric oxide and pro-inflammatory cytokines (Zarkesh-Esfahani et al. 2001, Dixit et al. 2003). The products of the inducible form of COX2 — prostaglandins and leukotrienes (also known as eicosanoids) — as well as nitric oxide, are all involved in the regulation of inflammation, chemotaxis and cytokine production, and therefore markedly impact the immune response. Moreover, leptin can induce chemotaxis of neutrophils and the release of oxygen radicals (such as superoxide anion and hydrogen peroxide) (Caldefie-Chezet et al. 2003, Zarkesh-Esfahani et al. 2004).These mediators can be particularly harmful to cells, as they can denature proteins and damage membrane lipids (by peroxidation of unsaturated fatty acids), carbohydrates and nucleic acids. At least in human neutrophils, leptin seems to mediate its effects through an indirect mechanism, probably involving the release of TNF from monocytes (Zarkesh-Esfahani et al. 2004). Leptin also affects natural killer (NK)-cell development and activation both *in vitro* and *in vivo* (Siegmund et al. 2002).

As NK cells express OBRb and *db/db* mice have a deficit of NK cells resulting from abnormal NK-cell development, it is possible that leptin might influence the development/maintenance of a normal peripheral NK-cell pool. Indeed, an important role of OBRb in NK-cell physiology is indicated by the ability of OBRb to influence NK-cell cytotoxicity through direct activation of signal transducer and activator of transcription 3 (STAT3) and the transcription of genes encoding IL-2 and perforin (Siegmund et al. 2002, Zhao et al. 2003, Tian et al. 2002). Last but not least, it has recently been shown that leptin can stimulate the production of growth hormone by peripheral-blood mononuclear cells (PBMCs) through protein kinase C (PKC)- and nitric oxide-dependent pathways (Dixit et al. 2003). This effect of leptin on the production of growth hormone might be important in immune homeostasis, given the fact that this cytokine-like hormone has marked influences on immune responses by controlling the survival and proliferation of immune cells. The effects of leptin on adaptive immune responses have been extensively investigated on human CD4+ T cells. Addition of physiological concentrations of leptin to a Mixed Lymphocytes Reaction (MLR) induces a dose-dependent increase in CD4+ T-cell proliferation (Lord et al. 1998). However, leptin has different effects on proliferation and cytokine production by human naive (CD45RA+) and memory (CD45RO+) CD4+ T cells (both of which express OBRb). Leptin promotes proliferation and IL-2 secretion by naive T cells, whereas it minimally affects the proliferation of memory cells (on which it promotes a bias towards Th1-cell responses). Furthermore, leptin increases the expression of adhesion molecules, such as intercellular adhesion molecule 1 (ICAM1, CD54) and very late antigen 2 (VLA2, CD49B), by CD4+ T cells, possibly through the induction of pro-inflammatory cytokines such as interferon-α (IFN-α). Increased expression of adhesion molecules could then be responsible for the induction of clustering, activation and migration of immune cells to sites of inflammation (Lord et al. 1998). Another important role of leptin in adaptive immunity is highlighted by the observation that leptin deficiency in *ob/ob* mice is associated with immunosuppression and thymic atrophy — a finding similar to that observed in acute starvation (Lord et al. 1998). Acute caloric deprivation causes a rapid decrease of serum leptin concentration accompanied by reduced Delayed-Type-Hypersensitivity (DTH) responses and thymic atrophy, which are reversible with administration of leptin (Lord et al. 1998, Howard et al. 1999, Lord et al. 2001). The thymic atrophy in *ob/ob* mice (or wild-type starved animals) affects the cortex of the thymus, in which most CD4+CD8+ T cells are found, and leptin replacement reduces the rate of apoptosis of such cells (Howard et al. 1999). Despite the evidence of direct effects of leptin on immune responses *in vitro*, a major problem remains

whether leptin can influence immune responses *in vivo*. This task is particularly difficult because of the complexity of the network of interactions that link leptin to several endocrine pathways. For example, the immune abnormalities associated with high cortisol levels and hyperglycaemia in obese *ob/ob* or *db/db* mice could simply be a consequence of obesity rather than direct effects of leptin (Lord et al. 1998, Howard et al. 1999). To help clarify this issue, studies of food restriction, which can reduce cortisol and glucose levels in *ob/ob* mice, have shown that only leptin replacement can fully restore normal immune responses in *ob/ob* mice, whereas experimentally induced reduction of serum levels of cortisol and glucose cannot reverse immune deficiency abnormalities. Although still controversial, these observations seem to indicate that the immune abnormalities in *ob/ob* mice cannot be simply ascribed to high circulating levels of cortisol and glucose, and that leptin might instead have direct effects on the immune system that are independent of the metabolic abnormalities associated with leptin (Lord et al. 1998, Howard et al. 1999). Another important issue is whether the effects of leptin during acute starvation can be ascribed to its central or peripheral actions. Recent experiments have shown that peripheral leptin has a dominant role in maintaining T-cell-mediated immune responses in rodents. Direct injection of leptin into the central nervous system (CNS) cannot compensate for the immunosuppression that is associated with starvation-induced hypoleptinaemia (Zhang et al. 2002). These findings might have therapeutic implications, as they indicate a lack of direct action of leptin on the CNS for reversal of starvation-induced immunosuppression, despite the beneficial effects of leptin on hormonal stress associated with starvation (reduced secretion of ACTH and glucocorticoids). In this regard, it is notable that T cells are sensitive to the supply of cellular nutrients, such as glucose (Frauwirth et al. 2002), because they do not have glycogen stores and, therefore, depend on the import of extracellular glucose to meet their metabolic needs (Khaled et al. 2002). By stimulating glucose uptake through extracellular signal-regulated kinase 1 (ERK1)/ERK2- and phosphatidylinositol 3-kinase (PI3K)-dependent pathways, leptin might help to restore the impaired T-cell function caused by starvation (Lord et al. 1998). In this context, it is worth mentioning that other long-chain helical cytokines similar to leptin (such as IL-3, IL-7 and IL-15) are important in promoting the uptake and metabolism of glucose (Khaled et al. 2002). In the case of IL-3, withdrawal of this cytokine from the culture medium leads to decreased lymphocyte uptake of glucose through reduced cell-surface expression of glucose transporter proteins. It can be hypothesized that similar effects might occur in the case of leptin, given the fact that leptin can also regulate cellular expression of the same transporters (Berti and Gammeltoft

1999). The effects on lymphocyte metabolism are also important because they are linked in part to pro-survival effects in both physiological and pathological conditions (such as in autoreactivity). For example, leptin might help to promote lymphocyte survival by upregulating lymphocyte surface expression of glucose transporters, such as GLUT1 and GLUT4. Other pro-survival effects of leptin could result from the ability of leptin to upregulate expression of the anti-apoptotic proteins BCL-2 and BCL-XL (which protect T cells from apoptosis and thymocytes from glucocorticoid-induced apoptosis) (Fujita et al. 2002, Shimabukuro et al. 1998, Najib and Sanchez-Margalet 2002).

1.5 Leptin in organ-specific autoimmunity: multiple sclerosis and type 1 diabetes

Considering that congenital deficiency of leptin can associate with increased frequency of infection and related mortality (Ozata et al. 1999), it was hypothesized that a low concentration of serum leptin might contribute to increased susceptibility to infection by reducing T helper cell priming and by affecting thymic function (Lord et al. 1998, Howard et al. 1999). On the contrary, the Th1-promoting effects of leptin have been linked with clarity to an enhanced susceptibility to develop experimentally induced autoimmune disease including experimental autoimmune encephalomyelitis (EAE), type 1 diabetes (T1D), and antigen-induced arthritis (AIA) (Ozata et al. 1999). This aspect is also of interest in relation to the well-known gender bias in susceptibility to autoimmunity. Autoimmune diseases are frequently more prevalent in females, and females are relatively hyperleptinemic, whereas males are relatively hypoleptinemic (Friedman an Halaas 1998,Ozata et al. 1999). It has been speculated that leptin could in part contribute to the gender-biased susceptibility to autoimmunity, and some work in mice seems to support this possibility. While more experimental evidence is needed to unequivocally define the role of leptin in several autoimmune conditions, it is nonetheless exciting that new developments in the field are leading to several new lines of inquiry. In this context, it is worth mentioning that leptin may only represent one of the many factors derived from the adipose tissue and neuroendocrine system that – in addition to playing an important function in the regulation of food intake and metabolism – also affect significantly the immune response. These mediators include adiponectin, visfatin, neuropeptide Y (NPY), and ghrelin (Tilg and Moschen 2006).

Multiple sclerosis (MS) is a chronic, immune-mediated, human chronic inflammatory disorder of the central nervous system (CNS) (Williams et al. 1994). The most studied model

of MS in animals is EAE, in which autoimmunity to CNS components is induced in susceptible strains of mice through immunization with self-antigens derived from basic myelin protein. The disease is characterized by autoreactive T cells that traffic to the brain and to the spinal cord and injure the myelin sheaths of CNS, with the result of chronic or relapsing-remitting paralysis (depending on the antigen and the strain of mice used). It has long been known that myelin-reactive Th1 CD4+ cells can induce and/or transfer disease, and Th1 cytokines are elevated in the CNS inflammatory lesions of EAE. In contrast, Th2 cytokines typically associate with recovery from EAE and/or protection from the disease (Williams et al. 1994). It has been shown that leptin is involved in both the induction and in the progression of EAE (Matarese et al. 2001, Sanna et al. 2003). Genetically, leptin-deficient mice are resistant to induction of both active and adoptively transferred EAE. This protection is reversed by leptin administration and associates with a switch from Th2- to Th1-type responses and IgG1 to IgG2a isotype switch. Similarly, in susceptible wild-type C57BL/6J mice, leptin worsens disease by increasing IFN-γ release and IgG2a production (Matarese et al. 2001). Importantly, a surge of serum leptin anticipates the onset of clinical manifestations of EAE. The peak of serum leptin correlates with inflammatory anorexia, weight loss, and the development of pathogenic T cell responses against myelin. Lymphomononuclear infiltrates in the CNS of EAE mice indicate in situ production of leptin in active inflammatory lesions, thus representing a significant local source of leptin (Sanna et al. 2003). Systemic and/or in situ leptin secretion was instead lacking in EAE-resistant mice. Taken together, these data suggest an involvement of leptin in CNS inflammation in the EAE model of MS.

In the human disease, it has been reported that the secretion of leptin is increased in both serum and cerebrospinal fluid (CSF) of naive-to-treatment patients with MS, an aspect that positively correlates with the secretion of IFN-γ in the CSF and inversely correlates with the percentage of circulating Treg cells, a subset of lymphocytes that is reduced in patients with MS as compared with healthy matched controls. Of note, the number of peripheral Treg cells in patients with MS inversely correlates with the serum levels of leptin, suggesting a link between the number of Treg cells and leptin secretion (Matarese et al. 2005). Considering that Treg cells are generated in the thymus, it is not known whether peripheral leptin or that produced in the perithymic adipose tissue could affect Treg cells generation/function in autoimmunity prone subjects. This aspect is not defined yet and is object of current extensive investigation. In any case, the fact that increased leptin secretion occurs in acute phases of MS and correlates with CSF production of IFN-γ is of possible interest for the pathogenesis and clinical follow-up of

patients with MS. As mentioned before, increased leptin secretion is present both in the serum and in the CSF of patients with MS and does not correlate with body mass index (BMI) (Matarese et al. 2005). The increase of leptin in the CSF is higher than in the serum, suggesting possible secondary in situ synthesis of leptin in the CNS and/or an increased transport across the blood-brain-barrier following enhanced systemic production. A recent gene microarray analysis of Th1 lymphocytes from active MS lesions has shown elevated transcripts of many genes of the neuroimmunoendocrine axis, including leptin (Lock et al. 2002). Leptin transcripts were also abundant in gene expression profiles of human Th1 clones, confirming that leptin gene transcription is induced concomitantly with the polarization toward Th1 responses – which are often involved in T-cell-mediated autoimmune diseases including MS. Moreover, in situ secretion of leptin near inflammatory T cells and macrophages was observed in active EAE lesions (Sanna et al. 2003). A possible explanation for the in situ elevated levels of leptin in the CSF of patients with MS could be the inflammatory cell itself, as suggested by studies with autoreactive human myelin basic protein (hMBP)-specific T cells from patients with MS that produced leptin and upregulated the expression of leptin receptor after activation Sanna et al. 2003, Matarese et al. 2005). Both anti-leptin and anti-leptin receptor blocking antibodies reduced the proliferative responses of the hMBP-specific T cell lines to antigen stimulation, underlying a possibility of leptin-based intervention on this autocrine loop to block autoreactivity. Finally, recent reports have shown increased secretion of serum leptin before relapses in patients with MS during treatment with IFN-β, and a capacity of leptin to enhance in vitro secretion of TNF-α, IL-6, and IL-10 from peripheral blood mononuclear cells of patients with MS in acute phase of the disease but not in patients with stable disease (Batocchi et al. 2003). In view of all these considerations, we suggest that leptin could be one of the many proinflammatory factors that act in concert to promote the pathogenic (autoreactive) Th1 responses targeting neuroantigens in MS.

Moreover, leptin is also important in spontaneous autoimmune diabetes in non-obese diabetic (NOD) mice (Atkinson and Leiter 1999). Leptin accelerates autoimmune diabetes in females NOD/LtJ mice (Matarese et al. 2002). Fluctuations in serum leptin levels have been also observed in a study performed by our group in an animal model of CD4+ T cell-mediated autoimmune disease, such as type 1 diabetes (T1D). Non-obese diabetic (NOD/LtJ) female mice, spontaneously prone to the development of beta-cell autoimmunity, have higher serum leptin levels, as compared to NOD/LtJ males and non-susceptible strains of mice, and show a serum leptin surge preceding the appearance of

hyperglycaemia (Matarese et al. 2002). Furthermore, early in life leptin administration significantly anticipated the onset of diabetes and increased mortality and inflammatory infiltrates in beta-islets; this phenomenon correlated with increased secretion of IFN-γ in leptin-treated NOD mice (Matarese et al. 2002). More recently, it has been found that a natural leptin receptor mutants of the NOD/LtJ strain of mice (named NOD/LtJ-db5J) display reduced susceptibility to T1D (Lee et al. 2005). These data further support the role of leptin in the pathogenesis of T1D. These NOD-db5J mice are obese, hyperphagic and show hyperglycaemia associated with hyperinsulinaemia. The leptin receptor mutation affects the extracellular domain of the leptin receptor probably impairing the leptin-binding and/or receptor dimerization. This effect is likely able to alter the intracellular signalling machinery, thus impairing the pathogenicity of anti-islets autoreactive T cells. Indeed, these mice show mild-low grade infiltration of the islets. Further studies are needed to address the molecular machinery determining the phenotype of resistance observed in these mice as well as the possibility to interfere with T1D pathogenesis by blocking the leptin axis.

1.6 T regulatory cells

The adaptive immune system of higher vertebrates provides a more efficient and specific immune defense against infectious microorganisms than that afforded by the innate immune system. The hallmark of the adaptive immune system is the random generation of antigen receptors in developing lymphocyte clones through a process of somatic cell gene rearrangement mediated by the recombination-activating gene recombinase. The essentially unlimited specificities of this anticipatory recognition system provides an efficient counterbalance to the short reproduction cycles and high mutation rates of infectious microorganisms.

However, the diversity of antigen recognition afforded by the system also poses the threat of autoimmunity because of the generation of self-reactive receptors. Expanded and developed in the framework of the clonal selection hypothesis, the idea of immunological 'tolerance' proposed a requirement for the elimination of autoreactive lymphocyte clones during development (Burnet 1957, Talmage 1957). A vast body of experimentation over the past half century has elucidated both cellular and molecular mechanisms leading to deletion or functional inactivation of autoreactive T and B cells in the primary lymphoid organs, thymus and bone marrow, respectively, or in the periphery. Collectively, these mechanisms act in a cell-intrinsic way and are therefore dubbed 'recessive tolerance', as elimination of an individual autoreactive lymphocyte clone does not affect other self-

reactive clones. Over the past decade a population of so-called 'regulatory T cells' (Treg cells) has been linked to the prevention of autoimmunity. Treg cells act in a dominant, trans-acting way to actively suppress immune activation, thereby functioning as critical mediators of self-tolerance and immune homeostasis.

The idea of a dominant form of immunological tolerance involving a specialized population of 'suppressor' T cells acting both to terminate conventional immune responses and to prevent autoimmune pathology was proposed over 30 years ago (Gershon and Kondo 1971). Early studies envisaged suppressor cell cascades involving multiple suppressor factors, anti-idiotypic T cell networks, 'suppressor-inducer' and 'contra-suppressor' cells (Dorf and Benacerraf 1984). However, the mechanisms responsible for these suppressive phenomena were never fully characterized at the molecular or biochemical level. Moreover, key findings of those studies were found to be demonstrably inaccurate and the field of suppressor T cell biology was largely discredited (Moller 1988). The present renaissance of the study of dominant tolerance can be ascribed mainly to the observation that mice thymectomized early in life develop organ-specific autoimmunity and that cells derived from adult spleen can prevent this disease (Nishizuka and Sakakura 1969). Using this model or models of lymphopenia-induced autoimmunity, several groups made critical observations demonstrating that specific CD4+ T cell subsets, fractionated based on expression of various cell surface markers, including CD5 and CD45RB, are capable of preventing autoimmune disease (Sakaguchi 2004, Shevach 2000). Efforts to better define the subset of cells mediating suppression of autoimmunity culminated in the identification of CD4+ T cells constitutively expressing the interleukin 2 receptor-α (IL-2Rα) chain (CD25) as being highly 'enriched' in suppressor activity (Sakaguchi et al. 1995). These 'naturally arising' CD4+CD25+ Treg cells became the best candidates for the T cell population mediating dominant tolerance to self. To emphasize their origin and importance, Treg cell production has been called the 'third function' of the thymus (Seddon and Mason 2000). The identification of a cell surface marker (CD25) allowing the enrichment of Treg cells has greatly facilitated the more rigorous study of T cell–mediated dominant tolerance. Another important milestone in the field was the development of an *in vitro* T cell suppression assay (Thornton and Shevach 1998, Takahashi et al. 1998, Read et al. 1998). After T cell receptor (TCR) crosslinking *in vitro*, Treg cells are unable to proliferate or produce IL-2 but are able to inhibit proliferative responses and cytokine production by effector T cells. However, this *in vitro* anergy belies a more complex activity *in vivo*. Adoptive transfer experiments suggest that Treg cells are capable of selfrenewal, as transfer of relatively

small numbers of Treg cells afforded a long-lasting protection against autoimmunity. In addition, Treg cells are capable of robust MHC class II–dependent proliferation in lymphopenic conditions, after specific TCR stimulation or after transfer into mice genetically deficient in Treg cells (Fisson et al. 2003, Gavin et al. 2002, Klein et al. 2003, Walker et al. 2003, Annacker et al. 2001, Malek et al. 2002, Fontenot et al. 2003). Additionally, an *in vitro* protocol has been developed for the expansion of Treg cell populations after TCR and CD28 engagement in the presence of very high concentrations of IL-2, which could allow for isolation and cloning of antigen-specific Treg cells (Tang et al. 2004). Thus, despite their apparent *in vitro* anergy, Treg cell population are capable of robust expansion *in vivo* and their early description as anergic cells is misleading.

The important issue of the mechanisms of suppressive action by Treg cells remains unresolved. Although *in vitro* suppression is contact dependent and is insensitive to transforming growth factor-beta (TGF-β) or IL-10 blockade, both IL-10 and TGF-β have been linked to suppression mediated by Treg cells in several *in vivo* experimental models (Shevach 2002, Green et al. 2003, Asseman et al. 1999). Reverse signalling through crosslinking of B7 (CD80 and CD86) on the cell surface of antigen-presenting cells or activated T cells, mediated by cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) expressed by Treg cells, has been proposed as yet another effector mechanism of suppression (Paust et al. 2004). However, like IL-10 and TGF-β, CTLA-4 does not seem to be a nonredundant mechanism of suppression, as Treg cells isolated from mice with the targeted deletions of genes encoding each of these molecules are suppressive *in vitro* (Piccirillo et al. 2002, Tang et al. 2004). The interplay of these mechanisms in Treg cell function *in vivo* remains to be further defined. In addition, after activation, human Treg cells may directly kill activated CD4 and CD8 T cells in a perforin- or granzyme-dependent way (Grossman et al. 2004). Finally, it has been proposed that Treg cells may suppress immune activation by 'soaking up' T cell growth factors such as IL-2 (Barthlott et al. 2003). It is likely the predominant effector mechanism of Treg cell–mediated suppression may vary depending on the specific tissue and inflammation type being studied. Treg cell–specific gene targeting may help to elucidate the suppressive mechanisms operating *in vivo*. Other important, unresolved issues are whether Treg cells show constitutive suppressive activity *in vivo* and whether this activity requires maintenance by 'tonic' Treg cell TCR and cytokine signals or whether the induction of suppressive mechanisms requires stronger TCR and/or cytokine receptor stimuli. Evidence supporting the idea of involvement of TCR signals in inducing the suppressive function of Treg cells *in vivo* has begun to emerge from the

analysis of Treg cells derived from TCR transgenic mice in the presence or absence of cognate ligand. Indirect evidence for the involvement of specific TCR stimulation required for Treg cell–mediated suppression has come from the finding of much more efficient protection against organ-specific autoimmunity mediated by Treg cells found in the organ-draining lymph nodes than by Treg cells found in nondraining lymph nodes or spleen (Green et al. 2002).

As for the involvement of cytokines in affecting suppressive properties of Treg cells, early reports indicated that Treg cell–mediated *in vitro* suppression could be overridden by provision of large amounts of IL-2 (Shevach 2002). However, more recent studies have suggested that Treg cells are capable of suppressing IL-2 mRNA induction in responder cells even in the presence of large amounts of IL-2 (Thornton et al. 2004). Moreover, two groups have reported that Treg cell suppressive activity *in vitro* is dependent on IL-2, as it is abrogated in the presence of IL-2-neutralizing antibodies (Thornton et al. 2004, de la Rosa et al 2004). Those data and results, suggesting that the maintenance of CD25 expression on Treg cells depends on IL-2 (Curotto de Lafaille 2004), envisage the possibility of a relatively simple regulatory network whereby the maintenance and suppressive activity of Treg cells is conditional on IL-2 production by nonregulatory T cells. Furthermore, increased amounts of IL-2 resulting from immune activation may fuel the expansion of the Treg cell population.

Although CD25 expression has been useful in defining the Treg cell population in nonimmune mice and humans, accurate discrimination between Treg cells and recently activated nonregulatory T cells, which upregulate CD25, during immune activation associated with autoimmune pathology or infection is almost impossible. Increased expression of CD25, as well as GITR, CTLA-4 and lymphocyte activation gene 3, on activated nonregulatory T cells suggests that expression of these molecules does not functionally define the Treg cell population and raises the possibility that not all Treg cells express these molecules.

Therefore, a principal challenge is to identify a unique functional molecular marker of Treg cells. The identification of such a molecule should help to resolve a fundamental issue regarding the nature of dominant tolerance. Two general models for T cell–mediated immunosuppression have been considered: that Treg cells represent a dedicated functional lineage, or that Treg cells represent a ‘plastic’ phenotype. The first model suggests the existence of a factor responsible for specifying a Treg cell lineage, which therefore serves as the mediator of the genetic mechanism of dominant tolerance. The second argues that T cell–mediated immunosuppression is not the purview of a dedicated Treg cell lineage but is the consequence of a dynamic balance between cells expressing

different amounts of cytokine receptors and different cytokine production profiles (Stockinger et al. 2001). The identification of mutations in the gene encoding Foxp3 as the cause of the fatal human autoimmune disorder 'immune dysregulation, polyendocrinopathy, enteropathy, X-linked' (IPEX) and the analogous disease in a spontaneous mutant mouse, scurfy, was a breakthrough in the field and led to subsequent studies that argue for the idea of Treg cells as a dedicated functional lineage (Brunkow et al. 2001, Chatila et al. 2000, Wildin et al. 2001, Bennet et al. 2001, Sakaguchi 2005). At a very young age, human patients with this autoimmune syndrome present with massive lymphoproliferation, early-onset insulin dependent diabetes mellitus, thyroiditis, eczema, severe enteropathy and food allergies preventing normal food intake, and additional autoimmune pathologies such as autoimmune hemolytic anemia and thrombocytopenia, as well as severe infections. Similar sequelae are found in scurfy mutant and Foxp3-deficient mice, including severe dermatitis, aggressive lymphoproliferation resulting in gross enlargement of secondary lymphoid organs, lymphocytic infiltration of multiple organs, hypergammaglobulinemia and autoimmune haemolytic anemia. Affected males succumb to the IPEX syndrome between 3 and 4 weeks of age. Analysis of the scurfy mutant before the identification of the causative mutation demonstrated that the disease is mediated by T cells, with CD4+ T cells being the primary effectors of the disease (Godfrey et al. 1994, Godfrey et al. 1991, Blair et al. 1994). In addition to showing substantially increased production of a broad spectrum of proinflammatory cytokines, the *in vitro* responses of T cells isolated from scurfy mice show a decreased activation threshold and lesser dependence on costimulation (Clark et al. 1999, Kanangat et al. 1996) . Furthermore, transgenic overexpression of Foxp3 results in reduced numbers of peripheral T cells, and the remaining T cells show impaired responses to TCR ligation. Those studies along with phenotypic similarities to TGF-β– and CTLA4-deficient mice led to the proposal that Foxp3 may mediate a general mechanism of negative regulation of T cell activation. Foxp3 belongs to a large family of functionally diverse transcription factors based on its winged helix–forkhead DNA-binding domain (forkhead box (Fox)). In addition to the C-terminal forkhead domain, Foxp3 also contains a Cys2His2 zinc finger domain and a coiled-coil–leucine zipper motif. Homology among full-length human, mouse and rat Foxp3 is very high, suggesting a highly conserved function. At present there is very little understanding of the function of Foxp3 at the molecular level. Foxp3 binds DNA, localizes to the nucleus and can act as a transcriptional repressor (Schubert et al. 2001). Identification of consensus forkhead binding domains adjacent to NFAT transcription factor binding sites in the promoters of several

cytokine genes, including those encoding IL-2, IL-4 and tumor necrosis factor, led to the proposal of a model of Foxp3-mediated transcriptional inhibition or repression in which Foxp3 antagonizes NFAT function by competition for DNA binding sites (Schubert et al. 2001). Based on those and other studies, it has also been proposed that Foxp3 is induced in a variety of cell types as a general mechanism of negative immune regulation by repressing production of inflammatory cytokines. However, so far there has been no characterization of Foxp3 target genes or the transcriptional program specified by Foxp3. The devastating lymphoproliferative autoimmune disease resulting from Foxp3 deficiency affects mutant males but not heterozygous female carriers for both humans and mice. Those observations and the fact that random X-chromosome inactivation is maintained in T cells from heterozygous female carriers suggest that control of the pathology in heterozygous females is mediated by a cell-extrinsic mechanism consistent with the idea of dominant tolerance. That consideration and some resemblance between the range of target organs affected by the disease in Foxp3 mutant mice and that in mice depleted of CD25+CD4+ T cells prompted examination of the function of Foxp3 in Treg cell biology. Analysis of Foxp3 expression in T cells at both the mRNA and protein level has shown high expression in CD25+CD4+ Treg cells and low expression of Foxp3 in naive and, notably, recently activated CD25–CD4+ T cells (Fontenot et al. 2003, Khattri et al. 2003, Hori et al. 2003). Those results suggest that Foxp3 is a specific molecular marker of Treg cells and, unlike CTLA-4, GITR and lymphocyte activation gene 3, allows for discrimination between Treg cells and activated nonregulatory T cells. Analysis of the origin of CD4+CD25+ Treg cells in chimeric mice containing a 1:1 mixture of allelically marked bone marrow stem cells derived from Foxp3-deficient and wild-type mice showed that Foxp3-deficient bone marrow cannot give rise to CD4+CD25+ Treg cells, thus demonstrating that CD4+CD25+ Treg cell development is critically dependent on Foxp3 expression21. In agreement with that finding, transgene-driven Foxp3 overexpression in mice results in an increase in the CD4+CD25+ Treg cell subset and acquisition of suppressive properties by CD4+CD25– and CD8+ T cells, although these cells are not as efficient at inhibiting CD25–CD4+ T cell *in vitro* responses as are CD4+CD25+ Treg cells (Khattri et al. 2003). The essential involvement of Foxp3 in programming Treg cell function was further demonstrated by the acquisition of regulatory properties by CD4+CD25– T cells after retroviral transduction with Foxp3. However, in those experiments, only some of the Foxp3-expressing cells acquired the characteristics of CD4+CD25+ Treg cells, including high expression of CD25 and suppressor activity. One possible interpretation of those results is that the

acquisition of regulatory properties after expression of Foxp3 in peripheral nonregulatory T cells is conditional on TCR specificity or the expression of additional cofactors either pre-existing or acquired in the process of activation and differentiation. Although Treg cells have high expression of Foxp3, low expression of Foxp3 has been detected in CD4+CD25– and CD8+ T cells (Fontenot et al. 2001, Furtado et al. 2002). Do all T cells have low expression of Foxp3, or do subsets of cells with high expression of Foxp3 exists in these populations? Along the same lines, the issue of a cell-intrinsic role for Foxp3 in nonregulatory T cell function has not been conclusively resolved. Does low Foxp3 expression modulate T cell activation in nonregulatory T cells? Analysis of mice expressing a 'knock-in' allele encoding a green fluorescent protein–Foxp3 fusion protein suggests that Foxp3 expression is highly restricted to Treg cells and that the low expression of Foxp3 detected in CD4+CD25– and CD8+ T cells is due to the presence of Foxp3+ Treg cells in these populations.

Another principal issue in the field is the frequency of the conversion of mature, nonregulatory T cells to Treg cells in the periphery as well as the *in vivo* conditions necessary for such conversion. Does a subset of nonregulatory T cells upregulate Foxp3 and acquire Treg cell activity during the course of conventional immune responses as part of a negative feedback loop? Do experimental manipulations that generate increased numbers of Treg cells expand a preexisting Treg cell population or convert them from nonregulatory T cells? If nonregulatory T cells can acquire Treg cell phenotype, is this a transient phenotype or a terminal differentiation into Treg cells, and does such conversion occur during the course of a conventional immune response? Unlike mouse CD25–CD4+ T cells, human CD25–CD4+ T cells are reported to upregulate Foxp3 after *in vitro* activation (Walker et al. 2003). However, because Foxp3 expression was analyzed in bulk T cell populations in those studies, it is unclear if the observed increase in Foxp3 expression was due to expansion of a small population of Foxp3+ CD25– Treg cells or conversion of nonregulatory T cells. Although more work is required to reconcile the data in human T cells, one possibility is that the function of Foxp3 is different in mice and humans. From a general perspective, peripheral upregulation of Foxp3 after activation of human CD25–CD4+ T cells would support a model of dominant tolerance in which Treg cells are generated *de novo* from nonregulatory T cells as a consequence of immune activation and thereby act as negative feedback regulators of conventional immune responses. However, the salient conservation of Foxp3 at the protein and nucleic acid level, in both coding and noncoding sequence, and the notable similarity in autoimmune syndrome found in both mice and

humans deficient in Foxp3, challenge the idea of such scenario. In conclusion, induction of Foxp3 expression in developing thymocytes commits these cells to the Treg cell lineage. It must be noted that induction of Foxp3 expression in peripheral nonregulatory T cells in both humans and mice in physiological conditions is a relatively rare process. The issue of whether Foxp3 is required only at the commitment stage of Treg cell differentiation or is also needed for the maintenance of Treg cell function remains to be addressed. The devastating lymphoproliferative autoimmune syndrome found in both mice and humans deficient in Foxp3, and thus Treg cells, demonstrates that Treg cell–mediated dominant tolerance is a vital mechanism of immune homeostasis. We argue that the early onset of this syndrome indicates that this mechanism evolved specifically to control T cell self-reactivity and autoimmune inflammation.

2. Aims of the study

We believe that there is a strong relationship between metabolic state and Treg cells response that controls immunological tolerance. We hypothesize that the product of the adipose tissue, leptin, acts as a proinflammatory cytokine that promotes Th1 responses on one side and inhibits Treg cell expansion on the other, setting the basis for exaggerated immuno-inflammatory responses to altered self or non-self, and leading to autoimmunity in subjects with autoimmunity risk factors (ie. genetic predisposition, HLA, environment, sex, etc.). Capitalizing our joint effort and trans-disciplinary expertise in immunology, metabolism and autoimmune diseases, we have used several cellular and molecular approaches in animal models and in humans to:

1) Identify the precise relationship between leptin, metabolic state and Treg cells in the context of autoimmune disease susceptibility;
2) Dissect the required milieu for the physiological development of Treg cells and the role of leptin in this process;
3) Set-up the cellular and molecular basis for the use of leptin antagonists to modulate T cell immune responses in autoimmune disorders such as multiple sclerosis;
4) Investigate the possibility to expand Treg cells with leptin antagonists for novel immune therapeutic intervention in multiple sclerosis and type 1 diabetes.

All the above considerations will also help to understand how the environment, through the availability of nutrients, is able to influence susceptibility to autoimmune disorders. Being leptin a unique mediator linking nutritional status and immune response, we feel that the understanding of the cellular and molecular mechanisms underlying leptin's actions on proinflammatory responses will provide fundamental insights to set-up novel strategies for immune interventions in autoimmune disorders and chronic inflammation.

3. Materials and Methods

3.1 Purification, cultures and assays with T cells

Human $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were purified from human healthy donors PBL either by magnetic cell separation using the Dynal® $CD4^+CD25^+$ Treg Kit (Dynal-Biotech, Oslo, Norway) or by flow cytometry cell sorting (MoFlo™ high-performance cell sorter, Dako, Glostrup, Denmark), and rapidly cleaned with the Detach® reagent (Dynal-Biotech) from surface bound anti-CD25 mAb. Both magnetic beads-based and flow cytometry-based purification techniques yielded a highly expressing $CD25^+$ population (95-98% pure by FACS analysis), 90% of which expressed Foxp3. The Treg cells:effector ratio in the suppression experiments was 1:1. Cells were cultured (5×10^4 cells/well) in round-bottom 96-well plates (Becton-Dikinson Falcon, Franklin Lakes, New Jersey) with RPMI medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies Inc., Gaithersburg, Maryland) and 5% AB human serum (Sigma-Aldrich, St. Louis, Missouri). Cells were stimulated for 3 days in the presence of anti-CD3/CD28 Dynabeads® (0.1 bead/cell) (Dynal-Biotech). On the last day, [^{3}H] thymidine (0.5 μCi/well) (Amersham-Pharmacia Biotech, Cologno Monzese, Italy) was added to the cultures and cells harvested after 12 hrs. Radioactivity was measured with a β-cell-plate scintillation counter (Wallac, Gaithersburg, Maryland).

For suppression experiments in the mouse, Treg cells were isolated with the Regulatory T Cell Isolation Kit (Miltenyi Biotec, Gladbach, Germany) and stimulated with Dynabeads® mouse anti-CD3/CD28 (0.5 bead/cell; 5×10^4 cells/well). The Treg cells:effector ratio in the suppression experiments was 1:2. Purified cells (98% pure by FACS analysis) were cultured in round-bottom 96-well plates (Becton-Dikinson Falcon) with RPMI medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies Inc.) and 5% FCS or 5% mouse serum (Hyclone-Pierce, Rockford, Illinois). Cells were stimulated for 3 days, labelled with [^{3}H] thymidine (0.5 μCi/well) for the last 16 hours of culture and harvested similarly to what done for human T cell cultures. Incorporation of Bromodeoxyuridine (BrdU) and FACS analysis were performed in T cell cultures in serum free medium (X-VIVO) using the BrdU Flow Kit from BD-Pharmingen in accordance to the manufacturer's instructions.

3.2 IL-2 measurement

IL-2 measurement was done using the CTLL-2 cell line (kindly provided by Dr. Nick Davey, Imperial College London, UK) according to a previously described method (Ng et al.,

2001). Cells were maintained in culture in supplemented RPMI medium with 10% FCS (Hyclone-Pierce) and human recombinant (rh)–IL-2 (10 U/ml, Boehringer Mannheim, Mannheim, Germany). They were subcultured every 2 to 3 days. Cells were rested in medium without IL-2 overnight prior to use in the assays.

3.3 Reagents, leptin neutralizing antibodies and leptin measurement

For *in vitro* blocking experiments, human leptin neutralizing mAb (R&D Systems, Minneapolis, Minnesota) was used at a final concentration of 0.25 to 25 μg/ml; controls were irrelevant isotype-matched antibodies (Biovendor Laboratory Medicine Inc., Brno, Czech Republic). Human recombinant leptin was purchased from R&D Systems. Human IL-2 neutralizing mAb (MQ1-17H12) was from BD Pharmingen and was utilized at final concentration of 5-10 μg/ml. FITC-anti-human-CD4 and PE-anti-human-CD25 were from BD Pharmingen; the anti-human Foxp3 staining set was from eBiosciences (San Diego, California). Recombinant mouse GAD65 was purified from GAD65-producing cells that were kindly provided by Dr. Roland Tisch, (University of North Carolina, Chapel Hill, USA); recombinant GAD65 was tested for purity by SDS-PAGE and silver staining before the experimental use. The endotoxin content in the preparations was below the detection limit (about 10pg of endotoxin/μg of protein) of the Limulus amebocyte lysate (LAL) method. The 9F8 is an anti-human ObR mAb, generated in our laboratory in collaboration with dott. Richard Ross, that cross-react with mouse ObR with low affinity.
Human leptin specific ELISA was purchased from R&D Systems and measurements were performed according to the manufacturer's instructions (Matarese et al., 2005). Serum free media were RPMI (Life Technologies), HyQ-ADCF (Animal Derived Component Free, from Hyclone-Pierce) and X-VIVO (BioWittaker).

3.4 Mice and *in vivo* experiments

Six weeks-old female leptin-deficient C57BL6/J-*ob/ob* (*ob/ob*), C57BL6/J, leptin-receptor deficient C57BL/Ks-*db/db* (*db/db*), C57BL/Ks-*db/*+ lean controls (*db/*+) and female SJL/J mice were purchased from Harlan Italy s.r.l. (Corezzana, Italy); B10.Cg.Tg(TcrAND)53Hed/J (AND-TCR Tg) PCC-specific transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA); NOD/LtJ and NOD-*Lepr*^{db5J}/LtJ mutants were kindly provided by Dr. Ed Leiter from The Jackson Laboratory. Experiments were

conducted in accordance with the animal welfare guidelines under an approved protocol of the Istituto Superiore di Sanità, Roma, Italy. Mice were age-matched for individual experiments and housed with a 12-hour light/dark cycle in the animal facility at the Università di Napoli "Federico II". *Ob/ob* mice were injected intraperitoneally twice daily for 10 days with mouse recombinant leptin (R&D Systems) dissolved in 200 μl of PBS at a dose of 1μg/g of body weight. The mouse leptin blocking antibody was produced in our laboratory after immunization of C57BL/6J mice with mouse recombinant leptin (R&D Systems) emulsified in complete Freund's adjuvant (CFA) CFA (Difco Laboratories, Detroit, MI, USA); mouse leptin specific antibodies (of the IgM class) were affinity purified with recombinant mouse leptin (R&D Systems) bound to AminoLink® Plus Immobilization Gel (AminoLink® Plus Immobilization Kit from Pierce, Rockford, IL, USA) from serum and ascites of immunized mice. Affinity purified IgM were used as control (BD-Pharmingen). WT mice were treated for three days either with 100 μg of control mouse IgM or with mouse leptin Abs intraperitoneally in a total volume of 100 μl of PBS.

Adoptive transfer experiments were performed by labeling highly purified (98% pure by FACS analysis) $CD4^+$ T cells obtained from C57BL6/J WT mice (cells were purified using the mouse $CD4^+$ negative isolation kit from Dynal) with the fluorescent dye CFSE (5-, 6-carboxyfluorescein diacetate succinimidyl ester) from Molecular Probes Inc., Eugene, Oregon, used at 1μg/ml. Briefly, 10^7 CFSE-labeled $CD4^+$ T cells were adoptively transferred into mice intravenously. Four and seven days later, spleen cells were harvested from mice and stained with PE-anti-Foxp3 (eBioscience) and Cy-anti-CD4 (BD PharMingen). In experiments of adoptive transfer performed with CFSE-labeled AND-TCR Tg $CD4^+$ T cells (98% pure), after four and seven days spleen cells were harvested from recipient mice and stained with anti-clonotypic PE-anti-Vα11.1 (RR8.1), biotynilated-anti-Vβ3 (KJ25) (both from BD PharMingen) and APC-anti-Foxp3 (eBioscience). Flow cytometric analysis of CFSE dilution was performed by gating on $CFSE^+CD4^+Foxp3^+$ cells (in non-Tg mice) and on $CFSE^+V\alpha11.1^+/V\beta3^+Foxp3^+$ cells (in AND-TCR Tg mice) with a FACScalibur™ (Becton-Dickinson, San Diego, California) and analyzed by Cell Quest® software (Becton-Dickinson).

For active induction of EAE, mice (*n* = 6 per group) were immunized s.c. in the flank with 100 μl CFA (Difco; BD Diagnostics — Diagnostic Systems) emulsified with 100 μg PLP139–151 peptide on day 0 and with 200 ng pertussis toxin (Sigma-Aldrich s.r.l.) i.p. on days 0 and 1. Control mice (*n* = 5 per group) were injected with CFA emulsified with PBS plus pertussis toxin according to the same schedule. Individual mice were observed daily for clinical signs of disease for up to 40 days after immunization and after

adoptive transfer. Mice were weighed and scored daily in a blinded fashion according to clinical severity of symptoms on a scale of 0 to 6, with 0.5 points for intermediate clinical findings: 0, no abnormality; grade 0.5, partial loss of tail tonicity, assessed by inability to curl the distal end of the tail; grade 1, reduced tail tone or slightly gait; grade 2, tail atony, moderately clumsy gait, impaired righting or any combination; grade 3, hind limb weakness or partial paralysis; 4, complete hind limb paralysis or fore limb weakness; grade 5, tetraplegia or moribund state; grade 6, death. The data were plotted as daily mean clinical score for all animals in a particular treatment group. Scores of asymptomatic mice (i.e., scores of 0) were included in the calculation of the daily mean clinical score for each group. A relapse was defined as a sustained (more than 2 days) increase in clinical score by at least 1 full grade after the animal had improved previously by at least 1 full grade and stabilized for at least 2 days. The data are plotted as the relapse rate of all the animals of that group (total number of relapses per group divided by total number of mice in the group; see Table 1). The brains and spinal cords were dissected between 15 and 20 days after immunization, according to the clinical stage of disease, and fixed in 10% formalin. Paraffinembedded sections of 5 μm thickness were cut and stained with H&E (Sigma-Aldrich). Sections from 4–10 segments per mouse were examined blindly for the number of inflammatory foci by using a previously published scoring system (7, 8).
Mice were treated 3 or 4 times with 100 μg control mouse IgG1 or with anti ObR specific mAb (9F8) i.p. in a total volume of 100 μl of PBS. Treatment was initiated with PLP139–151 peptide for 3 consecutive days (days –1, 0, and 1), during the acute phase of the disease for 4 consecutive days (days 8–11) or during disease progression (14–17).

3.5 Western blots, biochemical analyses and confocal microscopy

Total cell lysates were obtained in 50 mM HEPES (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1.0% Triton X-100, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin. 50 μg of total proteins were subjected to SDS-PAGE under reducing conditions. After electrophoresis, proteins were transferred onto a nitrocellulose filter membrane (Protan, Schleicher & Schuell) by using a Trans-Blot Cell (Bio-Rad) and transfer buffer containing 25 mM Tris, 192 mM glycine, 20% methanol. Membranes were placed in 5% non-fat milk in phosphate buffered saline, 0.5% Tween 20 (PBST) at 4°C for 2h to block the non-specific binding sites. Filters were incubated with specific antibodies before being washed three

times in PBST and then incubated with a peroxidase-conjugated secondary antibody (Amersham Biosciences). After washing with PBST, peroxidase activity was detected by using the ECL system (Amersham Biosciences) or Femto (Pierce). The antibodies used were the following: anti-$p27^{Kip-1}$, anti-STAT3 and anti-phospho-STAT3 (Y705), anti-STAT1 and anti-phospho-STAT1 (Y701) (Cell Signaling Technology, Beverly, MA, USA); anti-phospho-ObR (Y1138) (Upstate Biotechnology, Lake Placid, NY, USA); anti-leptin, anti-ObR, anti-ERK 1/2 and anti-phospho-ERK 1/2 (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); anti-Foxp3 (eBioscience); anti-SOCS3 (Serotec Ltd, Kidlington, UK). The filters were also probed with an anti-tubulin antibody (Sigma) to normalize the amount of loaded protein. Only for the human leptin western blots, performed on a 15% SDS-PAGE gel to better visualize 16kDa leptin, normalization was performed against total ERK1/2. All filters were quantified by densitometric analysis of the bands utilizing the program ScionImage 1.63 for Mac (Scion Corporation, Frederick, Maryland, USA). Confocal microscopy was performed in parallel with proliferation and biochemistry at 1h and 36h on 98% pure Treg cells and $CD4^{+}CD25^{-}$ cells. Cells were washed in PBS and 10^4 cells were seeded on multitest slide (ICN Biomedicals Inc., Aurora, Ohio USA) dried on air and fixed for 1 minute in methanol, washed in PBS, permealized in PBS containing 0.2% Triton X-100 for 3 minutes, and incubated in blocking solution (PBS 1% BSA) for one hour. Cells were then washed 3 times in PBS and incubated overnight at 4°C with primary antibodies (rabbit polyclonal anti-leptin and mouse anti-ObR mAb, both from Santa Cruz Biotechnology Inc.) diluted 1:100 in blocking solution, washed in PBS and incubated with the secondary antibody (goat anti-rabbit 488 and goat anti-mouse 543, both from Molecular Probes Inc.) for 1 hour at room temperature diluted 1:100 in blocking solution, washed in PBS and finally mounted in PBS/Glycerol 1:1. Immunofluorescence analysis was performed with a confocal laser scanner microscope Zeiss LSM 510. The wavelength of the Argon ion laser was set at 488 nm, that of the HeNe laser was set at 543 nm. Fluorescence emission was revealed by BP 505-530 band pass filter for Alexa Fluor 488 and by BP 560-615 band pass filter for Alexa Fluor 543. Double staining immunofluorescence images were acquired simultaneously in the green and red channels at a resolution of 1024x1024 pixels.

3.6 Statistical analysis

Analyses were performed using the Mann-Whitney *U*-test for unrelated two-group analyses and the Kruskal-Wallis

ANOVA test for three or more group analyses using the StatView™ software (Abacus Concepts Inc., Cary, NC, USA). Results are expressed as mean ± SD. *P* values < 0.05 were considered statistically significant.

4. Results and Discussion

4.1 Human Treg cells express higher amounts of ObR than $CD4^+CD25^-$ effector T cells

Previous studies have shown that ObR is expressed on $CD4^+$ T cells and that leptin signalling during TCR stimulation is able to switch immune responses towards a Th1 phenotype (La Cava and Matarese, 2004). To better understand the impact of this molecule on the different T cell subsets, we sought to analyze the expression of ObR on Treg cells and correlate its expression with Foxp3. $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ effector T cells purified from human healthy donors (Figure 1A, left panel) were analyzed for Foxp3 expression by FACS analysis and immunoblot (Figure1A, right panel and Figure1B, respectively). As expected, Treg cells showed high amounts of Foxp3 protein whereas $CD4^+CD25^-$ did not show detectable amount of the protein in cell extracts (Figure 1A-B). Instead, ObR was expressed on both freshly isolated cellular subsets, although at significantly higher amounts ($P < 0.001$) in Treg cells as compared to the $CD4^+CD25^-$ T cell effectors (Figure 1C).

4.2 Leptin neutralization results in the proliferation of human Treg cells stimulated with anti-CD3 and anti-CD28

We decided to investigate the ability of leptin to affect human Treg cells proliferative and/or suppressive capacity. It is well estabilished that human Treg cells were hyporesponsive to *in vitro* anti-CD3 and anti-CD28 stimulation (Figure 1D) (Ng et al. 2001). Addition of exogenous recombinant leptin to the cultures did not alter Treg cells hyporesponsiveness (Figure 1D). However, addition of neutralizing leptin monoclonal antibody (mAb) reversed the hyporesponsiveness and promoted Treg cells proliferation (Figure 1D) ($*P < 0.0001$) in a dose-dependent fashion (Figure 1E). Analogous results were observed using different leptin or ObR-blocking antibodies (data not shown). Confirming specificity, addition of exogenous recombinant leptin to anti-CD3 and anti-CD28-stimulated Treg cells antagonized the proliferation induced by leptin mAb (Figure 1D) ($**P < 0.01$). Moreover, dose-dependent increase of the proliferation of stimulated Treg cells in the presence of leptin mAb (Figure 1E) was reversed by addition of increasing doses of recombinant leptin (Figure 1F). Interestingly, leptin mAb inhibited the proliferation of purified effector $CD4^+CD25^-$ T cells, a phenomenon that was reversed by addition of exogenous leptin (Figure 1G) ($*P < 0.01$ and $**P < 0.01$, respectively). Thus, neutralization of leptin had opposite effects on effector $CD4^+CD25^-$ T cells and Treg cells - it inhibited proliferation on the former lymphocyte subset

(Figure 1G) whereas it promoted expansion of the latter subpopulation (Figure 1D). This effect was also evident morphologically, as formation of cell clumps in the cultures of Treg cells stimulated with anti-CD3 and anti-CD28 and leptin mAb, but not in the cultures of Treg cells stimulated with anti-CD3 and anti-CD28 in the absence of leptin mAb (data not shown). Finally, in co-culture experiments, Treg cells efficiently suppressed the proliferation of $CD4^{+}CD25^{-}$ T cells, and leptin neutralization reversed the suppression by Treg cells (Figure 1H). Data obtained suggest that freshly isolated human Treg cells express high amounts of ObR that account for an inhibitory loop that constrains the expansion of this subset. While leptin neutralization inhibits the proliferation of effector $CD4^{+}CD25^{-}$ T cells, this condition leads to an expansion of the Treg cells, which maintain their suppressive phenotype in the resting phase. The opposite effects of leptin blockade on $CD4^{+}CD25^{-}$ T cells and Treg cells associate with a differential expression of intracellular leptin and cell-surface ObR in the two cell subsets.

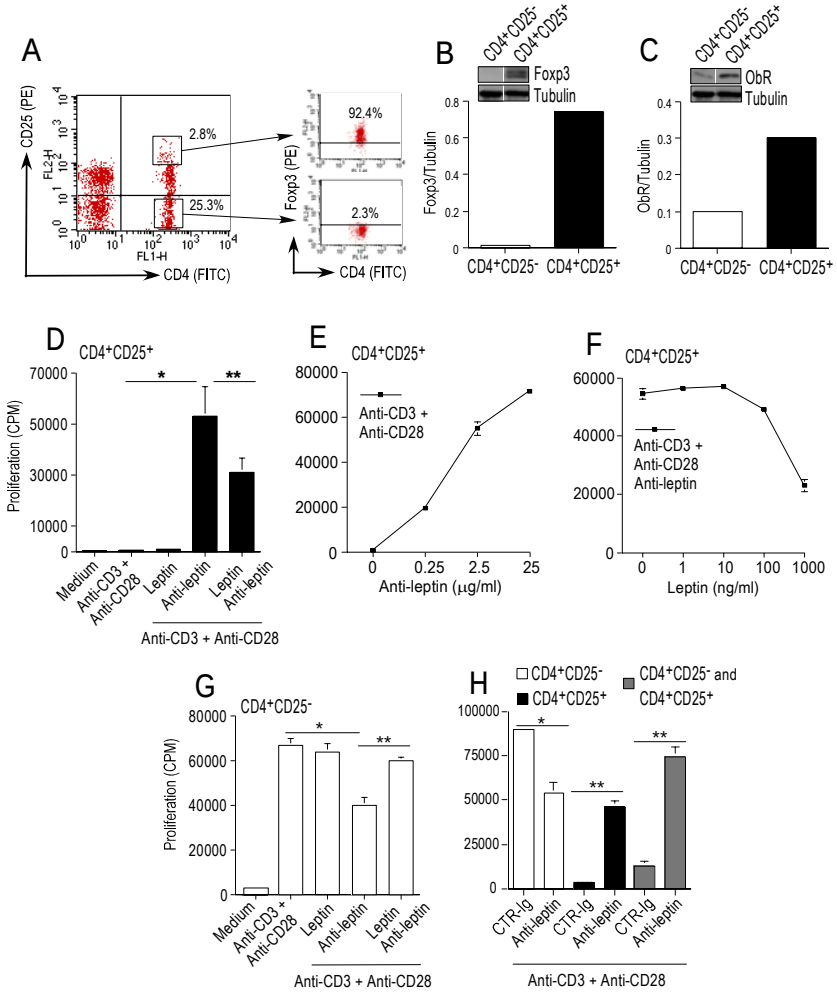


Figure 1. Human T_{reg} cells express high ObR and leptin neutralization reverses their hyporesponsiveness.

(A) Representative flow cytometry plot of human T cells stained for CD4, CD25 and Foxp3.

(B) and (C) Immunoblot analysis of sorted $CD4^+$ T cells on the basis of their CD25 expression. Graphs show quantitation of Foxp3 and ObR with respect to tubulin. One representative out of five independent experiments is shown.

(D) Proliferation of $CD4^+CD25^+$ T_{reg} cells treated with recombinant human leptin (250ng/ml) in the presence or absence of leptin mAb (10μg/ml). The data are shown as mean ± SD (n = 5, **P* < 0.0001; ** *P* < 0.01).

(E) Dose dependency of T_{reg} cells proliferation induced by leptin mAb. Proliferation was measured after treatment with indicated doses of leptin mAb. The data are shown as mean ± SD (n = 5).

(F) Proliferation of T_{reg} cells induced by a fixed dose of leptin mAb in the presence of increasing concentration of recombinant leptin. The data are shown as mean ± SD (n = 5).

(G) Proliferation of $CD4^+CD25^-$ effector T cells treated with recombinant human leptin (250ng/ml) in the presence or absence of leptin mAb (10μg/ml). The data are shown as mean ± SD (n = 5, **P* < 0.01; ** *P* < 0.01).

(H) Proliferation of $CD4^+CD25^-$ effector T cells, T_{reg} cells and of both cell types in co-culture in the presence or absence of leptin mAb (10μg/ml). The data are shown as mean ± SD (n = 5, **P* < 0.01; ** *P* < 0.0001).

4.3 Proliferated Treg cells have partial suppressive capacity that attains full activity when they enter the resting phase

The suppressive capability of Treg cells in the presence of leptin mAb was tested in co-culture experiments of Treg cells together with $CD4^+CD25^-$ T cells. Addition of exogenous leptin did not affect suppression of Treg cells on $CD4^+CD25^-$ T cells (Figure 2A). In contrast, suppression of proliferation was apparently abrogated in the presence of leptin mAb (Figure 2A, grey columns) (**P* < 0.0001). Concomitant addition of leptin together with the leptin mAb partly reduced the anti-leptin-induced reversal of suppression, confirming specificity for leptin neutralization in the co-culture experiments (Figure 2A, grey columns) (***P* < 0.01).

To understand whether Treg cells during anti-leptin-induced proliferation could exert suppressive capacity, we performed co-culture experiments using CFSE labelled $CD4^+CD25^-$ T cells in the presence of unlabeled Treg cells (Figures 2B-E). As expected, Treg cells suppressed the expansion of $CD4^+CD25^-$ effectors (Figure 2D) (**P* < 0.0001), and suppression was partially maintained in the presence of leptin mAb (Figure 2E) (***P* < 0.01) - albeit at a lower degree when compared with untreated Treg cells (Figure 2D). This suggested that Treg cells during anti-leptin-induced proliferation are partially functional in terms of suppressive capability. Moreover, the co-culture experiments of Treg cells with $CD4^+CD25^-$ T cells, in the presence of leptin mAb, indicated that the reversal of suppression (Figure 2A, grey

columns) was only apparent because $CD4^+CD25^-$ effectors were inhibited in part in their proliferation, as indicated by the CFSE dilution (Figure 2E). Thus, the high amount of [^{3}H] thymidine incorporation in co-culture of Treg cells with $CD4^+CD25^-$ T cells in the presence of leptin mAb (Figure 2A and Figure1H) has to be ascribed to both Treg cells proliferation and partly to $CD4^+CD25^-$ T cells. These phenomena were confirmed in terms of expression of the activation marker CD25 on CFSE-labelled $CD4^+CD25^-$ effector T cells. The anti-CD3 and anti-CD28 activation induced a substantial up-regulation of CD25 on the cell surface (Figure 2F) and was significantly inhibited in the presence of Treg cells (Figure 2H) (*P < 0.0001). A significant reduction of CD25 expression on effector T cells was observed in the presence of anti-leptin-expanding Treg cells (even if at lower extent when compared with untreated Treg cells) (Figure 2I) (**P < 0.01). In any case, the suppressive capacity of anti-leptin-expanding Treg cells was diminished of about 40% and a certain number of $CD4^+CD25^-$ T cells was still able to proliferate and divide (Figure 2E).

Next, we performed two-step experiments to address whether anti-leptin-proliferated Treg cells in the resting phase could retain their suppressive capacity over time. Eight days after the first expansion, stimulated Treg cells were still suppressive for $CD4^+CD25^-$ effector T cells in *in vitro* co-culture experiments (Figure 2L-N). Although effector $CD4^+CD25^-$ T cells cultured in the presence of leptin mAb proliferated upon re-stimulation and did not suppress allogeneic $CD4^+CD25^-$ T cells expansion in co-culture experiments (Figure 2M), the anti-leptin-expanded Treg cells maintained their hyporesponsiveness after re-stimulation and were capable to suppress proliferation of allogeneic $CD4^+CD25^-$ effector T cells (Figure 2N) (*P < 0.0001). Data obtained envisage an innovative therapeutic approach of Treg cells expansion, as the anti-leptin-expanded Treg cells, once in the resting phase, maintain their suppressive capacity over time.

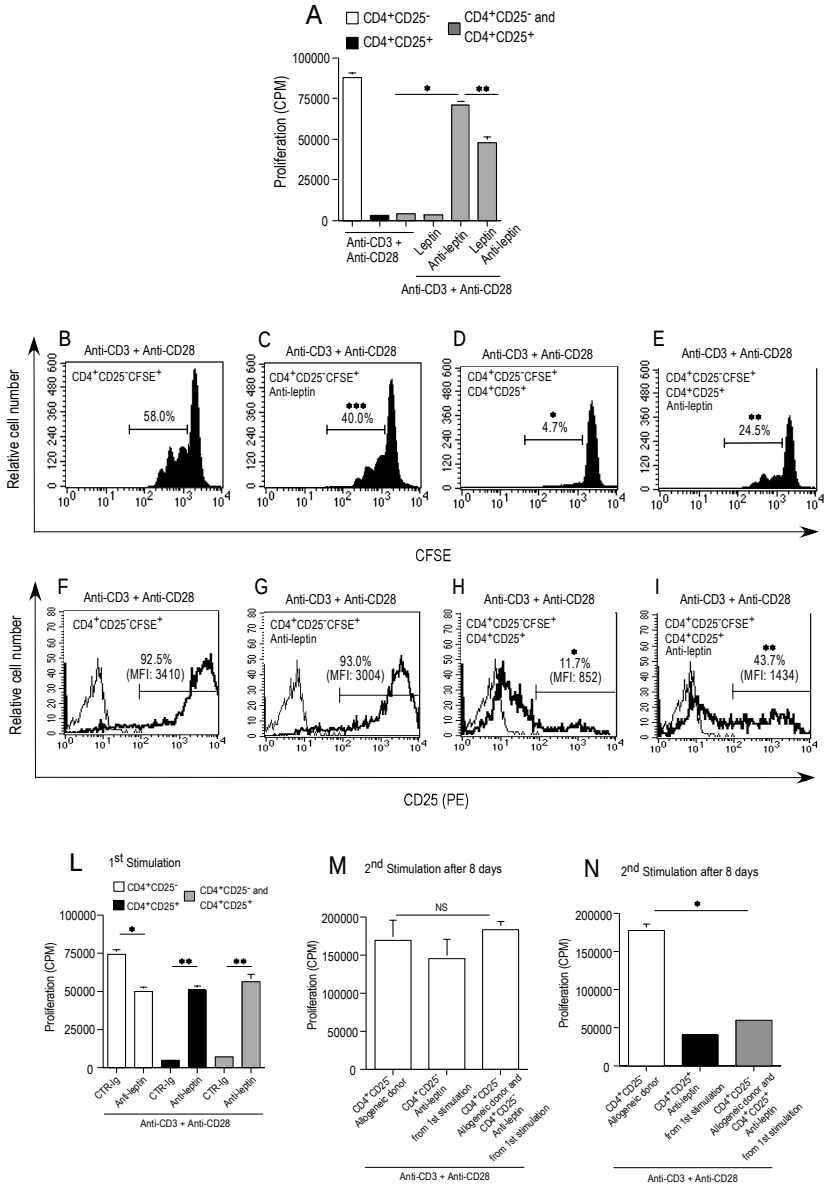


Figure 2. Human T_{reg} cells exhibit partial suppressive capacity upon leptin-mAb-induced proliferation.

(A) Proliferation of $CD4^+CD25^-$ effector T cells, T_{reg} cells and of both cell types in co-culture treated with recombinant human leptin (250ng/ml) in the presence or absence of leptin mAb (10μg/ml). The data are shown as mean ± SD (n = 3, $^*P < 0.0001$; $^{**}P < 0.01$).

(B-E) and (F-I) Proliferative response and CD25 expression analysis, respectively, of CFSE-labelled-$CD4^+CD25^-$ effector T cells alone or in co-culture with untreated or leptin-mAb-treated unlabelled T_{reg} cells. The thin line represents the isotype matched negative control and the thick line represents the CD25 staining. One representative out of three independent experiments is shown ($^*P < 0.0001$; $^{**}P < 0.01$; $^{***}P < 0.05$, as compared with $CD4^+CD25^-CFSE^+$).

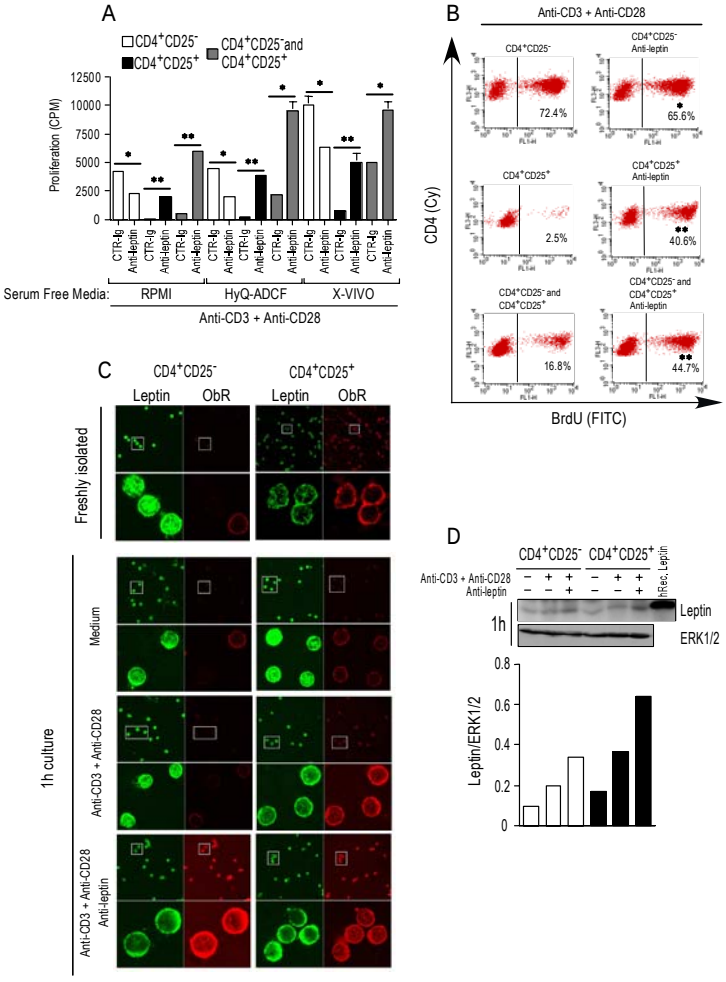
(L) Proliferation of CD4+CD25- effector T cells, Treg cells and of both cell types in co-culture stimulated with anti-CD3 and anti-CD28 in the presence or absence of leptin mAb (10μg/ml). The data are shown as mean ± SD (n = 5, $^*P < 0.01$; $^{**}P < 0.0001$).

(M) and (N) Suppressive activity of either CD4+CD25- or CD4+CD25+ Treg cells, treated with leptin mAb, eight days after anti-CD3 and anti-CD28 activation. The data are shown as mean ± SD (n = 5, *P < 0.0001; NS, Not significant).

4.4 Leptin production from human Treg cells

Leptin is present in media supplemented with human serum, such as the medium used in our experiments (see Methods). To test whether human Treg cells could expand in the absence of leptin, we stimulated Treg cells with anti-CD3 and anti-CD28 in three different types of serum- and leptin-free media (Figure 3A). Under these conditions, Treg cells maintained hyporesponsiveness even in the absence of exogenous leptin. Surprisingly, addition of leptin mAb to the cultures resulted in Treg cells proliferation (Figure 3A). This finding was also confirmed by BrdU incorporation in serum-free medium cultures (Figure 3B). This finding suggests the possibility that leptin may be produced by Treg cells, in a fashion similar to the production of leptin by $CD4^+$ T cells and monocytes in multiple sclerosis (Sanna et al. 2003; Matarese et al. 2005). To test this possibility, we examined leptin and ObR expression, on Treg cells and $CD4^+CD25^-$ T cells. Both freshly isolated Treg cells and $CD4^+CD25^-$ T cells stained positive for leptin and ObR, with different intensity patterns (Figure 3C). Moreover, after 1h culture, Treg cells showed higher leptin production than $CD4^+CD25^-$ T cells, and this tendency was maintained during anti-CD3 and anti-CD28 stimulation, both in the presence or in the absence of leptin mAb (Figure 3C). Parallel quantitation by immunoblotting analysis for leptin on cell lysates confirmed the difference (Figure 3D). Indeed, the presence of a basal production of leptin increased significantly after anti-CD3 and anti-CD28 stimulation (Figure 3D), and Treg cells always produced more leptin than $CD4^+CD25^-$ T cells (Figure 3D) (P < 0.05). Interestingly, leptin neutralization induced a compensatory leptin production and ObR upregulation in both Treg cells and $CD4^+CD25^-$ T cells, and again, to a higher amount in the Treg cells (Figure 3C-D). Additionally, to address the capacity of Treg cells and $CD4^+CD25^-$ T cells to secrete leptin, we performed a human leptin specific ELISA (see Methods) and confocal microscope analysis at different time points (12h and 36h) on culture supernatants (Figure 3E). At 12h the secretion of leptin was similar in both Treg cells and $CD4^+CD25^-$ T cells, whereas at 36h the amount of leptin secreted was significantly higher in the Treg cells, either unstimulated (*P < 0.03) or treated with anti-CD3 and anti-CD28 plus leptin mAb (**P < 0.05) (Figure 3E). The finding was confirmed by the observation of a reduced content of leptin in Treg cells at 36h (Figure 3E) in confocal microscopy and immunoblotting studies on cell lysates. Finally, these

results on leptin secretion were also confirmed by immunoblotting for leptin on culture supernatants (data not shown). It must be noted that in our experimental system, the culture media supplemented with human serum contained low - albeit significant - concentrations of human leptin (0.5-1ng/ml in the 5% human serum [HS]/95% RPMI). The contribution of HS-derived leptin *versus* Treg cells-derived leptin was analyzed in cultures with three different serum free media - all tested for the absence of leptin. The anti-leptin-induced expansion of the Treg cells in serum free conditions indicated that the Treg cells -derived leptin was sufficient to act as negative signal for the expansion of the Treg cells. It is possible to speculate that a leptin-mediated negative autocrine loop may operate on Treg cells, as both freshly isolated and cultured Treg cells stain positive for and secrete leptin. On the other hand, the leptin present in the culture medium and secreted by the $CD4^+CD25^-$ T cells could act as a positive signal for T cell proliferation. These differential effects induced by leptin could rely upon the different ObR levels and leptin secretory capacity, which would ultimately affect the intracellular signalling differentially.



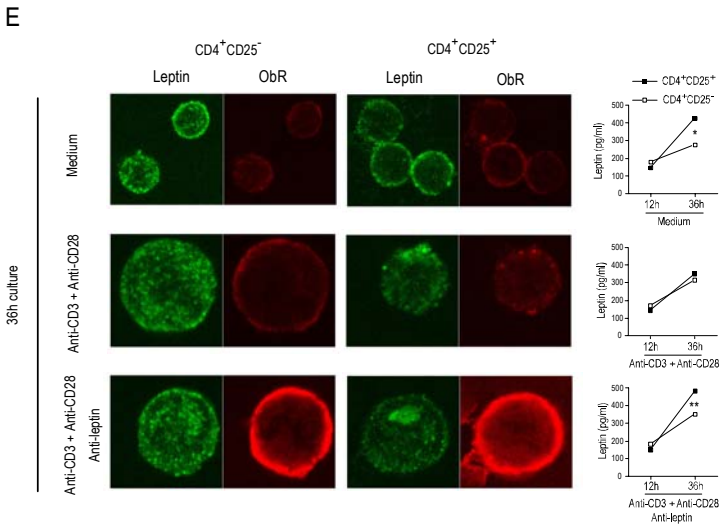


Figure 3. T_{reg} cells produce leptin and express high amounts of ObR.

(A) Proliferation of $CD4^+CD25^-$ effector T cells, T_{reg} cells and of both cell types in co-culture in three different serum- and leptin-free media in the presence or absence of leptin mAb. The data are shown as mean ± SD (n = 6, $*P < 0.01$; $**P < 0.001$).

(B) Flow cytometry plot of BrdU incorporation of $CD4^+CD25^-$ effector T cells, T_{reg} cells and of both cell types in co-culture in serum free medium (X-VIVO), in the presence or absence of leptin mAb during anti-CD3 and anti-CD28 stimulation. One representative out of three independent experiments is shown ($*P < 0.05$; $**P < 0.001$).

(C) Confocal microscopy of freshly isolated and 1h-stimulated T_{reg} cells and $CD4^+CD25^-$ effectors stained for leptin (in green) and leptin receptor (ObR) (in red). One representative out of three independent experiments is shown.

(D) Immunoblot for leptin on cell lysates from T_{reg} cells and $CD4^+CD25^-$ effectors. The graph shows quantitation of leptin with respect to total ERK1/2. One representative out of three independent experiments is shown.

(E) Confocal microscopy for leptin and ObR at 36h after anti-CD3 and anti-CD28 stimulation, and human leptin specific ELISA performed on cell culture media at 12h and 36h after stimulation (right column). The data are shown as mean ± SD (n = 3, $*P < 0.03$ and $**P < 0.05$).

4.5 Leptin-mAb-induced proliferation of Treg cells is IL-2 dependent, and IL-2-supported expansion of Treg cells is not affected by leptin

To test whether the leptin-mAb-induced proliferation of Treg cells was IL-2 dependent, we evaluated the effects of IL-2 neutralization on the Treg cells proliferative responses and IL-2 production. Addition of human IL-2 neutralizing mAb enhanced the inhibitory effects of leptin mAb treatment on the $CD4^+CD25^-$ T cell proliferation and IL-2 secretion (Figures 4A-B, white columns) ($*P < 0.01$ and $**P < 0.05$). IL-2 mAb reduced both Treg cells proliferation and IL-2 production induced by the neutralization of leptin (Figures 4A-B, black columns) ($*P < 0.001$). Moreover, the apparent reversal of

suppression in co-culture experiments due to anti-leptin-induced Treg cells proliferation was inhibited by anti-IL-2 (Figures 4A-B, grey columns) (*P < 0.01). IL-2 secretion was evaluated *via* CTLL-2 proliferation (Figure 4B).

Next we compared IL-2-induced proliferation *versus* anti-leptin induced proliferation on Treg cells. Addition of exogenous IL-2 reversed Treg cells unresponsiveness upon anti-CD3 and anti-CD28 stimulation (Figure 4C, left panel, *P < 0.01), as expected. Of interest, IL-2-induced proliferation was less than that observed upon leptin blockade (Figure 4C, left panel, **P < 0.05). To also address whether anti-leptin treatment affected the ability of Treg cells to proliferate in the presence of IL-2 but in the absence of TCR stimulation, we measured the proliferation of unstimulated Treg cells cultured with leptin mAb or IL-2 and anti-leptin plus IL-2. No proliferation was observed in the absence of TCR engagement in all the above conditions (Figure 4C, left panel). Finally, we tested whether leptin could inhibit IL-2-induced expansion of Treg cells. The anti-proliferative effect of increasing doses of recombinant leptin on Treg cells proliferation induced by IL-2 was not significant (Figure 4C, right panel), suggesting that IL-2 signalling may be independent and dominant on the leptin-mediated inhibitory effects on the cell proliferation. Previous experimental evidence suggests that the growth, homeostasis and function of the Treg cells is dependent on IL-2 *in vitro* and *in vivo* (Setoguchi et al. 2005; Fontenot et al. 2005). Our data also indicated that leptin neutralization could induce IL-2 secretion by Treg cells and that leptin mAb-induced-proliferation was IL-2-dependent. When comparing the efficiency of leptin neutralization and IL-2 to expand Treg cells *in vitro*, we observed that leptin mAb had a better efficiency than recombinant IL-2 (as indicated by Treg cells proliferation and number after eight days culture). Conversely, addition of increasing doses of recombinant leptin to the cell cultures did not affect the IL-2-mediated proliferation of the Treg cells, suggesting that the control of leptin on the expansion of the Treg cells was independent and not overcome by IL-2 signalling. McHugh et al. (McHugh et al. 2002) demonstrated that cultures of Treg cells with neutralizing glucocorticoid-induced TNF receptor (GITR) mAb allowed the Treg cells to respond to exogenous IL-2 in the absence of TCR-stimulation. We also tried to induce Treg cells proliferation in the absence of TCR-stimulation by adding high concentration of IL-2 and anti-leptin, but found lack of proliferation, indicating that anti-leptin-induced proliferation of the Treg cells needs concomitant TCR-engagement.

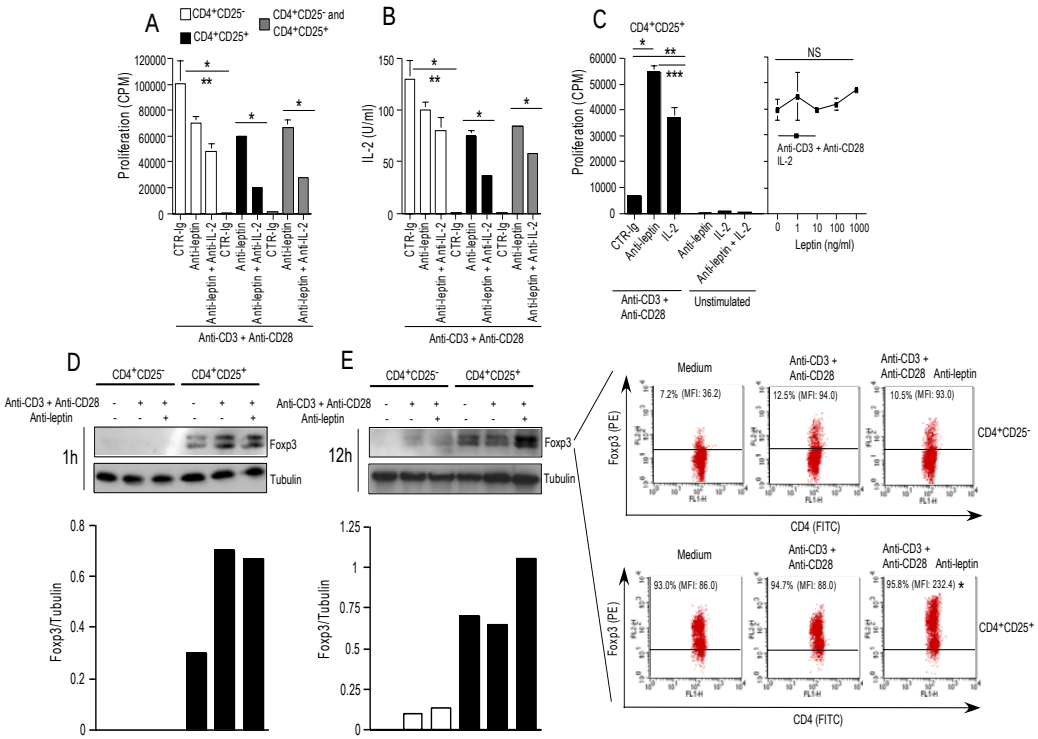


Figure 4. Leptin-mAb-induced proliferation of human T_{reg} cells is IL-2-dependent and Foxp3 expression is increased during proliferation.

(A) Proliferation of $CD4^+CD25^-$ effector T cells, T_{reg} cells and of both cell types in co-culture in the presence or absence of leptin mAb and IL-2 neutralizing mAb. The data are shown as mean ± SD (n = 5, $^*P < 0.01$; $^{**}P < 0.05$, white columns; $^*P < 0.001$ black columns and $^*P < 0.01$ grey columns, respectively).

(B) IL-2 secretion of $CD4^+CD25^-$ effector T cells, T_{reg} cells and of both cell types in co-culture in the presence or absence of leptin mAb and IL-2 neutralizing mAb. The data are shown as mean ± SD (n = 5, $^*P < 0.01$ and $^{**}P < 0.05$, white columns; $^*P < 0.001$ black columns and $^*P < 0.01$ grey columns, respectively).

(C) Proliferation of T_{reg} cells in the presence of either leptin mAb or recombinant IL-2 (left), stimulated or not with anti-CD3 and anti-CD28. The data are shown as mean ± SD (n = 5, $^*P < 0.0001$; $^{**}P < 0.01$; $^{***}P < 0.05$). Addition of scalar doses of recombinant leptin to proliferating T_{reg} cells (right) stimulated with anti-CD3 and anti-CD28 in the presence of recombinant IL-2. The data are shown as mean ± SD (n = 5, NS = Not significant).

(D) Immunoblot analysis of $CD4^+CD25^-$ effector T cells and T_{reg} cells in the presence or absence of leptin mAb, at 1h stimulation with anti-CD3 and anti-CD28. The graph shows quantitation of Foxp3 with respect to tubulin. One representative out of five independent experiments is shown.

(E) Immunoblot analysis and flow cytometry plot of $CD4^+CD25^-$ effector T cells and T_{reg} cells in the presence or absence of leptin mAb, at 12h stimulation with anti-CD3 and anti-CD28. The graph shows quantitation of Foxp3 with respect to tubulin. One representative out of five independent experiments is shown ($^*P < 0.01$, as compared with anti-CD3 and anti-CD28).

4.6 Treg cells expanded with leptin mAb have increased expression of Foxp3

We next addressed whether leptin neutralization could affect the amount of Foxp3 in Treg cells. Foxp3 expression was evaluated after leptin neutralization at 1h and 12h during anti-CD3 and anti-CD28 stimulation. As shown in Figure 4D, Foxp3 expression was increased at early time points after anti-CD3 and anti-CD28 stimulation ($P < 0.01$) and leptin mAb treatment did not alter Foxp3 protein amounts. Conversely, at 12 hours, Treg cells that proliferated after leptin mAb showed increased Foxp3 amounts when compared to those activated with anti-CD3 and anti-CD28 alone ($P < 0.01$) (Figure 4E). As expected, Foxp3 was undetectable in $CD4^+CD25^-$ T effectors after 1h of stimulation in all the experimental conditions (Figure 4D), whereas at 12h there was a little expression of Foxp3 after anti-CD3 and anti-CD28 stimulation either in the presence or in the absence of leptin mAb that did not reach statistical significance (Figure 4E). Foxp3 - the master gene for Treg cells development and function (Sakaguchi et al. 2005) - was induced in leptin-neutralized Treg cells at late time points (12h), supporting the evidence that removal of leptin from cultures medium not only expanded the Treg cells but also allowed the maintenance of their phenotype. The phenomenon might possibly be ascribed to the increased IL-2 secretion induced by leptin neutralization, as others have shown that IL-2 is capable to upregulate Foxp3 expression in human Treg cells (Zorn et al. 2006).

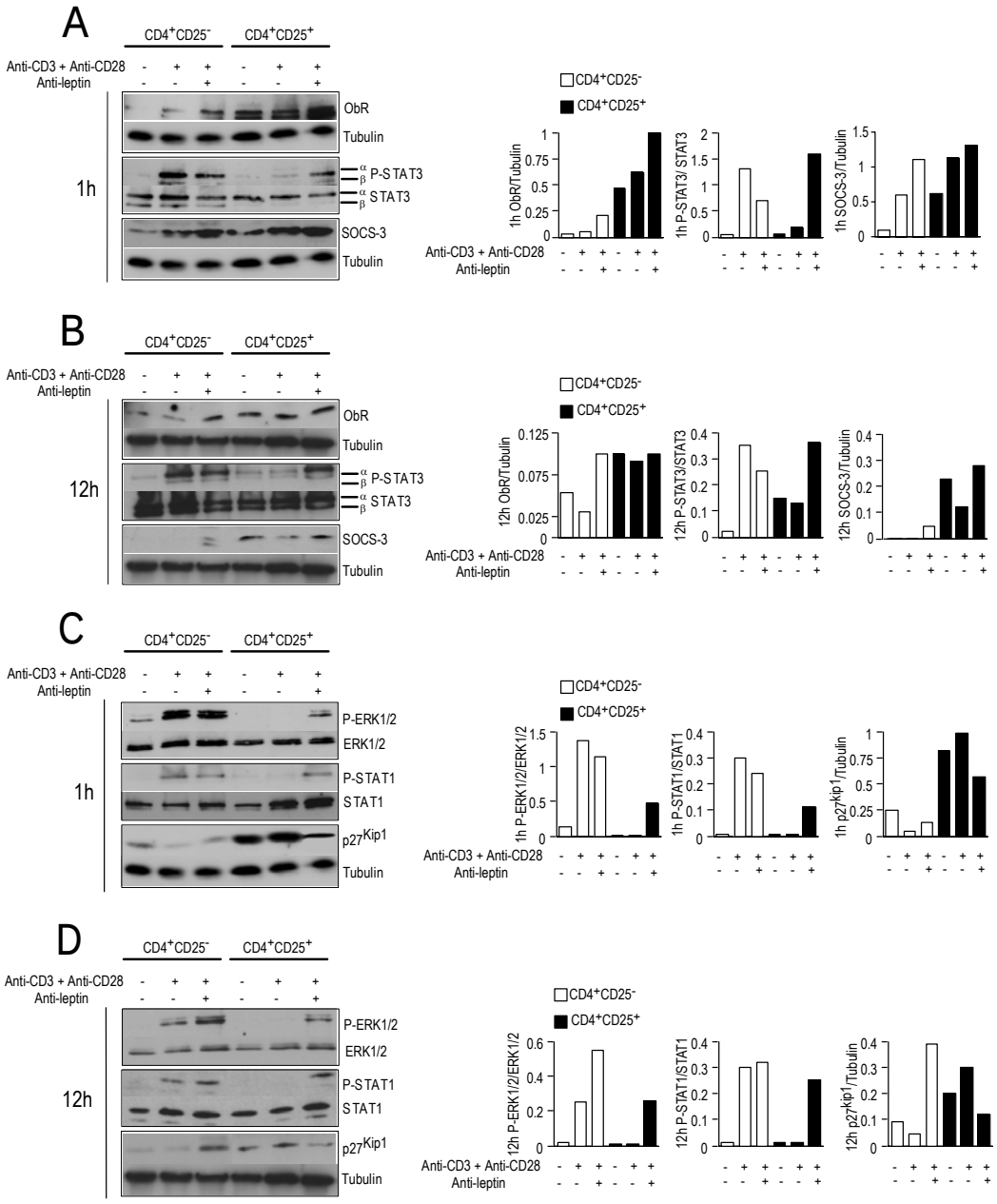


Figure 5. Molecular effects of leptin neutralization on human T_{reg} cells.

(A) and (B) Immunoblot for ObR, STAT3 and SOCS3 on $CD4^+CD25^-$ T cells and T_{reg} cells in the presence or absence of leptin mAb stimulated with anti-CD3 and anti-CD28 at 1h and 12h, respectively. Graphs show quantitation of each specific protein. One representative out of five independent experiments is shown.

(C) and (D) Immunoblot for ERK1/2, STAT1 and $p27^{kip1}$. Graphs show quantitation of each specific protein. One representative out of five independent experiments is shown.

4.7 The effect of leptin neutralization on ObR and STAT3 activation pathway

The molecular effects of leptin mAb were studied at early (1h) or late time points (12h) on highly purified human Treg cells or effector $CD4^+CD25^-$ T cells, activated or not with anti-CD3 anti-CD28. We first analyzed the leptin-ObR-STAT3 biochemical pathway: 1h and 12h cultures confirmed that in unstimulated cells the ObR was expressed at higher amounts in Treg cells than in $CD4^+CD25^-$ T cells (Figure 5A-B and Figure 3C). *In vitro* stimulation with anti-CD3 and anti-CD28 induced an upregulation of the ObR expression on both cell types and was more evident at 1h than at 12h (Figure 5A-B and Figure 3C). Leptin neutralization on both Treg cells and $CD4^+CD25^-$ T cells further upregulated the expression of ObR particularly at 1h, suggesting that leptin blockade might induce a compensatory upregulation of its receptor.

As readout of ObR activity, we measured phospho-STAT3 (P-STAT3) amounts, because STAT3 is known to participate in the intracellular signalling pathways of ObR (La Cava and Matarese, 2004). Activation of $CD4^+CD25^-$ T cells induced strong STAT3 phosphorylation at 1h and 12h and anti-leptin treatment reduced the P-STAT3 amounts, particularly at 1h (Figure 5A-B). Conversely, stimulation of Treg cells was not associated with a marked increase of P-STAT3, whereas anti-leptin induced amounts of P-STAT3 at 1h and 12h comparable to those observed in proliferating $CD4^+CD25^-$ T cells (Figure 5A-B). Of note, at 1h, $CD4^+CD25^-$ T cells expressed both STAT3 spliced isoforms (Maritano et al. 2004) - STAT3α and STAT3β - whereas Treg cells mainly expressed the STAT3α isoform. This phenomenon was not as evident after 12h when the two spliced isoforms were similar in both cell subsets, suggesting that the tissue culture conditions could induce STAT3β in Treg cells, independently on the different type of stimulation. Finally, in unstimulated or anti-CD3 and anti-CD28-stimulated Treg cells, at both 1h and 12h, the amounts of P-STAT3 were higher than those observed in unstimulated $CD4^+CD25^-$ cells, suggesting an increased "basal" leptin-mediated signalling in Treg cells. This result could be secondary to higher basal expression of ObR on Treg cells (Figure 5A-B, Figure 1C and Figure 3C). No induction of P-STAT3 was observed in Treg cells treated with leptin mAb alone without TCR stimulation.

We also investigated in our system the expression of suppressor of cytokine signalling 3 (SOCS3), a key negative regulator of cytokine signalling, including leptin (Kinjyo et al. 2006; La Cava and Matarese, 2004). SOCS3 in anti-CD3 and anti-CD28 stimulated $CD4^+CD25^-$ T cells increased markedly and was further increased by leptin neutralization at 1h (Figure 5A). This finding inversely correlated with the corresponding P-STAT3 expression. At a later time point (12h), SOCS3 was undetectable and could be found at low

amounts upon leptin neutralization (Figure 5B). At 1h, SOCS3 in Treg cells was markedly higher than in $CD4^+CD25^-$ cells in all the experimental conditions. Specifically, SOCS3 expression was high in unstimulated Treg cells and increased after anti-CD3 and anti-CD28 stimulation despite little STAT3 activation. Treatment with anti-leptin, which induced P-STAT3, did not alter the SOCS3 amounts (Figure 5A). At 12h, basal SOCS3 expression was higher in the Treg cells than in $CD4^+CD25^-$ cells, and was markedly reduced after anti-CD3 and anti-CD28 stimulation. In conclusion, leptin mAb treatment - which associates with STAT3 activation in Treg cells – also induced SOCS3 expression (Figure 5B).

4.8 The effect of leptin neutralization on biochemical pathways involved in T cell activation and anergy

To evaluate whether leptin neutralization could affect T cell activation and anergy, we studied tyrosine-phosphorilation of the extracellular-signal regulated kinases 1 and ERK2 (ERK1/2). Leptin neutralization in the presence of anti-CD3 and anti-CD28 in $CD4^+CD25^-$ T cells, increased ERK1/2 phosphorylation as compared to anti-CD3 and anti-CD28 stimulation alone, particularly at 12h (Figure 5C-D). Treatment of Treg cells with anti-CD3 and anti-CD28 did not induce phospho-ERK1/2 (P-ERK1/2), confirming their anergic state. However, leptin mAb induced high amounts of ERK1/2 tyrosine-phosphorylation at both 1h and 12h, concomitantly with the induction of Treg cells proliferation and a reversal of their hyporesponsiveness (Figure 5C-D). Moreover, we investigated whether leptin neutralization could alter STAT1, another transcription factor whose activity is required for the development and function of Treg cells (Nishibori et al. 2004). We found that leptin neutralization did not affect STAT1 phosphorylation (P-STAT1) in $CD4^+CD25^-$ effectors at both time points. In contrast, P-STAT1 was markedly induced in the leptin-neutralized Treg cells (Figure 5C-D). The fact that leptin neutralization in $CD4^+CD25^-$ T cells reduced the levels of STAT3 phosphorylation and had little effect on STAT1 needs an explanation. This partial effect on the ObR-STAT3-mediated signalling could be ascribed to the fact that also other cytokines activate STAT3 (Doganci et al. 2005). As such, removal of leptin could only inhibit in part STAT3 phosphorylation. In this context, it is interesting to note that also SOCS3, a key negative regulator of STAT3-activating cytokines (Kinjyo et al. 2006) was differentially expressed in the Treg cells and in the $CD4^+CD25^-$ T cells. The regulation of STAT3 signalling by SOCS3 in $CD4^+CD25^-$ effectors was bi-phasic, with a first peak at 1h and a rapid degradation at 12h, as previously described (Wormald et al. 2006). Leptin neutralization increased SOCS3 amounts concomitantly with

a reduced phosphorylation of STAT3. This is also consistent with the SOCS3 inhibition of IL-2 production and T cell proliferation which we observed in our work (Matsumoto et al. 2003). In Treg cells, SOCS3 was highly expressed in basal conditions and markedly induced during acute stimulation, in agreement with the anergic state of the Treg cells and their reduced IL-2 secretion.

Last, we studied the modulation of the cyclin-dependent kinase inhibitor p27 ($p27^{kip1}$), a molecule involved in the control of cell-cycle, T cell anergy and known to block the cell-cycle progression in Treg cells (Li et al. 2005, Wells et al. 2001). $p27^{kip1}$ was down-modulated by anti-CD3 and anti-CD28 stimulation in $CD4^+CD25^-$ effectors whereas anti-leptin did not down-modulate $p27^{kip1}$ expression at 1h and markedly increased its expression at 12h (Figure 5C-D), explaining, at least in part, the inhibition of $CD4^+CD25^-$ T cells proliferation induced by leptin neutralization. In contrast, Treg cells showed elevated amounts of $p27^{kip1}$ before and after anti-CD3 and anti-CD28 stimulation, confirming their anergic state associated with cell-cycle arrest. Leptin mAb treatment induced degradation of this molecule at both 1h and 12h, which could explain reversal of their anergic state and subsequent proliferation (Figure 5C-D). Taken together these data suggest that, at the molecular level, Treg cells expressed high levels of ObR and of $p27^{kip1}$ but no phosphorylation of ERK1/2, STAT1, and little phosphorylation of STAT3. In contrast, leptin neutralization upon anti-CD3 and anti-CD28 stimulation and Treg cells expansion associated with a rapid degradation of $p27^{kip1}$ as well as a marked phosphorylation of ERK1/2, STAT1 and STAT3 (phosphorylation of STAT3 in Treg cells could be partly explained by both the induction of the ERK1/2, which is known to phosphorylate STAT3 independently of ObR (Quadros et al. 2004, Barboza et al. 2004), as well as by the secretion of STAT3-activating cytokines, ie. IL-2 and IL-6 (Dogangi et al. 2005). Recently, Zorn and collegues (Zorn et al. 2006) showed that IL-2 upregulates Foxp3 expression in human Treg cells through a STAT3-dependent mechanism, confirming that induction of Foxp3 (in our case, during leptin neutralization) may occur *via* a STAT3-dependent mechanism. Intriguingly, $CD4^+CD25^-$ T cells, after leptin neutralization, showed an increase of $p27^{kip1}$ associated with sustained ERK1/2 phosphorilation - a phenotype often observed in anergic T cells (Wells et al. 2001, Chen et al. 1999, Waiczies et al. 2005) and in effector T cells from mice treated with anti-leptin antibodies during autoimmune encephalomyelitis (De Rosa et al. 2006). Our results indicate that leptin neutralization can “unlock” the hyporesponsiveness of Treg cells *via* a rapid degradation of the cell-cycle inhibitor $p27^{kip1}$, associated with the phosphorylation of ERK1/2. These biochemical events would allow the Treg cells to enter

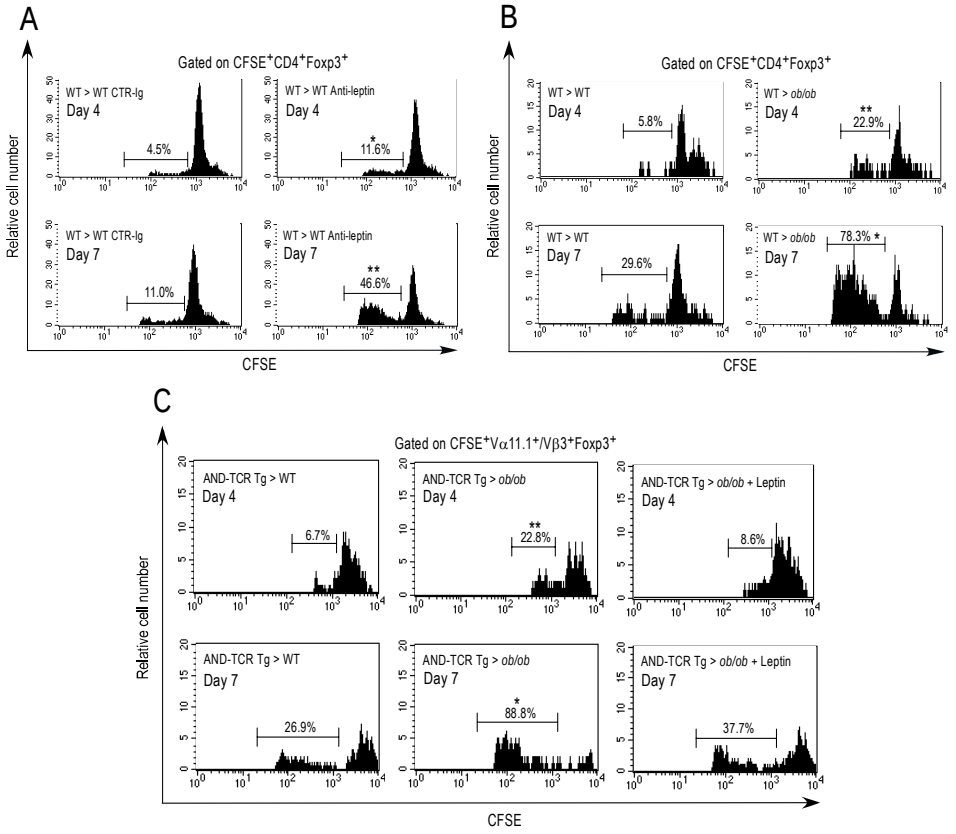
the G1/S phase of the cell-cycle, induce IL-2 gene transcription and, consequently, reverse their anergic state (Li et al. 2005). Our data also indicate that these changes associate with the activation of STAT1 and STAT3, two transcription factors related with cytokine signalling and proliferation of the Treg cells (Doganci et al. 2005, Nishibori, et al. 2004).

4.9 Leptin deficiency promotes proliferation of Treg cells in mice

Treg cells, despite their *in vitro* hyporesponsiveness, can expand *in vivo* in normal, non-lymphopenic hosts (Trenado et al. 2003, Gavin et al. 2002). We used non-irradiated, non-lymphopenic recipient mice to avoid a homeostatic expansion of the Treg cells which would occur in lymphopenic hosts (Trenado et al. 2003, Gavin et al. 2002). The *in vivo* proliferative capacity of Treg cells in anti-leptin-treated wild-type (WT) mice *versus* control-Ig-treated mice was tested using CFSE-labelled-$CD4^+$ T cells from normal WT mice and measuring the CFSE dilution in the $CD4^+Foxp3^+$ cells (Figure 6A). Mouse Treg cells expanded *in vivo* more robustly and earlier (day 4 and day 7 post transfer) when adoptively transferred into leptin-neutralized WT mice (Figure 6A) (*P < 0.01; **P < 0.001). This result was confirmed by adoptive transfer of CFSE-labelled-$CD4^+$ T cells from normal WT mice into leptin-deficient *ob/ob* mice, in which the Treg cells expanded more robustly and earlier (day 4 and day 7 after transfer) when compared with cells transferred into normal WT mice (Figure 6B) (**P < 0.001 and *P < 0.01, respectively). We also confirmed our data in a transgenic antigen-specific system utilizing donor mice carrying the (TcrAND)53Hed transgene (AND-TCR Tg mice) expressing the clonotypic Vα11.1/Vβ3 TCR chains, specific for the carboxy-terminal fragment of pigeon cytochrome c (PCC) (Kaye et al. 1989). Assessment 4 and 7 days after adoptive transfer revealed that transferred CFSE-labelled clonotypic AND-TCR Treg cells proliferated more vigorously in *ob/ob* mice as compared with normal WT controls (Figure 6C) (**P < 0.001 and *P < 0.01, respectively). This phenomenon was reversed by recombinant leptin administration to leptin-deficient *ob/ob* mice (Figure 6C). To avoid interference of $CD4^+$ T cell lymphopenia on the Treg cells expansion *in vivo*, in the adoptive transfer experiments in *ob/ob* mice, we utilized 6 weeks-old mice (in which the leptin deficiency has not yet determined significant reduction of the $CD4^+$ T cell number). The *in vivo* results were also confirmed by CFSE dilution experiments gating on the $CD4^+CD25^+$ Treg cells in both leptin neutralized WT and leptin-deficient *ob/ob* mice, respectively (Figure 6D and F,

respectively). Foxp3 expression was increased in the Treg cells of the leptin neutralized WT mice (Figure 6E).

Taken together, *in vivo* studies in leptin-deficient *ob/ob* mice show higher percentage and absolute number of circulating Treg cells. They can expand up to 90% *in vivo* in normal non-lymphopenic hosts by day 28 post transfer (Trenado et al. 2003). Adoptive transfer experiments of CFSE-labelled Treg cells from WT mice into leptin-deficient *ob/ob* mice showed a significant *in vivo* expansion of Treg cells. It is also noteworthy that chronic leptin deficiency allows higher expansion of Treg cells when compared with acute leptin neutralization induced by leptin mAb (the percentage of proliferating Treg cells in *ob/ob* mice was always higher than that observed in leptin neutralized WT mice). Importantly, *in vivo* leptin neutralization also determined an increased Foxp3 expression in the Treg cells, suggesting maintenance of their suppressive phenotype after expansion *in vivo*. Taken together, our data suggest that the presence of ObR may be crucial in the control of the expansion of Treg cells because stimulated Treg cells from *db/db* mice proliferated better than Treg cells from controls *in vitro*, whereas the suppressive capability of Treg cells from *db/db* mice was similar to that of control mice.



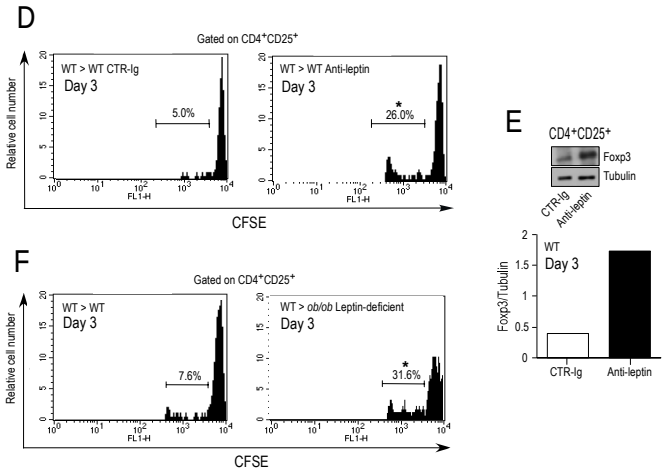


Figure 6. *In vivo* leptin neutralization or congenital leptin deficiency associate with proliferation of T_{reg} cells.

(A) Proliferation measured as CFSE dilution of CFSE-labelled-T_{reg} cells obtained from WT mice and transferred into control (CTR)-Ig or mouse leptin neutralizing Ab-treated WT mice. The histogram shows the percent of $CFSE^+$ T_{reg} cells (gated on $CD4^+Foxp3^+$ cells) that had divided 4 and 7 days after transfer. One representative out of three independent experiments with 3 mice per group is shown (**P* < 0.01; ***P* < 0.001).

(B) CFSE dilution profile of CFSE-labelled-T_{reg} cells (gated on $CD4^+Foxp3^+$ cells) obtained from WT mice and transferred into WT or leptin-deficient *ob/ob* mice, 4 and 7 days after transfer. One representative out of three independent experiments with 3 mice per group is shown (***P* < 0.001; **P* < 0.01).

(C) CFSE dilution profile of PCC-specific CFSE-labelled AND-TCR T_{reg} cells (gated on $Va11.1^+/Vb3^+Foxp3^+$ cells) adoptively transferred into WT, *ob/ob* and *ob/ob* treated with recombinant leptin, 4 and 7 days after transfer. One representative out of two independent experiments with 4 mice per group is shown (***P* < 0.001; **P* < 0.01).

4.10 Proliferative potential and functional capacity of T_{reg} cells from leptin receptor-deficient mice

We studied the *in vitro* proliferation and suppressive capacity of $CD4^+CD25^-$ effectors and Treg cells from congenitally leptin-receptor-deficient *db/db* mice and normal *db/*+ heterozygous controls (Figure 7A-C). Stimulation with anti-CD3 and anti-CD28 of $CD4^+CD25^-$ effector T cells from *db/db* mice was less effective in inducing proliferation than stimulation on $CD4^+CD25^-$ from *db/*+ mice (Figure 7A) (**P* < 0.001). Conversely, *in vitro* stimulation of Treg cells from *db/db* mice induced significantly higher proliferation than that of Treg cells from *db/*+ heterozygous controls (Figure 7B) (***P* < 0.01). These data suggested that the absence of the ObR impaired the expansion of $CD4^+CD25^-$ effectors and enhanced the proliferative potential of Treg cells. The ObR-deficiency seemed to affect the proliferative potential of the Treg cells rather than their qualitative or functional activity, as Treg cells from *db/db* mice suppressed the proliferation of $CD4^+CD25^-$ T cells in a fashion similar to that of Treg cells from *db/*+ control mice (Figure 7C), confirming the finding

that addition of exogenous leptin did not alter the suppressive capacity of Treg cells *in vitro* (Figure 2A).

Subsequent studies were performed analyzing antigen-specific responses in an autoimmune disease model: the *in vitro* proliferative capacity of Treg cells against the pancreatic autoantigen glutamic acid decarboxylase (GAD)65 was evaluated in autoimmune diabetes nonobese diabetic (NOD/LtJ) mice *versus* leptin-receptor-mutant NOD-*Lepr*^{db5J}/LtJ mice (Lee et al. 2005) (Figure 7D). These recently-isolated ObR mutant mice display obesity, hyperphagia, and resistance to the development of pancreatic β-islets infiltration (Lee et al. 2005). We found that after five days of splenocyte cultures, Treg cells from NOD-*Lepr*^{db5J}/LtJ proliferated more robustly against mouse GAD65 than Treg cells from NOD/LtJ females, suggesting an increased autoantigen-specific proliferative potential of the Treg cells from mice with impaired ObR signalling (Figure 7D) (*P < 0.01).

4.11 Leptin-deficient mice have increased numbers of Treg cells that can be reduced by administration of leptin

Mice with genetic deficiency of leptin (*ob/ob*) or leptin-receptor (*db/db*) have reduced susceptibility to autoimmunity (Matarese et al. 2001, Sanna et al., 2003, Siegmund et al. 2004, Faggioni et al. 2000, Lee et al. 2005). Since Treg cells play a central role in regulating autoimmunity (Shevach 2002, Sakaguchi, 2005), we tested whether genetic deficiency of leptin associated with effects on Treg cells. A significant increase of the percentage of peripheral Treg cells was observed in *ob/ob* as compared to WT (Figure 7E-F, respectively) (*P < 0.02). Administration of leptin reduced the elevated number of Treg cells in the *ob/ob* mice to a number comparable to that found in the WT mice (Figure 7E-F) (*P < 0.02).

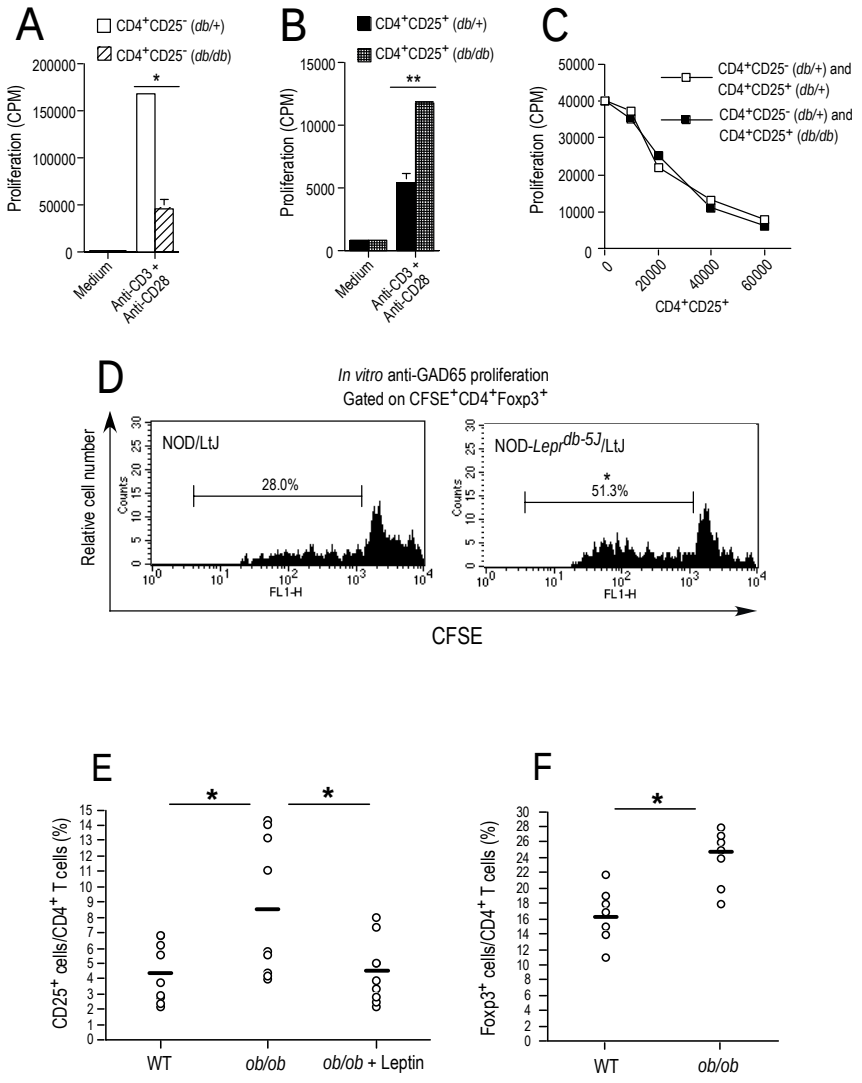


Figure 7. ObR deficiency increases T_{reg} cells proliferative potential, does not alter their suppressive capacity and impairs $CD4^+CD25^-$ proliferation.

(A) Proliferation of $CD4^+CD25^-$ effector T cells from *db*/+ and leptin receptor-deficient *db/db* mice stimulated with anti-CD3 and anti-CD28. The data are shown as mean ± SD (n = 5, **P* < 0.001).

(B) Proliferation of T_{reg} cells from *db*/+ and *db/db* mice stimulated with anti-CD3 and anti-CD28. The data are shown as mean ± SD (n= 5, ***P* < 0.01).

(C) Proliferation of $CD4^+CD25^-$ effector T cells from *db*/+ mice in the presence of increasing number of either *db*/+ or *db/db* T_{reg} cells, stimulated with anti-CD3 and anti-CD28. The data are shown as mean ± SD (n = 5).

(D) CFSE dilution of T_{reg} cells (gated on $CD4^+Foxp3^+$ cells) from leptin receptor mutant NOD-*Lepr*$^{db\text{-}5J}$/LtJ mice stimulated with mouse recombinant GAD65, after five days culture. One representative out of five independent experiments is shown (**P* > 0.01).

(E) Percentage numbers of peripheral CD4+CD25+ Treg cells in WT, leptin deficient *ob/ob* mice, and recombinant leptin-treated *ob/ob* mice. One representative out of three independent experiments with 8 mice per group is shown (**P* < 0.02).

(F) Percentage numbers of peripheral CD4+Foxp3+ Treg cells in WT and leptin deficient *ob/ob* mice. One representative out of three independent experiments with 7 mice per group is shown (**$P < 0.02$).

4.12 *In vivo* leptin-receptor neutralization improves EAE clinical score and delays disease progression

To test whether interference with the leptin axis could modify the *in vivo* generation and/or the expansion of Treg cells, we evaluated the absolute number and the percentage of Treg cells during EAE in mice treated or not with a leptin-receptor blocking antibody (9F8 mAb).

We first analyzed the capacity of 9F8 to affect induction and progression of EAE after immunization with proteolipid protein 139–151 ($PLP_{139–151}$) myelin peptide. Treatment with 9F8 was initiated prior to immunization (from day –1 to day 1 relative to immunization), during the acute phase of the disease (days 8–11), or during the disease course (days 14-17) for 3 and 4 consecutive days, respectively (Figure 8A; see Methods for details). In all the conditions leptin-receptor neutralization led to a statistically significant reduction in EAE clinical score and percentage of initial body weight loss). These effects led to significant clinical improvement and delayed disease progression during the following 40 days of observation (Figure 8A), indicating that leptin-receptor blockade inhibited both development and progression of EAE. Moreover, observation of animals over a longer period of time (90–120 days) revealed a significant reduction in relapse rate, suggesting that this protection was long lasting.

Surprisingly, when we compared the percentage of Treg cells from spleen and lymphnodes of EAE affected mice, treated or not with 9F8, we did not find increased number of Treg cells after *in vivo* leptin-receptor neutralization (data not presented). Starting from these preliminary results, we hypothesized that alteration of the leptin axis did not modify the *in vivo* generation and/or expansion of Treg cells, and that amelioration of disease clinical signs observed after leptin-receptor neutralization was not to be ascribed to an *in vivo* induction of tolerance. However, a more detailed analysis of the *in vitro* expansion of Treg cells during a specific $PLP_{139–151}$ T cell activation, showed that the percentage of these cells increased when lymphocytes derived from EAE affected CTR mice, that did not receive *in vivo* treatment with leptin-receptor neutralizing mAb, were cultured *in vitro* in the presence of 9F8 (Figure 9A-B). On the contrary, no statistically significant difference was observed when lymphocytes derived from EAE affected 9F8 treated mice (in all the experimental conditions) were cultured *in vitro* with $PLP_{139–151}$, in the presence or in the absence of 9F8 mAb.

The evidence that disturbances in the leptin axis determinate an increased *in vitro* expansion of Treg cells after

a $PLP_{139–151}$ specific T cell activation, led us to suppose that also *in vivo*, after leptin-receptor neutralization, there was an increased expansion of Treg cells that, together with a reduced activation of autoreactive T cells and with a decrease of pro-inflammatory cytokines, could explain the amelioration of EAE clinical signs and disease progression. Probably, by analyzing the percentage of peripheral Treg cells in EAE affected mice, treated or not with 9F8, we did not evaluate whether an increased expansion of this subset was accompanied with a robust migration of Treg cells into CNS. This possible explanation needs further characterization and will be confirmed by future immunohistochemical analyses of the CNS sections derived from the different groups of mice.

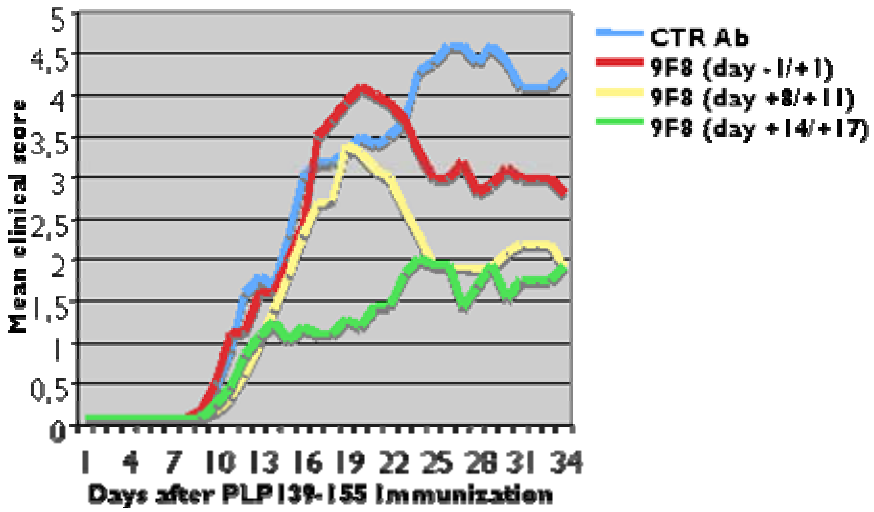


Figure 8. 9F8 treatment during active EAE reduces clinical severity of the disease

(A) Mean clinical score of SJL/J female mice treated with 9F8 mAb, injected either from day –1 to day 1, from days 8-11 or from days 14-17 (100 µg/d i.p.). Leptin-receptor blockade significantly reduced clinical score and body weight loss. Data are from 1 representative experiment out of 3.

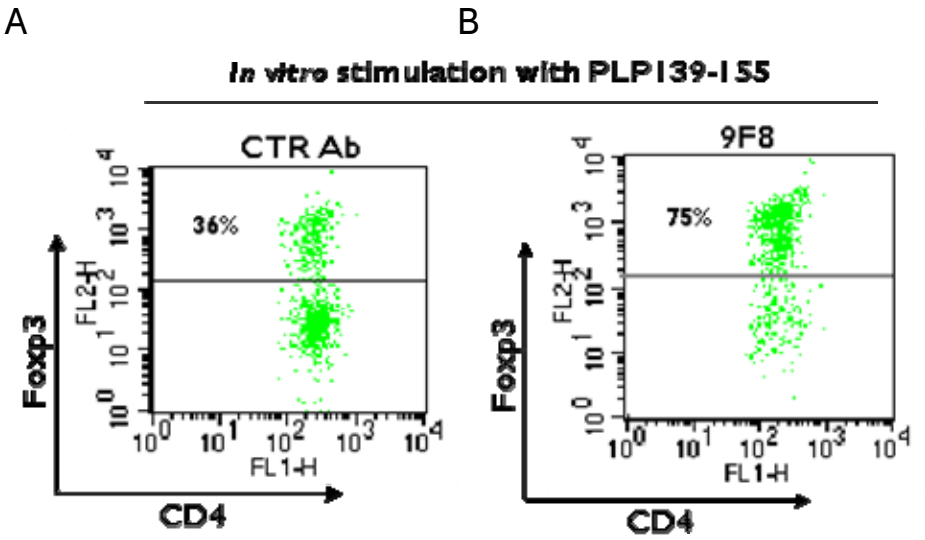


Figure 9. *In vitro* 9F8 treatment of lymphocytes derived from EAE affected mice, during a a specific $PLP_{139–151}$ T cell activation, lead to an increase of Treg cells percentage.

Flow cytometry plots of CD4+Foxp3+ cells after 8 days of a specific $PLP_{139–151}$ T cell activation, in the absence (A) or in the presence (B) of 9F8 in the culture medium. One representative out of three independent experiments is shown (**P* < 0.05; ***P* < 0.001).

5. Conclusions

In this study we establish a unique link between Treg cells and leptin by showing that leptin can modulate the hyporesponsiveness and proliferation of Treg cells both *in vitro* and *in vivo*. Our results indicate that leptin neutralization can "unlock" the hyporesponsiveness of Treg cells *via* a rapid degradation of the cell-cycle inhibitor $p27^{kip1}$, associated with the phosphorylation of ERK1/2. These biochemical events would allow the Treg cells to enter the G1/S phase of the cell-cycle, induce IL-2 gene transcription and, consequently, reverse their *in vitro* anergic state (Li et al. 2005).

Incidentally, this study also describes a novel strategy to expand human peripheral Treg cells, *via* leptin neutralization, although this approach needs to be further investigated. Current strategies to expand Treg cells employ *ex vivo* addition of cytokines to cultured cells during TCR-stimulation (Tang et al., 2004; Jiang et al., 2003). Since neutralization of the leptin present in human serum of tissue cultures and of leptin produced by Treg cells may be sufficient to determine an expansion of the Treg cells, it might be possible that leptin neutralization, in addition with exogenous IL-2, may improve the protocols of expansion for Treg cells. We are currently investigating this possibility. The find that leptin can act as a negative signal for the proliferation of Treg cells envisions new possibilities of anti-leptin-based approaches for the immunotherapy of conditions characterized by low numbers of Treg cells. Leptin might act as an endogenous "sensing" factor linking the environment (availability of nutrients) to circulating Treg cells number. Since nutritional deprivation increases susceptibility to infection and associates with amelioration of clinical manifestations of autoimmunity (Kuchroo et al. 2003, Payne 2001), it will be important to address how this relates to the influence of leptin on Treg cells and whether anti-leptin-based intervention can be applied to tune cognate T cell responses in immune regulation.

We are currently performing experiments of Treg cells expansion from naïve to treatment MS patients; our future goal is to expand in vitro myelin-specific Treg cells and reinfuse them in MS patients with the aim to induce long-term self-tolerance and ameliorate the disease progression.

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This work is dedicated to the memory of Eugenia Papa and Prof. Serafino Zappacosta.

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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.

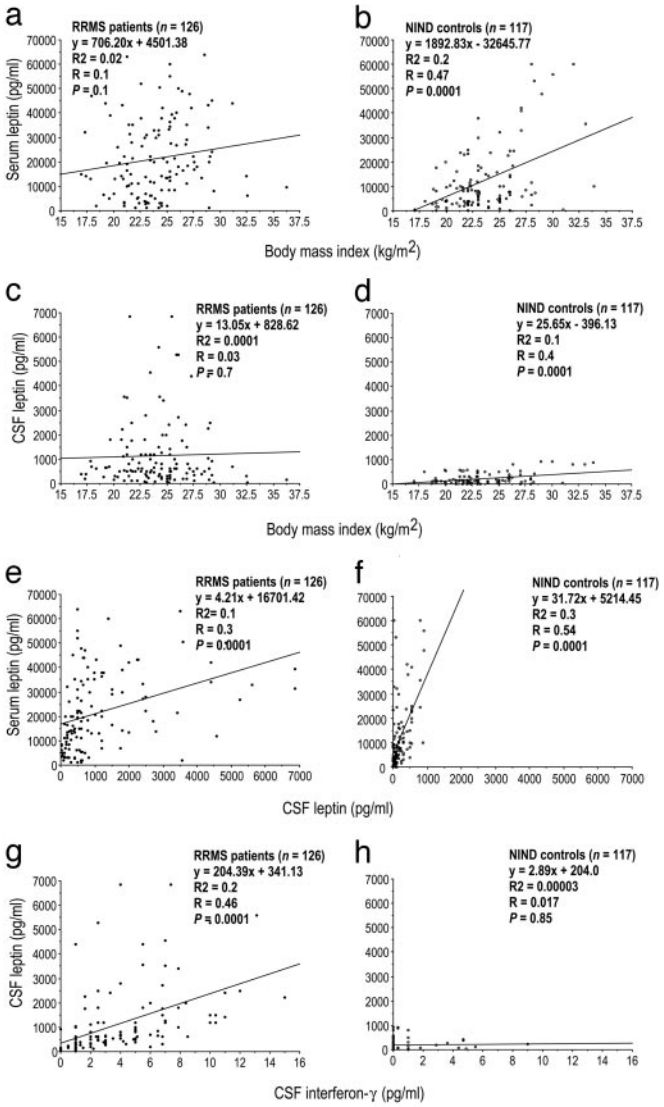

Fig. 1. Naïve-to-therapy RRMS patients show an increased secretion of leptin in serum and CSF that correlates with IFN-γ production in CSF. Statistical analyses of these data are summarized in Table 1. (*a* and *b*) Simple regression analysis between serum leptin and BMI in RRMS patients ($n = 126$) 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).

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

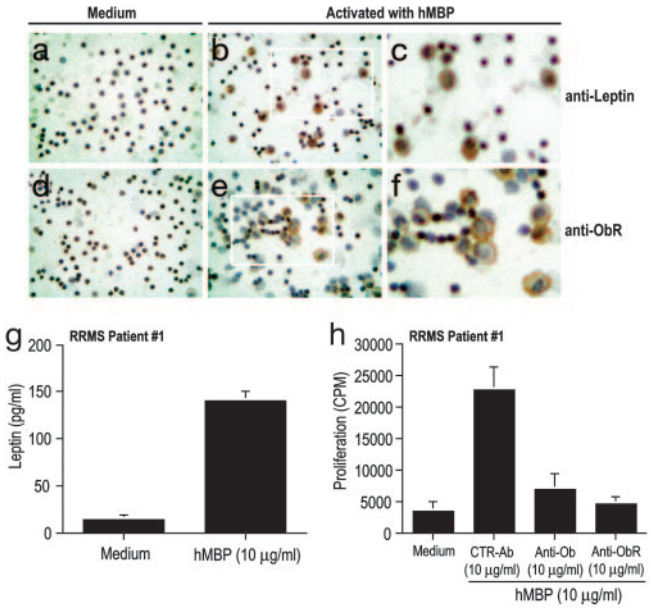

Fig. 2. T cell lines against hMBP derived from naïve-to-treatment RRMS patients produce immunoreactive leptin, up-regulate the ObR, and are inhibited in their proliferation by anti-leptin or anti-leptin-receptor blocking antibodies. (*a–c*) Expression of leptin in T cells from a naïve-to-treatment RRMS 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.

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*).

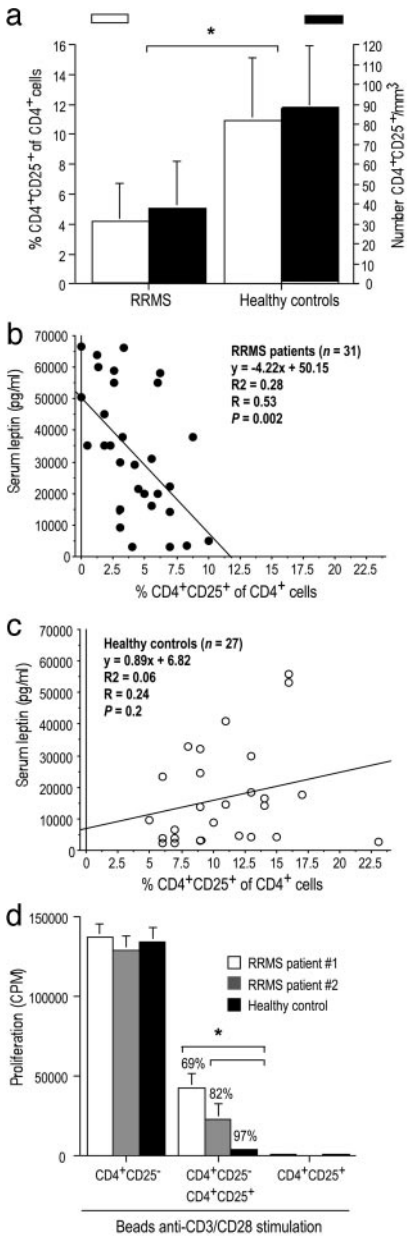

Fig. 3. Inverse correlation between serum leptin and circulating T_{Regs} in RRMS patients. (*a*) The immune phenotype of circulating lymphocytes in RRMS patients selected on the basis of their increase in serum leptin (RRMS patients with a serum leptin increase to ≥2.5-fold higher than the mean of serum 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

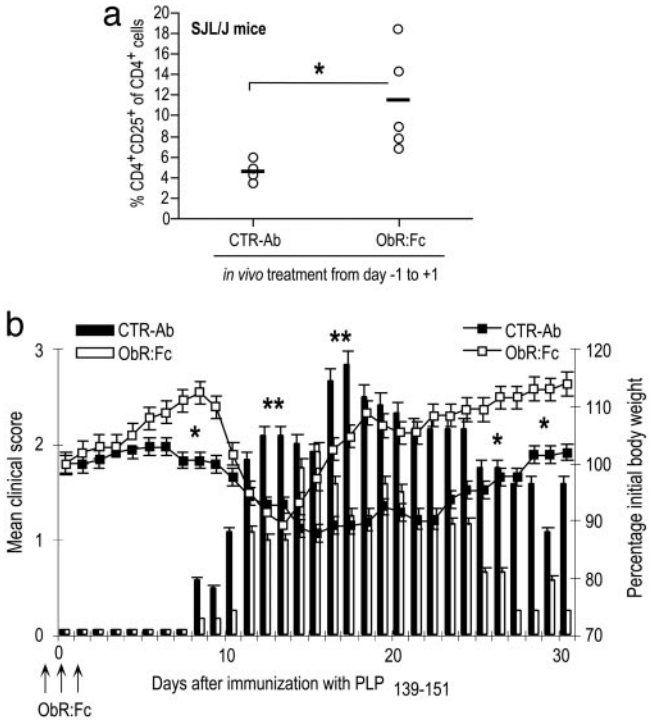

Fig. 4. Neutralization of leptin with ObR:Fc increases the number of T_{Regs} and ameliorates the clinical course of R-EAE. (*a*) Treatment of R-EAE-susceptible SJL/J female mice with ObR:Fc induced a significant increase in the circulating T_{Regs}. *, $P = 0.01$. (*b*) Mean clinical score (bars) and 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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Differential regulation of metabolic, neuroendocrine, and immune function by leptin in humans

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To elucidate whether the role of leptin in regulating neuroendocrine and immune function during short-term starvation in healthy humans is permissive, i.e., occurs only when circulating leptin levels are below a critical threshold level, we studied seven normal-weight women during a normoleptinemic-fed state and two states of relative hypoleptinemia induced by 72-h fasting during which we administered either placebo or recombinant methionyl human leptin (r-metHuLeptin) in replacement doses. Fasting for 72 h decreased leptin levels by ≈80% from a midphysiologic (14.7 ± 2.6 ng/ml) to a low-physiologic (2.8 ± 0.3 ng/ml) level. Administration of r-metHuLeptin during fasting fully restored leptin to physiologic levels (28.8 ± 2.0 ng/ml) and reversed the fasting-associated decrease in overnight luteinizing hormone pulse frequency but had no effect on fasting-induced changes in thyroid-stimulating hormone pulsatility, thyroid and IGF-1 hormone levels, hypothalamic–pituitary–adrenal and renin–aldosterone activity. FSH and sex steroid levels were not altered. Short-term reduction of leptin levels decreased the number of circulating cells of the adaptive immune response, but r-metHuLeptin did not have major effects on their number or *in vitro* function. Thus, changes of leptin levels within the physiologic range have no major physiologic effects in leptin-replete humans. Studies involving more severe and/or chronic leptin deficiency are needed to precisely define the lower limit of normal leptin levels for each of leptin's physiologic targets.

fasting | reproductive

Deficiency of the adipocyte-secreted hormone leptin (1) is associated with distinct abnormalities in energy-demanding processes such as neuroendocrine and immune function. Leptin-deficient *ob*/*ob* mice and humans with congenital complete leptin deficiency have abnormal neuroendocrine function, including hypogonadotropic hypogonadism, hypothalamic hypothyroidism, and/or growth-hormone-axis abnormalities (2–6) and impaired cell-mediated immunity (4, 7), which are improved with leptin replacement (4, 8). Similarly, starvation-induced decline of circulating leptin to very low levels in normal mice (9) and lean men (10) causes comparable neuroendocrine (9, 10) and immune defects (11, 12) that are significantly blunted or reversed with exogenous leptin.

We have shown that an 80% decline of leptin levels from ≈2 to 0.3 ng/ml in men mediates the fasting-induced suppression of gonadotropin and thyroid-stimulating hormone (TSH) pulsatility as well as sex steroid, insulin-like growth factor-1 (IGF-1), and thyroid hormone levels (10). Importantly, although observational studies have proposed that leptin regulates the hypothalamic–pituitary–gonadal axis only when serum leptin levels fall below a "threshold" of ≈2 ng/ml (13), the role of decreasing leptin levels to approximately, but not below, this threshold in leptin-replete humans with higher baseline leptin levels (e.g., normal-weight women) has not yet been directly studied.

To elucidate whether such a threshold exists, below which leptin has a "permissive" effect to regulate neuroendocrine and immune function [including peripheral blood mononuclear cell (PBMC) subpopulations, T cell proliferation, and cytokine production], we assessed pituitary hormone pulsatility and hormone levels of several neuroendocrine axes and markers of immune function in normal-weight women during a normoleptinemic-fed condition and two hypoleptinemic 72-h fasting states, with administration of either placebo (to achieve a low leptin level close to the proposed threshold) or recombinant methionyl human leptin (r-metHuLeptin) (to replace leptin to physiologic levels). To further investigate the question of a threshold leptin level in regulating immune function, we studied the effect of a range of leptin levels on T cell proliferation *in vitro*.

Results

Seventy-Two-Hour Fasting Suppresses Serum Leptin Levels out of Proportion to Changes in Body Weight and Fat Mass, and r-metHuLeptin Replacement Restores Leptin Levels Without Affecting Metabolic Parameters. In the baseline fed state, body weight increased slightly without significant changes in percent or total fat mass or fat-free mass, whereas serum leptin levels and insulin levels increased, and free fatty acid (FFA) levels decreased (Table 1). Complete fasting for 72 h significantly decreased serum leptin levels to ≈20% of baseline, out of proportion to the slight decreases in body weight and fat mass (Table 1). Leptin pulsatility on the third day of fasting was markedly suppressed (Fig. 1*a*) with loss of normal diurnal variation and decreased 24-h mean levels (20.5 ± 1.6 to 2.8 ± 0.2 ng/ml), peak height (23.8 ± 2.1 to 3.4 ± 0.3 ng/ml), valley mean (20.2 ± 1.6 to 7.8 ± 0.2 ng/ml), integrated area (29,286 ± 2,354 to 3,997 ± 277), and pulse mass (2.6 ± 0.8 to 0.5 ± 0.1) (all $P < 0.05$ vs. fed) but not pulse frequency or interpulse interval. r-metHuLeptin during fasting fully corrected the fasting-induced suppression of leptin to levels that were higher than baseline but within the physiological range for women (24-h mean: 42.4 ± 4.0 ng/ml vs. trough level on day 4 at 8 a.m. in Table 1) (Fig. 1*a*). Similar decreases in body weight and fat mass were observed as during fasting alone, with a slightly greater decrease in fat-free mass with r-metHuLeptin (Table 1). Resting metabolic rate was not affected by fasting or r-metHuLeptin, and r-metHuLeptin did not alter fasting-induced changes in insulin, respiratory quotient, or FFA (Table 1).

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Abbreviations: ACTH, adrenocorticotropic hormone; FFA, free fatty acid; FSH, follicle-stimulating hormone; T3, triiodothyronine; IGF-BP, IGF-binding protein; LH, luteinizing hormone; PBMC, peripheral blood mononuclear cell; PRA, plasma renin activity; TSH, thyroid-stimulating hormone.

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Table 1. Weight, body composition, resting metabolic rate (RMR), and hormone levels at the beginning (Day 1) and end (Day 4) of a fed state ($n = 7$), 72-h fasting with placebo ($n = 6$), and 72-h fasting with r-metHuLeptin ($n = 7$) mean ± SE

	Baseline fed state ($n = 7$)		Fasting + placebo ($n = 6$)		Fasting + leptin ($n = 7$)		Overall	Baseline
	Day 1	Day 4	Day 1	Day 4	Day 1	Day 4	*P*	*P*
Weight, kg	57.0 ± 2.3	58.0 ± 2.2*	56.9 ± 3.1	55.4 ± 2.8*	56.2 ± 2.2	54.1 ± 2.0*	0.01†‡§	0.61
Fat mass, %	29.9 ± 1.5	30.0 ± 1.4	31.2 ± 1.9	29.3 ± 2.2*	30.0 ± 1.6	29.1 ± 1.9	0.07†‡	0.85
Fat mass, kg	17.2 ± 1.3	17.3 ± 1.2	18.0 ± 1.9	16.4 ± 1.8*	17.0 ± 1.4	15.9 ± 1.5*	0.01†‡§	0.45
Fat-free mass, kg	39.9 ± 1.4	40.7 ± 1.7	38.9 ± 1.6	39.0 ± 1.6	39.2 ± 1.3	38.2 ± 1.3*	0.03†‡¶	0.68
RMR, kcal/d	1,267 ± 40	1,259 ± 36	1,285 ± 82	1,352 ± 43	1,330 ± 52	1,344 ± 34	0.26	0.12
Respiratory quotient	0.87 ± 0.03	0.91 ± 0.01	0.88 ± 0.02	0.74 ± 0.01*	0.88 ± 0.03	0.72 ± 0.01*	0.01†‡§	0.83
Leptin, ng/ml	11.4 ± 1.6	16.7 ± 1.2*	14.7 ± 2.6	2.8 ± 0.3*	12.2 ± 1.6	28.8 ± 2.0*	0.002†‡§¶	0.85
Insulin, μIU/ml	6.72 ± 1.21	9.28 ± 1.51*	6.06 ± 0.81	1.22 ± 0.24*	7.91 ± 1.54	1.23 ± 0.30*	0.01†‡§	0.85
FFA, mmol/l	0.07 ± 0.01	0.04 ± 0.00*	0.05 ± 0.01	0.94 ± 0.16*	0.04 ± 0.01	0.83 ± 0.08*	0.01†‡§	0.08
Estradiol, pg/ml	57.0 ± 12.1	64.5 ± 11.3	60.9 ± 13.1	66.1 ± 27.2	61.2 ± 11.1	52.8 ± 10.2	0.31	0.61
FSH, mIU/ml	6.00 ± 0.61	5.39 ± 0.90	5.83 ± 0.47	4.92 ± 0.51	5.81 ± 0.44	5.66 ± 0.69	0.22	0.85
Testosterone, ng/dl	74.6 ± 11.8	74.2 ± 10.9	78.5 ± 11.9	68.5 ± 9.8	66.4 ± 8.1	67.6 ± 7.1	0.85	0.61
fT3, pg/ml	2.81 ± 0.10	2.60 ± 0.08	2.98 ± 0.17	1.66 ± 0.11*	2.78 ± 0.16	1.69 ± 0.08*	0.01†‡§	0.68
Reverse T3, ng/dl	15.0 ± 0.7	12.4 ± 1.0*	16.9 ± 2.3	33.5 ± 3.7*	15.8 ± 0.9	34.1 ± 2.0*	0.01†‡§	0.61
Free T4, ng/dl	1.19 ± 0.07	1.12 ± 0.06	1.22 ± 0.09	1.18 ± 0.08	1.17 ± 0.08	1.17 ± 0.08	0.83	0.54
IGF-1, ng/ml	265.3 ± 17.1	346.9 ± 24.5*	260.8 ± 19.5	162.2 ± 12.5*	239.3 ± 25.3	147.6 ± 10.7*	0.01†‡§	0.31
IGF-BP3, μg/ml	4.78 ± 0.11	5.11 ± 0.22*	4.94 ± 0.14	4.22 ± 0.33	4.66 ± 0.25	4.06 ± 0.21*	0.04†‡§	0.51
Cortisol, μg/dl	16.9 ± 3.4	16.7 ± 3.1	17.5 ± 0.9	17.6 ± 3.0	16.9 ± 1.9	14.5 ± 2.1	0.51	0.85
PRA, ng/ml/hr	4.16 ± 0.71	2.28 ± 0.75	2.45 ± 0.80	6.03 ± 2.42	6.50 ± 3.18	9.42 ± 2.52	0.03	0.22
Aldosterone, pg/ml	60.0 ± 11.7	38.4 ± 3.0	66.0 ± 12.8	112.1 ± 23.5	66.1 ± 11.7	132.6 ± 27.0	0.01†‡§	1.0
24-hr urine cortisol, μg		19.6 ± 1.9		28.6 ± 3.3		25.0 ± 2.5	0.03‡	—
24-hr urine sodium, mEq		195.4 ± 26.4		167.8 ± 9.9		153.2 ± 24.6	0.51	—

Overall *P* value for comparison of change from day 1 to day 4 and baseline *P* value for comparison of day 1 values across the three conditions by nonparametric ANOVA *, $P < 0.05$ vs. Day 1; †, $P < 0.05$ by one-way ANOVA; ‡, $P < 0.05$ for fed vs. fasting + placebo; §, $P < 0.05$ for fed vs. fasting + r-metHuLeptin; ¶, $P < 0.05$ for fasting + placebo vs. fasting + r-metHuLeptin.

r-metHuLeptin Restores the Fasting-Induced Decline in Overnight Luteinizing Hormone (LH) Pulse Frequency but Does Not Alter the Suppression of TSH Pulsatility and IGF-1 Levels or Mild Activation of the HPA Axis. Overnight LH peak frequency decreased significantly during fasting (7.3 ± 0.4 vs. 4.5 ± 1.1 peaks per 12 h, $P < 0.05$) and was fully corrected with r-metHuLeptin (6.4 ± 0.5 peaks per 12 h, $P = 0.04$ by ANOVA) (Table 2 and Fig. 1*b*). A similar trend that did not reach statistical significance was observed for 24 h (see Table 4, which is published as supporting information on the PNAS web site) but not daytime (data not shown) LH pulsatility. There were no changes in estradiol, follicle-stimulating hormone (FSH), or testosterone with fasting or r-metHuLeptin (Table 1).

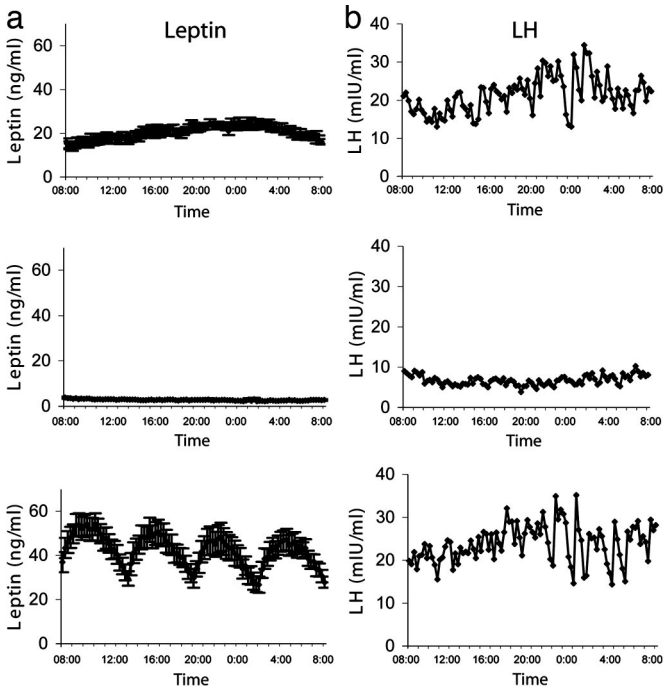

Fig. 1. Twenty-four-hour profile (8 a.m.–8 p.m.) of average ($n = 7$) leptin (*a*) and representative LH (*b*) levels on day 3 of a baseline fed state (*Top*), 72-h fasting with placebo (*Middle*), or 72-h fasting with replacement-dose r-metHuLeptin (*Bottom*).

Thyroid hormones were stable at baseline (Table 1), and frequently sampled TSH levels showed typical diurnal variation and pulsatility (Fig. 3, which is published as supporting information on the PNAS web site). Seventy-two-hour fasting significantly decreased free triiodothyronine (T3), increased reverse T3, and markedly suppressed several parameters of TSH pulsatility, whereas free thyroxine remained stable. r-metHuLeptin did not alter these fasting-induced changes of the thyroid axis (Fig. 3 and Tables 1 and 2).

IGF-1 decreased by 40% after 72-h fasting alone and with r-metHuLeptin, with similar findings in IGF-binding protein 3 (IGF-BP3) (Table 1). Neither serum cortisol levels nor adrenocorticotropic hormone (ACTH) pulsatility were altered by fasting or r-metHuLeptin (Tables 1 and 2). However, there was an overall significant difference in 24-h urine cortisol, with higher levels in both fasting states vs. the fed state ($P = 0.03$ for fed vs. fasting). Plasma renin activity (PRA) and aldosterone tended to increase with fasting but with no effect of r-metHuLeptin (Table 1). There was an overall significant change for PRA and aldosterone but no significance by post hoc tests or change from days 1 to 4. Twenty-four-hour urine sodium (Table 1), nitrogen, and volume (data not shown) were not affected by fasting or r-metHuLeptin.

Seventy-Two-Hour Fasting Reduces the Number of Cells of the Adaptive Immune Response, but r-metHuLeptin Has Minimal Effects to Restore Their Number with No Effect on Innate Immunity or *in Vitro* T Cell Function. Acute starvation resulted in a decline of total $CD3^+$ T lymphocytes by 838 ± 268 cells per mm^3 (461 ± 344 after adjustment for controls) ($P = 0.04$ for days 1 vs. 4). r-metHuLeptin during fasting blunted this decline to 302 ± 185 cells per mm^3

Table 2. Pulsatility characteristics (mean ± SE) of frequently sampled (every 15 minutes) overnight LH (8 p.m.–8 a.m.) and 24-h TSH and ACTH (8 a.m.–8 a.m.) on day 3 of a baseline fed state ($n = 7$), 72-h fasting with placebo (P) ($n = 6$), or 72-h fasting with r-metHuLeptin (RL) ($n = 7$)

	Fed	Fast + P	Fast + RL	*P*
Overnight LH, IU/liter				
Mean level	10.57 ± 2.59	7.61 ± 0.58	11.51 ± 2.76	0.31
Peak frequency	7.29 ± 0.42	4.50 ± 1.06	6.43 ± 0.48	0.04*†
Peak interval	80.86 ± 6.94	119.51 ± 20.54	95.91 ± 12.18	0.07
Peak width	50.15 ± 3.98	91.20 ± 28.61	66.96 ± 11.09	0.35
Peak height	13.31 ± 3.21	10.44 ± 1.00	15.00 ± 3.48	0.82
Valley frequency	8.00 ± 0.31	5.50 ± 1.06	7.14 ± 0.59	0.047†
Valley mean level	9.37 ± 2.24	6.28 ± 0.84	10.30 ± 2.69	0.31
Integrated area	7,462 ± 1830	5,358 ± 428	8,101 ± 1940	0.31
Pulse mass	1.35 ± 0.37	4.01 ± 2.15	2.35 ± 0.49	0.07
TSH, μIU/ml				
Mean level	1.42 ± 0.20	0.35 ± 0.08	0.51 ± 0.07	0.006*†‡
Peak frequency	8.29 ± 0.68	11.33 ± 0.92	9.57 ± 0.87	0.048†
Peak interval	151.1 ± 11.4	114.8 ± 10.8	141.4 ± 12.6	0.006†
Peak width	105.25 ± 11.5	68.18 ± 4.16	86.49 ± 11.26	0.042†
Peak height	1.75 ± 0.28	0.41 ± 0.08	0.59 ± 0.08	0.006*†‡
Valley frequency	9.14 ± 0.70	11.67 ± 0.88	9.71 ± 0.87	0.013†
Valley mean level	1.25 ± 0.20	0.32 ± 0.07	0.46 ± 0.06	0.006*†‡
Integrated area	2,005 ± 283	495 ± 109	728 ± 98	0.006*†‡
Pulse mass	0.65 ± 0.14	0.04 ± 0.02	0.13 ± 0.04	0.050*†‡
ACTH, pg/ml				
Mean level	10.48 ± 1.28	9.89 ± 2.01	9.33 ± 1.23	0.31
Peak frequency	9.57 ± 1.07	12.17 ± 0.87	10.14 ± 0.70	0.17
Peak interval	139.6 ± 15.2	105.3 ± 5.4	123.7 ± 5.6	0.51
Peak width	88.87 ± 11.13	67.63 ± 4.92	83.39 ± 6.35	0.61
Peak height	13.52 ± 1.74	13.51 ± 3.09	12.70 ± 1.92	0.14
Valley frequency	10.14 ± 1.06	12.50 ± 0.67	10.57 ± 0.75	0.54
Valley mean level	8.71 ± 1.12	8.74 ± 1.75	7.79 ± 0.93	0.07
Integrated area	14,703 ± 1856	14,029 ± 2846	12,915 ± 1,760	0.07
Pulse mass	6.17 ± 0.86	4.22 ± 2.82	2.85 ± 0.76	0.25

P value for nonparametric ANOVA across the three conditions. *, $P < 0.05$ by one-way ANOVA; †, $P < 0.05$ for fed vs. fasting + placebo; ‡, $P < 0.05$ for fed vs. fasting + r-metHuLeptin.

(201 ± 350 adjusted) ($P = 0.14$ for days 1 vs. 4). The decline in $CD3^+$ T lymphocytes was greater during 72-h fasting alone vs. with r-metHuLeptin ($P = 0.04$) although not different after adjustment for controls ($P = 0.50$). We found similar findings in $CD4^+$ and $CD8^+$ T lymphocytes and $CD19^+$ B lymphocytes, with greater declines during 72-h fasting alone vs. with r-metHuLeptin, although the differences were not statistically significant (Table 3 and Table 5, which is published as supporting information on the PNAS web site). Naïve and memory $CD3^+$, $CD4^+$, and $CD8^+$ subpopulations decreased more with fasting than with r-metHuLeptin during fasting, although these differences were not significant after adjustment for controls, except an increase in naïve $CD8^+/CD45RA^+$ cells with r-metHuLeptin vs. decrease during fasting (Tables 3 and 5). Decreases in PBMCS were not due to nonspecific factors, because $CD3^-/CD16^+CD56^+$ natural killer (NK) cells, representing innate immunity, increased slightly with fasting. The proliferative response of T cells to several polyclonal stimuli decreased after 72-h fasting but was not significantly different from fasting with r-metHuLeptin (OKT3, −19,246 ± 5,251 vs. −8,217 ± 14,773 cpm, $P = 0.47$; PHA, −36,214 ± 11,808 vs. −25,823 ± 10,362 cpm, $P = 0.72$; PMA/Iono, −31,545 ± 20,328 vs. +4,829 ± 18,050 cpm, $P = 0.47$; PPD, −445 ± 4,620 vs. −4,125 ± 4,843 cpm, $P = 0.72$). Stimulated cytokine (IFN-γ, IL-10, and IL-4) production by PBMCs did not change with 72-h fasting or r-metHuLeptin, nor did serum glucose levels (data not shown).

Table 3. Change in PBMC subpopulations (mean ± SE) from days 1 to 4 of 72-h fasting with placebo or replacement-dose r-metHuLeptin ($n = 5$)

	Actual		Adjusted for controls	
PBMC subpopulation	Fasting + placebo	Fasting + leptin	Fasting + placebo	Fasting + leptin
$CD3^+$	−838 ± 268†	−302 ± 185*	−461 ± 344	−201 ± 350
$CD4^+$	−534 ± 184†	−247 ± 138	−327 ± 246	−139 ± 273
$CD8^+$	−257 ± 77†	−57 ± 52	−128 ± 93	−43 ± 88
$CD3^+/CD45RA^+$	−443 ± 124†	−134 ± 134	−197 ± 188	+14 ± 243
$CD3^+/CD45RO^+$	−396 ± 177	−168 ± 85	−274 ± 175	−215 ± 152
$CD4^+/CD45RA^+$	−234 ± 76†	−149 ± 72	−126 ± 104	−71 ± 151
$CD4^+/CD45RO^+$	−300 ± 119†	−98 ± 74*	−201 ± 143	−68 ± 129
$CD8^+/CD45RA^+$	−209 ± 78†	+15 ± 72	−71 ± 98	+85 ± 104*
$CD8^+/CD45RO^+$	−96 ± 111	−70 ± 6	−73 ± 74	−147 ± 67
$CD19^+$	−152 ± 51†	−82 ± 24	−86 ± 69	−71 ± 70
$CD3^-/CD16^+CD56^+$	+80 ± 27	+91 ± 34†	+27 ± 26	+56 ± 53

*, $P < 0.05$ vs. fasting alone; †, $P < 0.05$ vs. day 1.

Changes in IGF-1 Correlate with Changes in Several PBMC Subpopulations in Response to 72-h Fasting Alone and with r-metHuLeptin. To evaluate whether changes in PBMC subpopulations are related to changes in hormones known to be affected by fasting and/or leptin, we analyzed the correlation between changes in PBMC subpopulations with changes in hormones relevant to immune function, including leptin, insulin, FFA, estradiol, FSH, testosterone, free T3, free thyroxine, IGF-1, IGF-BP3, and cortisol. The most significant correlations were observed for IGF-1, and borderline significant correlations were noted for free T3 (see Table 6, which is published as supporting information on the PNAS web site). Correlations with other hormones were not significant after adjustment for controls (data not shown).

Complete Leptin Depletion from Human Serum or Neutralization in Culture Medium Significantly Impairs *in Vitro* T Cell Proliferation and Is Reversible with Leptin Replacement. We then investigated whether the lack of major changes in PBMC proliferation and cytokine production may have been due to the continued presence of leptin at physiological levels despite being suppressed to ≈20% of baseline (2.8 ± 0.3 ng/ml), i.e., above a putative threshold level for immune function. T cells were first stimulated with OKT3, PHA, or PMA/Iono in 5% human serum, which corresponds to a leptin level of ≈1 ng/ml. In contrast to the lack of effect of 72-h fasting (with leptin level ≈2.8 ng/ml) on T cell function, complete removal of leptin from serum with anti-human leptin Ab dramatically suppressed the proliferative response to essentially no proliferation (Fig. 2 *a–c*). Specificity of the system was confirmed by adding recombinant human leptin back to leptin-depleted serum, which restored proliferation to a level comparable with that in the basal state. The restoration of proliferation was less efficient with PMA/Iono compared with OKT3 and PHA and was reachable in all conditions only with high doses of leptin (ranging from 50 to 100 ng/ml). To further dissect the role of leptin present in culture medium on T cell proliferation, we added anti-leptin neutralizing Abs to culture medium during mixed lymphocyte reactions, which inhibited T cell proliferation between 45% and 60% compared with medium alone or control Ab, suggesting that leptin levels above a certain threshold are necessary for sustaining *in vitro* T cell proliferation (Fig. 2*d*).

Discussion

Maintenance of normal neuroendocrine and immune function depends critically on the availability of adequate energy stores. Evidence from animal and human models supports a central role for leptin as a signal of energy sufficiency and mediator of the adaptation to starvation (9, 10, 14). Congenital complete leptin deficiency (3–5) or acute hypoleptinemia to <1 ng/ml in leptin-replete humans induces changes in reproductive, thyroid, and IGF axes (10) and immune function (12), whereas increasing serum

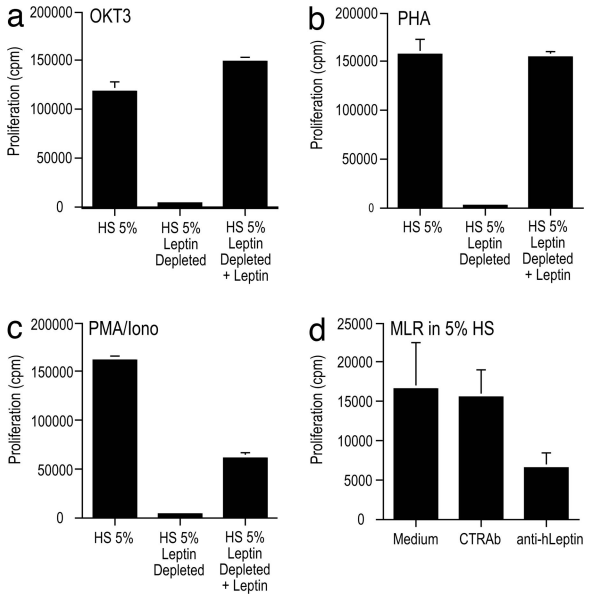

Fig. 2. Leptin depletion or neutralization inhibits polyclonal T cell proliferation and mixed lymphocyte reactions (MLR), respectively. (*a–c*)The proliferative response of T lymphocytes to polyclonal stimuli (OKT3, PHA, and PMA/Iono) from controls is completely inhibited in medium with human serum depleted of leptin. Addition of recombinant human leptin (at 100 ng/ml final concentration) completely reverses this phenomenon. (*d*) Anti-leptin blocking Abs partially inhibit the antigen-specific proliferative response of T cells during MLR. HS, human serum.

leptin levels to >2–3 ng/ml with r-metHuLeptin corrects, fully or in part, these abnormalities (4, 10). However, whether a critical leptin threshold of ≈3 ng/ml exists, above which leptin has no major additional physiological effect on neuroendocrine and/or immune function, has been proposed based on observational studies (13) but not yet tested in an interventional fashion.

We studied women who have substantially higher baseline leptin levels than lean men. Although 72-h fasting results in a similar percent decline of leptin levels as in men (≈15–20% of baseline), the absolute levels achieved are ≈10-fold higher (2.8 ng/ml) (13). Leptin levels after r-metHuLeptin (28.8 ng/ml) were higher than baseline, but remained well within the physiologic range and below levels (≈400–4,000 ng/ml) achieved with pharmacologic dosing (15, 16). Decreasing serum leptin levels to 2.8 ng/ml and increasing leptin back to a high physiologic range had only modest effects on LH pulsatility and did not substantially alter any other neuroendocrine or immune parameter. This alteration in levels also had no statistically significant effect on metabolic variables. Larger, longer-term studies are needed to more fully elucidate this finding.

The unique aspect of this study lies in the *in vivo* exploration of decreasing leptin from mid- to low-physiologic levels but not to the extremely low levels of severe leptin deficiency (10). Previous leptin administration data in congenital (4) and lipoatrophic (17) leptin-deficient subjects and leptin-replete subjects with short-term relative leptin deficiency (10) or 10% reduced body weight (18) suggest that a leptin level of ≈2–3 ng/ml appears to be necessary for the regulation of neuroendocrine axes (particularly the hypothalamic–pituitary–gonadal axis). Importantly, these data provide insight into the "normal range" of leptin levels, an issue of considerable diagnostic and therapeutic importance when r-metHuLeptin gains a place in the therapeutic armamentarium. Our findings are consistent with the existence of a threshold leptin level for regulation of neuroendocrine and immune function, whereas an upper limit to leptin's effect on metabolic parameters may exist (i.e., leptin resistance). Given the pleiotropic nature of leptin and the complexity of the leptin system, it remains to be determined whether different thresholds exist for other physiological functions influenced by leptin and/or whether differences in populations with varying degrees or duration of leptin deficiency exist.

We studied women in the midfollicular phase of their menstrual cycles, an important time for development of dominant follicles. Decreasing leptin to low-physiologic levels decreased overnight LH pulse frequency only modestly by 40% but not other pulsatility parameters, FSH, or sex steroid levels. This finding stands in distinct contrast to r-metHuLeptin's ability to (*i*) restore several LH pulsatility parameters and the 40% decline in testosterone with the same duration but greater magnitude of fasting-induced hypoleptinemia in lean men (10) and (*ii*) normalize LH pulsatility and ovulatory cycles in women with hypothalamic amenorrhea and chronic leptin deficiency (14). Although follicle development was not directly evaluated, the maintenance of normal menstrual cycles argues against substantial disruption of follicular growth and ovulation. Interestingly, normal-weight women have decreased LH pulse frequency during short-term starvation despite maintaining ovulatory cycles in several (19, 20) but not all (21–23) studies. Partial restoration of leptin levels with r-metHuLeptin during 4-day fasting was associated with a decrease in overnight LH pulses in normal-weight women, but fed-state leptin levels were not reached (23). Potential effects of r-metHuLeptin may have been obscured by interindividual differences, which our study and others (19, 20, 24, 25) have minimized by studying the same subjects under different conditions at the same times of their menstrual cycles.

It has been suggested that LH pulsatility is disrupted at a threshold of energy availability deep in negative energy balance (26). Low energy availability, but not the stress of exercise alone, suppressed leptin rhythm (27) and altered LH pulsatility (26) in eumenorrheic, sedentary women. Similarly, 72-h fasting disrupted follicle growth and lengthened the follicular phase in lean women with body fat <20% (24), but not in normal-weight women with body fat similar to that of our subjects (≈27%) (25). Taken together, these studies suggest that lean women with lower leptin levels may be more vulnerable to energy deficit than normal-weight women. Thus, evaluation of very lean women during short-term starvation may provide insights into the relative contribution of a critical leptin level vs. duration of hypoleptinemia.

In this study, 72-h fasting caused marked suppression of TSH pulsatility, decrease in free T3, and increase in reverse T3. Humans with complete functional leptin deficiency due to defects in the leptin or leptin-receptor gene have altered TSH pulsatility (28) or central hypothyroidism (6). In small, uncontrolled studies, r-metHuLeptin increased free thyroxine and free T3 levels in leptin-deficient children (4) and reversed the decrease in thyroid hormones in subjects during weight loss (18). Although normalizing leptin from very low levels (≈0.2 ng/ml) blunted the fasting-induced suppression of TSH pulsatility in lean men (10), increasing leptin from ≈2.8 to ≈28.8 ng/ml had no effect on TSH pulsatility in women. We propose that these differences in TSH pulsatility may be related to a threshold leptin level similar to that for the hypothalamic–pituitary–gonadal axis.

Short-term fasting decreased IGF-1 levels with no effect of r-metHuLeptin and no major alterations in IGF-BP3. We have shown that r-metHuLeptin in fasting men modestly blunted the starvation-induced decrease in IGF-1 levels (10) and increased IGF-1 and IGF-BP3 levels over 2–3 mo in leptin-deficient women with hypothalamic amenorrhea (14). In congenital leptin-deficient humans, r-metHuLeptin for 18 mo increased IGF-BP1 and IGF-BP2 but not IGF-1 or IGF-BP3 (5). Thus, although the role of leptin in regulating IGF-1 requires further study, the above observations suggest that leptin may need to decrease below a certain level for IGF-1 to decrease because of leptin deficiency.

We found mild activation of the HPA axis with 72-h fasting but no effect of r-metHuLeptin. Leptin-deficient humans have elevated basal cortisol and ACTH levels and disturbed diurnal rhythm (3), and long-term r-metHuLeptin in three leptin-deficient adults in-

creased cortisol levels, but these studies were uncontrolled (5). In healthy humans, regulation of the HPA axis appears to be independent of leptin (10) in contrast to leptin's reversal of starvation-induced HPA activation in mice (9). We report herein a lack of change in ACTH pulsatility in response to r-metHuLeptin. Although observational human studies have shown an inverse relationship between pulsatility of ACTH and cortisol with that of leptin (29), our interventional studies do not support a direct role for leptin in regulating the HPA axis; whether pulsatile or longer duration of r-metHuLeptin is required remains to be studied. Consistent with our prior findings (10), fasting-induced changes in PRA and aldosterone appear to be independent of leptin.

In this study, acute starvation in leptin-replete humans determines a specific change in peripheral lymphocyte distribution that differentially affects the number of circulating cells of the adaptive and innate immune response. Restoration of leptin levels had no major effect on fasting-induced changes in immunophenotypes, except for naïve $CD8^+CD45RA^+$ cells, indicating that decreasing leptin levels to ≈2.8 ng/ml has a minor role in mediating the effects of short-term starvation on PBMCs. Similarly, the *in vitro* proliferative and cytokine-producing capacity of T cells was not affected, suggesting that this degree of short-term leptin deficiency disrupts immune function only minimally, i.e., induces partial changes in immune cell distribution but no impairment of T cell proliferation against classical polyclonal and recall antigens.

Leptin reverses the immunosuppression associated with 48-h starvation in normal mice (8, 11), increases thymic or splenic cellularity in leptin-deficient *ob*/*ob* mice (8), and, by using a similar protocol for acquiring PBMCs and analyzing immune function, improves severely impaired T cell function in children with congenital leptin deficiency (4). Thus, our findings highlight the contrast between short-term mild and long-term severe leptin deficiency on immune function. Relative leptin deficiency after leptin withdrawal in mice depleted of fat after high-dose leptin administration reduces thymic and splenic cellularity (30). The bioequivalence of 48-h starvation in mice is likely closer to a few weeks in humans, and, thus, 72-h fasting in humans may be insufficient for substantial alterations in immune function to occur, but further studies are needed to clarify this role of leptin over a wider range of leptin levels. This robustness of the immune system is consistent with evidence that acute starvation does not affect susceptibility to infectious diseases, whereas more long-term starvation profoundly alters inflammatory immune responses and infectious disease susceptibility in mice and humans (31). Indeed, both anorexic and malnourished subjects (particularly those with protein energy malnutrition) have impaired T cell-mediated immune responses and very low leptin levels (31).

We studied T cell function *in vitro* to differentiate between insufficient duration vs. degree of hypoleptinemia and found a striking reduction of T cell proliferation to polyclonal stimuli when leptin was absent from culture medium that was fully restored when leptin was added back at low doses. Importantly, the use of autologous serum in the *in vivo* immune studies preserves the existing environment of the cells and avoids introducing exogenous, confounding factors. The lack of change in serum glucose levels, verified viability of immune cells, and observed proliferative response argue against a substantial change in metabolic environment due to shipment, because T cells become anergic in low-glucose settings (32).

The simultaneous assessment of neuroendocrine and immune function in this paradigm provides a unique opportunity to investigate whether leptin regulation of immune function may be mediated in part by leptin-associated changes of hormones that influence immune function. We observed a significant correlation between IGF-1 and PBMC subpopulations. The IGF-1 receptor is expressed on immune cell types including activated T cells, B cells, NK cells, and monocytes and exerts effects on T cells and antigen-presenting cells (33), supporting the notion that IGF-1 may have immunomodulatory effects. Importantly, we did not find any correlation with cortisol, similar to the mouse model of acute leptin deficiency after leptin withdrawal (30). Correlations cannot prove causality and must be interpreted with caution in cross-sectional analysis. Further clinical and mechanistic investigations into the role of these hormones acting alone or in concert with leptin to affect immune function are needed.

In summary, our findings suggest that leptin serves a permissive role in regulating neuroendocrine and immune function. Given the beneficial effect of leptin replacement in ameliorating these defects in more severe and/or chronic leptin deficiency, we propose that, similar to other hormone deficiency syndromes, a leptin-deficiency syndrome exists. The lower limit of normal leptin levels appears to be ≈3 ng/ml (by using the assay reported herein), but the exact normal range remains to be defined precisely. Neuroendocrine alterations during more chronic relative hypoleptinemia in the setting of obesity (e.g., during weight loss) may involve different physiological mechanisms and adaptations. Whether changes of another putative factor are responsible for leptin-independent changes that occur when leptin remains above this threshold, and/or whether duration or an interaction of degree and duration of hypoleptinemia are important remains to be studied.

Methods

Study Design. This protocol was approved by our Institutional Review Board, and clinical quality r-metHuLeptin (Amgen, Thousand Oaks, CA) was administered under an Investigational New Drug application to the Food and Drug Administration. All subjects were healthy without immunologic or endocrine disease based on examination and routine blood tests. Seven women (age = 22.4 ± 1.2 yr) with body mass index <25 kg/m^2 and regular menstrual cycles (length 26–32 days) not on oral contraceptives for at least 6 mo participated in three separate studies in our General Clinical Research Center: a baseline isocaloric fed state as described in ref. 10 and two 72-h fasting studies scheduled in random order in double-blind fashion with administration of r-metHuLeptin (dose 0.08 mg/kg/day on day 1, increased to 0.2 mg/kg/day on days 2–3 to account for declining leptin levels with additional fasting, divided into 4 equal doses given s.c. every 6 h, starting at 8 a.m. on day 1), during one fasting study, or placebo (same schedule and volume as the corresponding r-metHuLeptin dose), during the other fasting study. Each subject completed three studies, separated by at least 8 wk to permit recovery of hematocrit, leptin levels, and weight to baseline, except one subject for the fasting/placebo study. For each subject, the frequent sampling was matched to a cycle day within 2 days of the cycle day of the other two frequent sampling studies and within menstrual cycle days 6–11. During fasting studies, subjects ate a snack the night before day 1 and then had only calorie-free liquids and daily multivitamin, NaCl (500 mg), and KCl (40 meq) until 10 a.m. on day 4. Leptin, insulin, FFA, estradiol, FSH, testosterone, free T3, reverse T3, free thyroxine, IGF-1, IGF-BP3, cortisol, PRA, and aldosterone were measured at 8 a.m. on days 1 and 4 and 24-h urine cortisol, sodium, and urea nitrogen on day 3. Starting at 8 a.m. on day 3, blood samples for leptin, LH, TSH, and ACTH were drawn every 15 min for 24 h through an indwelling peripheral i.v. line. At 8–9 a.m. on days 1 and 4, resting metabolic rate (DeltaTrac II Metabolic Monitor; SensorMedics) and body composition (bioelectric impedance analysis; RJL Systems, Clinton Township, MI) were measured. For five consecutive subjects completing both fasting studies, a blood sample obtained at 8 a.m. on days 1 and 4 of the fasting studies was shipped to Naples, Italy, by express courier to be processed within 48–72 h for immune assays along with a blood sample from a matched healthy, nonsmoker, fed control to account for any potential external effects. Processing of PBMCs within this time frame assures their viability, according to standard immunology procedures (34) and verified by our extensive studies in healthy

controls and leptin-deficient subjects using a similar shipment protocol (4). As described in ref. 10, hormone levels were run in duplicate by using standard immunoassays and within the same run for a given subject, and the program CLUSTER 8.0 was used to characterize leptin, LH, TSH, and ACTH pulsatility.

Immunophenotypic Analysis, T Cell Proliferation, and Cytokine Production. Viability of cells was verified at 85–95% by using trypan blue staining and annexin-5 binding during flow cytometry, and serum glucose levels were measured as a marker of metabolic activity. Immunophenotypic analysis of peripheral blood (see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site) and T cell cultures in medium supplemented with 5% (vol/vol) autologous serum were performed as described (4). Cell culture supernatants (100 μl) were removed 48–60 h after stimulation and frozen at −80°C until assayed for IFN-γ, IL-4, and IL-10 (PharMingen).

***In Vitro* Leptin Neutralization and Depletion of Leptin from Human Serum.** Mixed lymphocyte reactions were performed on isolated PBMCs (stimulator cells) and their corresponding HLA-mismatched irradiated (30-Gy) stimulators in the absence and presence of purified polyclonal rabbit anti-human leptin Ab (anti-hOb, 10–20 μg/ml; provided by Radek Sokol, BioVendor, Brno, Czech Republic) with affinity-purified rabbit polyclonal IgG as a control. Responder and stimulator cells were cultured for 5 days in RPMI medium 1640, supplemented with 5% human AB serum (Sigma) at a 1:1 ratio. For leptin depletion from serum, a protein G-Sepharose affinity column (Amersham Pharmacia) was used after adhesion on G protein of a polyclonal rabbit anti-human leptin Ab (BioVendor). Human AB serum was passed through the columns three times and collected. Anti-human leptin-specific ELISA and Western blotting to measure the serum content of leptin indicated that the amount of leptin was below the assay detection limit after depletion. Human recombinant leptin (R & D System) was used to verify specificity.

Statistical Analysis. Data (mean ± SE) were analyzed by using SPSS 8.0 (SPSS, Chicago). Changes of variables from days 1 to 4 were compared by using Wilcoxon signed-rank paired tests or with nonparametric ANOVA with post hoc tests by least-significant difference across the three conditions. Immune data were adjusted for controls by subtracting the change in a parameter for the matched control from that for the subject, and data were analyzed by absolute values as well as adjusted for controls. $P < 0.05$ was considered significant. Bivariate Spearman correlation analyses were performed on changes in hormone levels and PBMC subpopulations from days 1 to 4, and $P < 0.01$ was considered significant to correct for multiple comparisons.

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Adipokines, Metabolism and the Immune Response in the Regulation of Inflammation

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Abstract: The white adipose tissue (WAT) represents the most important structure of the organism able to provide energy stores and heat insulation. Recently, its has been postulated that the adipose tissue can be considered as a complex, essential, and highly active metabolic and endocrine organ. Indeed, it is able to respond to different signals from the endocrine organs and from the nervous the immune systems. More recently, the adipose tissue has also been hypothesised to represent an "extension" of the immune system, for its capacity to contain immune cells, lymph nodes, thymus, but above all, for its ability to produce a series of cytokines and chemokines typical of the immune system (generally named adipokines). A better understanding of the immune and endocrine function of the adipose tissue will lead to the development of innovative therapeutic strategies in inflammatory disorders.

INTRODUCTION

The classical view of the adipose tissue as a static reservoir of lipids necessary in time of reduced food availability in the environment, is no longer valid. Today, adipocytes have been shown to be able to secrete hormones and cytokines both necessary for the endocrine and immune function [1]. This has induced a series of novel hypothesis on the role of the adipose tissue as a key regulator of the immune function and to consider as a secondary "immune organ" [2]. Indeed, it is well-known that immune cells such as lymphocytes and monocytes/macrophages are present in the adipose tissue and also, the capacity of adipocytes to secrete a huge number of cytokines, chemokines, and factors classically considered of immune origin [1, 2]. Here, is presented an overview of the immune functions of adipose tissue. These functions may be divided into two broad categories: 1) secreted products that have a classical metabolic effects on important immune functions; 2) homing of the immune cells in classical sites embedded in abundant adipose tissues such as the bone marrow, thymus and lymph nodes.

LEPTIN, ADIPOSE TISSUE AND IMMUNITY

Several recent observations show that leptin is a hormone/cytokine that is involved in the immune-neuroendocrine crosstalk and functions as a key signal, coupling the metabolic axis to the immune system [3]. Leptin, the product of the *obese* gene, is a 167-aminoacid peptide hormone mainly synthesised by adipocytes, that regulates-body weight by stimulating energy expenditure through increased thermogenesis and by suppressing food intake [4]. In addition, leptin is sexually dimorphic, its serum concentration being higher in females than in males with similar body fat mass. Leptin belongs structurally to the family of long-chain helical cytokines and has a similarity with interleukin-6 (IL-6), IL-12, IL-15, granulocyte colony-stimulating factor (G-CSF), oncostatin M (OSM), prolactin and growth hormone [4]. The similarity of both functions, as a hormone and as a cytokine, reinforces the concept that leptin links the endocrine to the immune system [3, 4]. The effects of leptin are mediated by the long form receptor, known as OBRb, that is a member of the class I cytokine receptor family. This form is expressed by hypothalamus in areas that are responsible for secretion of neuropeptides and neurotransmitters regulating appetite. In addition to the long form of OBRb, there are alternatively spliced forms, named short forms, that seem to mediate the transport and degradation of leptin [3, 4]. The wide tissue distribution of OBRb, expressed by the ovary, pancreatic β cells, endothelial cells, $CD34^+$ haematopoietic bone-marrow precursors, monocytes/macrophages, T and B cells, suggests a pleiotropic effect of leptin on the nutritional status, metabolism and neuroimmunoendocrine axis [3, 4].

In recent years, a number of studies has investigated the effect of leptin on innate and adaptive immune responses; specifically, leptin can promote the activation of monocytes/macrophages, chemotaxis and activation of neutrophils as well as the development and activation of natural killer cells, regulating in this way the innate immune response [3]. The modulation of adaptive immune response by leptin is shown by increasing the expression of adhesion molecules on $CD4^+$T cells, promoting proliferation and secretion of IL-2 by naïve $CD4^+$T cells, increasing the production of interferon-γ (IFN-γ) by memory T cells, with little effect on proliferation. Similarly to other pro-inflammatory cytokines, it

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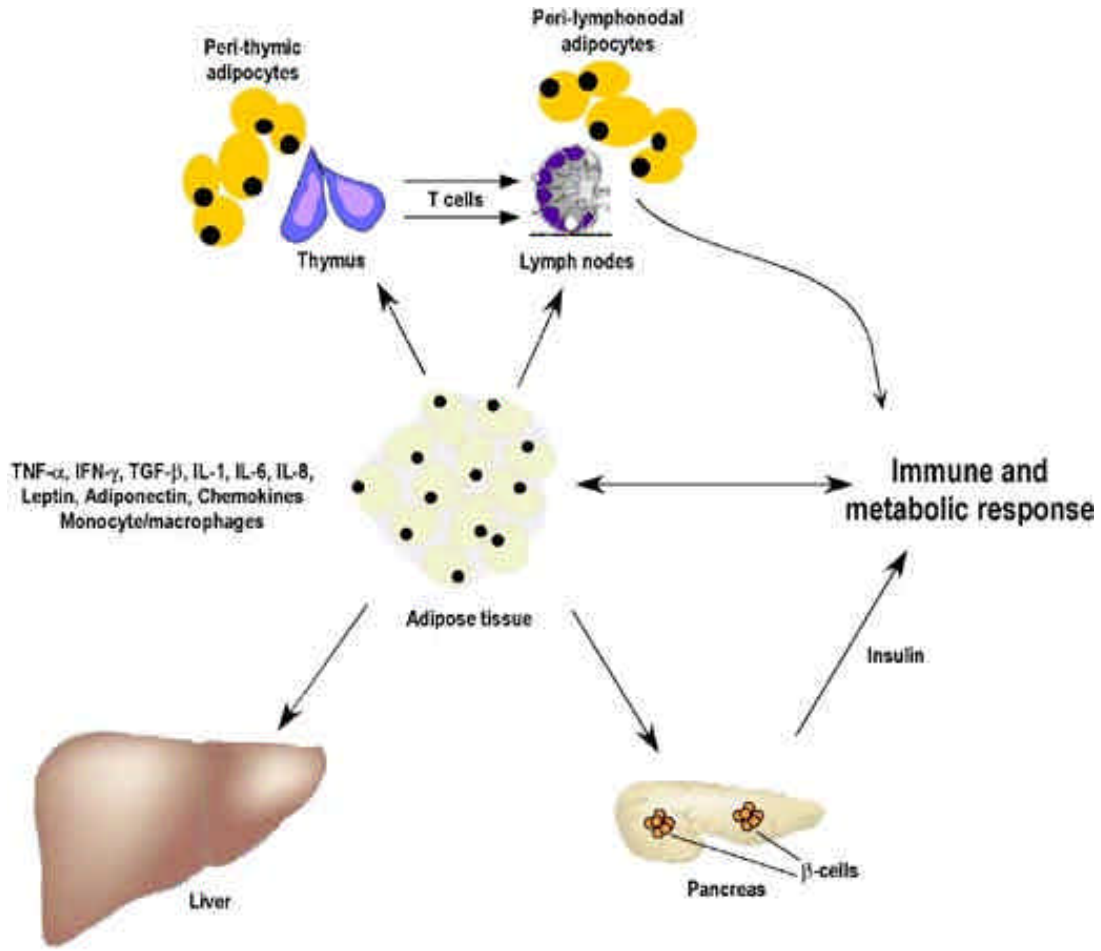

Fig. (1). Adipose tissue as immune and endocrine organ.
Adipocytes are able to secrete cytokines and hormones that participate of the immune response and metabolism.

has been shown that leptin promotes T helper 1 (Th1)–cell differentiation, and can modulate the onset and progression of autoimmune responses in several animal models of disease [3]. These effects of leptin on the immune response modulation have been shown in leptin-deficient (*ob/ob*) mice and humans, with congenital deficiency of leptin, in which both metabolic disturbances and immune abnormalities, including decreasing inflammatory cytokine secretion and thymic hypotrophy have been observed. These abnormalities are reversed by the administration of recombinant leptin in both mice and humans. Also, the possible involvement of leptin in autoimmunity has been shown in studies on *ob/ob* mice protected against several experimental autoimmune diseases and the administration of exogenous leptin to autoimmune disease genotypically susceptible mice, anticipates the onset and accelerates the progression of autoimmune responses [3]. The functional inhibition of the leptin axis could be considered as a possible novel therapeutic approach in conditions of increased leptin secretion such as autoimmune disorders and chronic inflammation. In this context, it is important to note that leptin could contribute to atherosclerosis and to increased risk of cardiovascular disorders in overweight and obese individuals. Indeed, leptin being secreted in proportion to body fat, its high concentrations in overweight individuals could, at least in part, contribute to the low-grade chronic inflammatory condition observed in obese subjects [5]. Obese subjects show also higher c-reactive protein (CRP), IL-6, IL-8, and TNF-α, all cytokines promoting inflammation [5]. These factors in conjunction with increased leptin may contribute to the increased endothelial activation and secretion of inflammatory mediators and cardiovascular disorders in obese subjects. Approaches aimed at reducing leptin secretion (i.e. diet low caloric diet) are already employed in the treatment of atherosclerosis. Future strategies able to neutralise leptin action with monoclonal antibodies could be also considered as novel therapeutic approaches [5].

CLASSICAL CYTOKINES, CHEMOKINES AND HORMONES IN ADIPOSE TISSUE

Cytokines are proteic messengers important in the homeostasis and function of immune cells [6]. They are classically secreted by lymphocytes, macrophages and antigen presenting cells (APCs) such as dendritic cells (DCs). Recently, it has been shown that also other tissues such as liver, epithelial cells, and neural cells are also an important source of cytokines responsible of the acute phase response. More

recently, it has been shown that adipocytes are also important producers of cytokines such as: IFN-γ, TNF-α, transforming growth factor-β (TGF-β), leukaemia inhibiting factor (LIF), IL-1, IL-6, IL-8, and chemokines, such as monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1 (MIP-1) [1].

In addition to adipokines, several cytokines exert their action in both immune and metabolic pathways. One of such cytokines is IL-1, which consists of two molecular forms - IL-1α and IL-1β, and is produced by monocytes/macrophages, epithelial, endothelial, and glial cells [7]. IL-1 is a major mediator of inflammation induced in the brain during fever. IL-1 effects on metabolism include induction of anorexia, and also inhibition of the synthesis of fatty acids and adipocytes differentiation [7]. Moreover, IL-1 can act as a hypoglycaemic agent, suggesting an anti-diabetic role for IL-1. Interestingly, IL-1 also promotes release of neuropeptides important in both energy homeostasis and immune function such as the corticotropin releasing hormone (CRH), melanocortins, and α-melanocyte stimulating hormone (α-MSH). Within the IL-1 gene family, is also included the IL-1 receptor antagonist (IL-1Ra), which binds IL-1 receptors without agonistic activity [7, 8]. IL-1Ra-deficient mice show growth retardation, are lean, resistant to high-fat-diet-induced obesity, and have reduced lipoprotein lipase (LPL) activity and low serum insulin [7, 8].

IL-6 is another important cytokine involved in the regulation of energy status and innate and adaptive immunity. Structurally, IL-6 belongs to the long-helical cytokine family and mainly regulates B cell development, antibody production, haematopoiesis and adipocyte/metabolic function. In non-inflammatory conditions, IL-6 is largely produced by the adipose tissue and the circulating levels of IL-6 correlate with body mass index (similarly to leptin), insulin sensitivity and glucose tolerance [9]. Both IL-6 and leptin can exert inhibitory feedback on the body fat mass by acting directly on hypothalamic nuclei that express their respective receptors, and high doses of IL-6 increase triglycerides and glucose levels. Interestingly, IL-6-deficient mice develop mature onset obesity and decreased glucose tolerance, produce reduced amounts of inflammatory cytokines, and are resistant to autoimmune diseases such as EAE and arthritis [10, 11].

TNF-α and IFN-γ are two cytokines pivotal for the host defence from infections and for the development of Th1 responses, and are involved as well in the pathogenesis of autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and type-1 diabetes [6, 12-15]. TNF-α is secreted by activated macrophages and by adipocytes and can affect adipogenesis *via* its action on the phosphorylation (and functional inhibition) of the peroxisome proliferator-activated receptor-γ (PPAR-γ) [12, 13]. TNF-α can also augment lipolysis and adipocyte apoptosis. A role of TNF-α in the insulin resistance in obesity is suggested by the finding that patients with insulin resistance have increased levels of TNF-α, and by the observation that TNF-α-deficient mice are protected from obesity-induced insulin resistance [12, 13]. Regarding IFN-γ, it seems that, in addition to its immune functions, this cytokine may play a central role during hypermetabolic and anorectic responses associated with inflammation, as IFN-γ-receptor-deficient mice are protected from LPS-induced anorexia [14, 15].

Another adipokine capable to significantly influence energy balance and T cell immunity is the proteic hormone insulin [16]. Insulin enhances uptake of nutrients - thus raising the levels of intermediary cellular metabolism, while promoting lymphocyte activation, T cell responsiveness and cytokines action. Since insulin increases energy requirements and protein synthesis, which are necessary for appropriate T cell function, defects of insulin action (in conditions such as insulin resistance, type 1 and type 2 diabetes) result in inappropriate immune responses [16]. Moreover, during hyperglycaemia and ketoacidosis, insulin promotes secretion of inflammatory cytokines and favours T cell activation. Although resting T cells are devoid of insulin receptors, these receptors become expressed on T cells after activation with antigens or mitogens. After binding to its receptor on activated T cells, insulin exerts its classical effects such as stimulation of glucose uptake and oxidation, amino acid transport, lipid metabolism, and protein synthesis [16].

GLUCOSE, AMINO ACID, LIPID METABOLISM AND IMMUNE RESPONSE

Recently it has been shown that not simply the secretion of common mediators at the interface between metabolism and immune response are able to affect T cell function but also the glycidic, amino acidic and lipid metabolic response within T cells and APCs is able to profoundly affect the outcome of the immune response [2].

Indeed, a rapid and sustained increase of metabolism is required to support proliferation, differentiation, and effector functions of T cells. Access to nutrients *via* increased expression of glucose transporters (GLUTs) and activation of glycolysis is an important means by which costimulatory receptors can stimulate T cell growth [2, 17]. Not surprisingly, several glycidic and amino acid intracellular pathways have recently been found crucial for T cell function and for the induction of tolerance/anergy. More specifically, it has been found that glucose metabolism has important implications on T cell activation and proliferation through the CD28 costimulation. It is well documented that T cells increase glucose uptake and glycolysis during an immune response *via* the CD28 costimulatory molecule, which recruits phosphstidylinositol-3'-kinase (PI3K) and Akt, suggesting that CD28 favours the glycolytic flux, thus providing T cells with their energetic, biosynthetic and metabolic needs [2, 17]. Similarities exist between the signalling pathways downstream of the insulin receptor and CD28. In particular, stimulation of CD28 promotes glucose uptake, glycolysis, and expression of glucose transporters (GLUT1), which depend on PI3K activity [2, 17]. These findings suggest that CD28 may be associated with regulation of glucose utilisation as well, and that CD28 might influence T cell activation and metabolism similarly to the insulin receptor.

Another metabolic event that significantly affects T cell responses and tolerance is the catabolism of the essential aminoacid tryptophan. Mammals have an intracellular heme-containing enzyme - indoleamine 2,3-dioxygenase (IDO), which catalyses the oxidative catabolism of tryptophan. IDO is expressed at basal levels in the thymus, gut, lung, and the

maternal-foetal interface, and becomes upregulated during infection and inflammation [18]. The role of IDO in the suppression of microbial infections has long been acknowledged for the ability of this enzyme to reduce availability of tryptophan to infected tissues, with resulting deprivation of an essential aminoacid to cells and thus resolution of inflammation. More recently, IDO has been shown capable to induce T cell hyporesponsiveness, by reducing available free tryptophan to T cells. T cells are sensitive to tryptophan deprivation specifically during the G_0-S transition phase of the cell cycle. T cell cycle arrest clearly prevents clonal expansion and may determine cell death by apoptosis and/or tolerance (ignorance, anergy, immune deviation, and/or generation of regulatory T cells) [18]. Interesting work by Munn and Mellor has suggested that cells expressing IDO during pregnancy can regulate T cell immunity during pregnancy and that placental cells synthesising IDO may protect the foetus from maternal immune alloreactivity. Indeed, blockade of tryptophan catabolism during pregnancy enables maternal T cells to reject the foetus as an allograft. The effects of the IDO could be relevant in a myriad of other conditions. IDO-induced depletion of tryptophan could determine reduced growth of viruses, bacteria and parasites but also of proliferation of T cells. Tryptophan catabolism and IDO upregulation could consume anion superoxide, thus clearing inflammatory sites from free radicals, while degradation of tryptamine, serotonin and melatonin would induce changes in the neuroendocrine function. Moreover, tryptophan is the precursor of kynurenines, which are produced by IDO for the transcriptional control of IFN-γ [18]. These molecules could be relevant in immune homeostasis because of the central role of IFN-γ and its regulation in cell-mediated immunity and in the resolution of infections. Immunoactive/neuroactive kynurenines also affect immune and neuronal homeostasis by controlling apoptosis of T cells and neurotoxicity, respectively. In addition to tryptophan, also arginine metabolism is crucial for a correct immune response particularly in protection from infections and the control of the immune response [19, 20]. More specifically, it has been shown that L-arginine is metabolised by immune cells by two enzymes: the nitric oxide synthase (NOS), which oxidises L-arginine in two steps that generate nitric oxide (NO) and citrulline; and arginase, which converts L-arginine into urea and L-ornithine [19, 20]. Separate genes encode three different isoforms of NOS in mammalian cells: NOS1 and NOS3 are constitutively expressed in neuronal tissue and endothelium, respectively, and NOS2, the inducible form of NOS, is expressed in multiple tissues and cell types, including vascular endothelium, DCs and macrophages [19, 20]. Induction of NOS2 is controlled mainly by Th1 cytokines, such as IFN-γ and TNF-α. The enzymatic activity of NOS2 is sustained over a long period of time, enabling the production of 1000-fold higher amounts of NO than those produced by NOS1 and NOS3. Two distinct isoforms of arginase, encoded by two genes, are found in mammalian cells: type I arginase (Arg1), a cytosolic enzyme expressed at high levels in the liver as a component of the urea cycle, and type II arginase (Arg2), a mitochondrial enzyme expressed at lower levels in kidney, brain, small intestine and mammary gland, with limited expression in liver [19, 20]. Both isoforms are expressed in murine macrophages: Arg1 is induced by Th2 cytokines including IL-4, IL-13, TGF-β and IL-10, whereas Arg2 is induced by lipopolysaccharide (LPS), and intact bacteria. Discrepancies have been reported on the ability of LPS to induce Arg1, which probably reflect the use of either macrophage-like cell lines or primary cultured macrophages. The activity of NOS is regulated by cytokines and microbial products [19, 20]. It is important in protection from infections on one side and for the induction of tolerance on the other. Indeed, NO secretion is important for protection from mycobacterium tuberculosis infection; and depletion of arginine from the milieu together with induction of peroxynitrites generated by NOS2 cause activated T cells to undergo apoptosis. Therefore, metabolism of arginine is crucial also in the control of T cell tolerance, particularly for the myeloid suppressor cells (MSCs), able to control and block T cell activation [19, 20].

Finally, as previously mentioned, also lipid metabolism plays a relevant role in the control of the immune response and resistance to infections [21]. Of particular interest are the liver X receptors (LXRs). More specifically, the nuclear receptors LXRa and LXRb are important regulators of the bile acid synthesis in the liver and regulate the cholesterol absorption in the intestine. The physiological activators for the LXRs include oxysterols and intermediates in the cholesterol biosynthetic pathway [21]. In the monocyte/macrophage cell lineage, the scavenger receptor-mediated uptake of oxidised lipoproteins leads to the transcriptional activation of LXRs and induction of genes such as apoE, that enhances cholesterol removal form cells. Both forms of LXRs are expressed at high levels in macrophages, and their activation activates cholesterol efflux, indicating that the function in lipid metabolism is crucial. Macrophages are key cells of the innate and adaptive immune response. The function of LXRs in this context is not very clear. More recently, it has been shown that the LXR-dependent gene expression profile impacts macrophage function in setting of bacterial infection. Indeed, mice lacking the LXRs are highly susceptible to bacterial infection such as *Listeria Monocytogenes* (LM) [21]. Particularly, LXR-null macrophages undergo accelerated apoptosis when challenged with LM and exhibit reduced bacterial clearance *in vivo*. These data suggest that the LXR-dependent gene expression plays an unexpected role in innate immunity and suggest that common nuclear receptor pathways mediate macrophage responses to modified lipoproteins and intracellular pathogens. Together with previous work, these results position LXRs at the crossroads of lipid metabolism and innate immunity. These observations have implications for the pharmaceutical control of inflammation and the pathogenesis of atherosclerosis; however, the role of such crosstalk in normal physiology remains to be clarified. Therefore, drug compounds able to interfere with such biochemical pathways may have a large impact on both inflammation and infection susceptibility [21].

HIV INFECTION AND HAART AS MODEL FOR BIDIRECTIONAL COMMUNICATION BETWEEN IMMUNITY AND METABOLISM

HIV infection is associated with qualitative and quantitative defects of $CD4^+$ T cells. AIDS patients show a progressive decline in the $CD4^+$ T cell subset with a marked reduction of naïve T cells in peripheral blood. In addition, the reduction of $CD4^+$ T cells is associated with a progressive dis-

ruption of the T cell receptor (TCR)-Vβ repertoire. Several hypotheses have been proposed to explain these phenomena, such as the reduced thymic output of T lymphocytes, clonal anergy, increase of T cell apoptosis and trapping of activated cells in lymphoid tissues [22].

Highly active antiretroviral therapy (HAART) represents the most effective current approach to control HIV infection. A decrease of viral load with an improvement in immunological parameters and a decrease of opportunistic infections are well documented [22]. Despite these effects, HAART is still unable to fully reconstitute the immune system or to reduce drastically the HIV reservoir. Indeed, it is possible to observe a significant reduction in the viral load not associated with an optimal immune-reconstitution characterised by a significant increase in the $CD4^+$ T cell count. These patients are defined as "discordants", and represent a relevant clinical problem due to their increased susceptibility to opportunistic infections.

Leptin, the adipocyte-derived hormone, represents a key factor for the regulation of food intake, reproduction and basal metabolism [3, 4]. It is also an acute-phase reactant raised during infections and reduced by starvation. Recently, it has been shown that leptin can modulate T cell function *in vitro* and *in vivo*. *In vitro* it increases proliferation and IL-2 secretion by $CD4^+$ naive ($CD45RA^+$) T cells and enhances IFN-γ production by $CD4^+$ memory ($CD45RO^+$) T lymphocytes. The leptin-induced pattern of cytokines is classical of the T helper 1 (Th1) pro-inflammatory immune response with consequent suppression of the Th2 regulatory cytokines such as IL-4 [3, 4]. Furthermore, leptin is also able to reverse the immunodepression associated with acute starvation and provides protection from starvation-induced lymphoid and thymic atrophy. HIV-induced lymphoid atrophy resembles that observed in malnourished individuals with reduced adipose tissue and leptin levels. HIV patients show low adiposity with consequent low leptin levels. The failure of increase in production of leptin during the infections suggests that an impairment of the leptin response is present during the early phases of the HIV infection [3, 4]. More recently, it has been shown that in a group of HIV^+ children there is an increase in serum leptin during HAART [23]. Interestingly, the leptin levels were increased only in the patient s with a significant increase in $CD4^+$ T cells. Conversely, in patients in which leptin did not change during HAART, low number of $CD4^+$ T lymphocytes was observed [23]. The effects of leptin on the secretion of pro-inflammatory cytokines by memory T cells, its ability to expand naive T cells and its capacity to restore thymic function during starvation make leptin an attractive candidate for augmenting the immune-reconstitution in patients during HAART [23].

HAART has been associated with the development of lipodystrophy, changes in fat distribution, dyslipidaemia, insulin resistance and diabetes. These effects have been for long considered side-effects of HAART being associated with and increased cardiovascular risk. It is clear that HAART has important effects on adipose tissue metabolism and function. Considering adipose tissue as an important metabolic and immune organ, which plays a role in synthesizing and secreting a number of hormones and cytokines, it is important to understand whether or not the therapeutic action of HAART is only to be ascribed to the anti-HIV replicative capacity or also to the metabolic action on secretion of hormones and cytokines from the adipose tissue. The study of this specific aspect of the HIV infection will be of impact to understand the bidirectional communication between immunity and metabolism.

CONCLUDING REMARKS

There are still many questions concerning the role of several molecules at the interface between metabolism and immune regulation [2]. Significant leaps of knowledge have been done in the recent years; thanks to the expanding research on such molecules. While new information is unveiling complex networks that intimately connect metabolism and immunity, further research is needed. Nonetheless, these new acquisitions have prompted us to no longer regard the adipose tissue as a mere store of body fat but rather as an active participant in the regulation of essential body processes such as the immune homeostasis. From this evidence, the development of novel therapeutic targets is sure to emerge in the near future.

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ORIGINAL ARTICLE

Clinical and Molecular Genetic Spectrum of Congenital Deficiency of the Leptin Receptor

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ABSTRACT

BACKGROUND

A single family has been described in which obesity results from a mutation in the leptin-receptor gene (*LEPR*), but the prevalence of such mutations in severe, early-onset obesity has not been systematically examined.

METHODS

We sequenced *LEPR* in 300 subjects with hyperphagia and severe early-onset obesity, including 90 probands from consanguineous families, and investigated the extent to which mutations cosegregated with obesity and affected receptor function. We evaluated metabolic, endocrine, and immune function in probands and affected relatives.

RESULTS

Of the 300 subjects, 8 (3%) had nonsense or missense *LEPR* mutations — 7 were homozygotes, and 1 was a compound heterozygote. All missense mutations resulted in impaired receptor signaling. Affected subjects were characterized by hyperphagia, severe obesity, alterations in immune function, and delayed puberty due to hypogonadotropic hypogonadism. Serum leptin levels were within the range predicted by the elevated fat mass in these subjects. Their clinical features were less severe than those of subjects with congenital leptin deficiency.

CONCLUSIONS

The prevalence of pathogenic *LEPR* mutations in a cohort of subjects with severe, early-onset obesity was 3%. Circulating levels of leptin were not disproportionately elevated, suggesting that serum leptin cannot be used as a marker for leptin-receptor deficiency. Congenital leptin-receptor deficiency should be considered in the differential diagnosis in any child with hyperphagia and severe obesity in the absence of developmental delay or dysmorphism.

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The assessment of patients with severe early-onset obesity conventionally includes screening for potentially treatable neurologic and endocrine conditions and identifying known genetic conditions so that appropriate genetic counseling and, in some cases, treatment can be instituted.[1] Classically, patients with genetic obesity syndromes have been identified in childhood as a result of associated mental retardation and developmental abnormalities.[2] However, several monogenic disorders have been identified in which obesity itself is the predominant presenting feature. These disorders result from disruption of the hypothalamic leptin–melanocortin signaling pathway.[3-8]

Twelve subjects with congenital leptin deficiency due to loss-of-function mutations in the gene encoding leptin have been identified[3,4,9,10] (and unpublished data). Characteristic features include hyperphagia, obesity, hypogonadism, and impaired T-cell–mediated immunity. Treatment with recombinant human leptin reverses all aspects of the phenotype.[9,11,12] So far, only one mutation in the leptin-receptor gene (*LEPR*) has been reported, in three severely obese adult siblings from a consanguineous family of Algerian origin.[5] This mutation results in abnormal splicing of leptin-receptor transcripts and generates a mutant leptin receptor that lacks both transmembrane and intracellular domains. The mutant receptor circulates at high concentrations, binding leptin and resulting in very elevated serum leptin levels.[13] To determine the prevalence of pathogenic mutations in *LEPR* in severely obese patients, we studied 300 subjects with severe, early-onset obesity.

METHODS

SUBJECTS

When we began the study, the Genetics of Obesity Study (GOOS) cohort consisted of 2100 unrelated probands with severe obesity of early onset (before 10 years of age); severe obesity was defined as a standard-deviation score for the body-mass index (BMI) (the weight in kilograms divided by the square of the height in meters) of more than 3. We calculated BMI standard-deviation scores using reference data from the U.K. population.[14] The mean (±SD) score in the GOOS cohort is 4.2±0.8. Of the 2100 subjects, 1800 were reported to have a history of hyperphagia. Of these 1800 subjects, 300 were selected to determine the prevalence of leptin-receptor mutations: all 90 subjects in the GOOS cohort from consanguineous families, as well as 210 additional subjects who were impartially selected. The mean BMI standard-deviation score for the group screened was 4.5±1.2. Mutations in known obesity genes were ruled out with the use of biochemical analysis (mutant leptin and prohormone convertase 1 genes) and direct nucleotide sequencing (of the genes encoding pro-opiomelanocortin and the melanocortin 4 receptor [MC4R]); there were no mutations in additional candidate genes for obesity (*SIM1*, *NHLH2*, *CPE*, *MCHR1*, and *MCHR2*).

Subjects with mutations in *LEPR* and their relatives were invited to participate in clinical studies at the Wellcome Trust Clinical Research Facility at Addenbrooke's Hospital, Cambridge, United Kingdom. All studies were approved by the Anglia and Oxford multiregional ethics committee and the local–regional ethics committee of Cambridge. Each subject, or his or her parent if the subject was a child younger than 16 years, provided written informed consent; the minors provided oral consent. All clinical studies were conducted in accordance with the principles of the Declaration of Helsinki.

In adults, overweight and obesity were defined according to the World Health Organization criteria: a BMI of 25.0 to 29.9 and a BMI of 30.0 or higher, respectively. Because there are no internationally recognized definitions of overweight and obesity in persons under 18 years of age, we used criteria proposed by the International Obesity Task Force and supported by a recent International Consensus on Childhood Obesity[15]: an age-adjusted BMI above the 91st percentile and an age-adjusted BMI above the 99th percentile, respectively.

DETECTION OF MUTATIONS AND GENOTYPING

Genomic DNA was isolated from leukocytes derived from whole blood, and the coding region of the *LEPR* gene was amplified with the use of the polymerase chain reaction and was then sequenced (see the Supplementary Appendix, available with the full text of this article at www.nejm.org). We also sequenced *LEPR* in impartially selected nonobese control subjects: 100 alleles from each of three population-derived cohorts — of white European origin,[16] of South Asian origin,[17] and of Turkish origin.[18] (See the Supplementary Appendix for details of our studies of mutant-receptor function.)

BODY COMPOSITION, GROWTH, AND ENERGY BALANCE

We used anthropometric methods and whole-body dual-energy x-ray absorptiometry (DPX software, Lunar) to determine body composition, as previously described.[8,11] We measured resting metabolic rate using indirect calorimetry after a 12-hour overnight fast and using an open-circuit, ventilated, canopy measurement system (Europa Gas Exchange Monitor, Nutren Technology). After adjustment for body composition, the resting metabolic rate was compared with that predicted by age- and sex-specific equations.[19,20] Semiquantitative assessment of eating behavior was undertaken in subjects younger than 18 years, as previously described.[8,9]

METABOLIC AND ENDOCRINE STUDIES

Fasting blood samples were analyzed for levels of leptin, glucose, insulin, thyrotropin, free thyroxine, insulin-like growth factor 1 (IGF-1), follicle-stimulating hormone, luteinizing hormone, estradiol, and testosterone, with the use of standard assays.[11]

LYMPHOCYTE COUNT AND FUNCTION

Lymphocytes were isolated from fresh whole blood, and cell phenotypes were measured by cytofluorometric (fluorescence-activated cell-sorting) analysis, as reported previously.[9] Proliferative responses to antigenic stimuli were measured.[9] Lymphocyte counts and proliferative responses in subjects with leptin-receptor deficiency were compared with those in 46 control subjects matched for age (from 8 years to adult).

STATISTICAL ANALYSIS

Clinical data are expressed as means ±SD. Differences between groups were compared with use of the unpaired Student's t-test. All reported P values are from two-sided tests, and P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

DETECTION OF MUTATIONS AND FAMILY STUDIES

We identified five nonsense and four missense mutations in 8 of the 300 probands (Table 1 and Fig. 1A). Seven probands were homozygous for such mutations, and one proband (Subject 1 in Family 8) was a compound heterozygote for a missense mutation (R612H) and a nonsense mutation. None of these mutations were found in alleles from control subjects. Six of the probands were from consanguineous families (Fig. 2A). In three pedigrees (Families 1, 2, and 4), additional homozygous family members were identified, all of whom had severe, early-onset obesity (Fig. 2A).

FUNCTIONAL ANALYSIS OF MUTANT RECEPTORS

All frame-shift mutations occurred in the N-terminal domain of the leptin receptor and were pre-

Table 1. Leptin-Receptor Mutations in Subjects with Severe Early-Onset Obesity.

Mutation	Family No.	No. of Affected Subjects	Race or Ethnic Group*
Homozygous frame-shift			
4-bp deletion in codon 22	1	3 (1 deceased)	Bangladeshi
11-bp deletion in codon 70	2	2	Turkish
66-bp deletion in codon 514	3	1 (deceased)	Iranian
Homozygous nonsense			
W31X	4	3	Southern European
Homozygous missense			
A409E	5	1	Turkish
W664R	6	1	Norwegian
H684P	7	1	White (United Kingdom)
Compound heterozygous			
1-bp deletion in codon 15 and R612H	8	1	White (United Kingdom)

* Race or ethnic group was assigned by the physician.

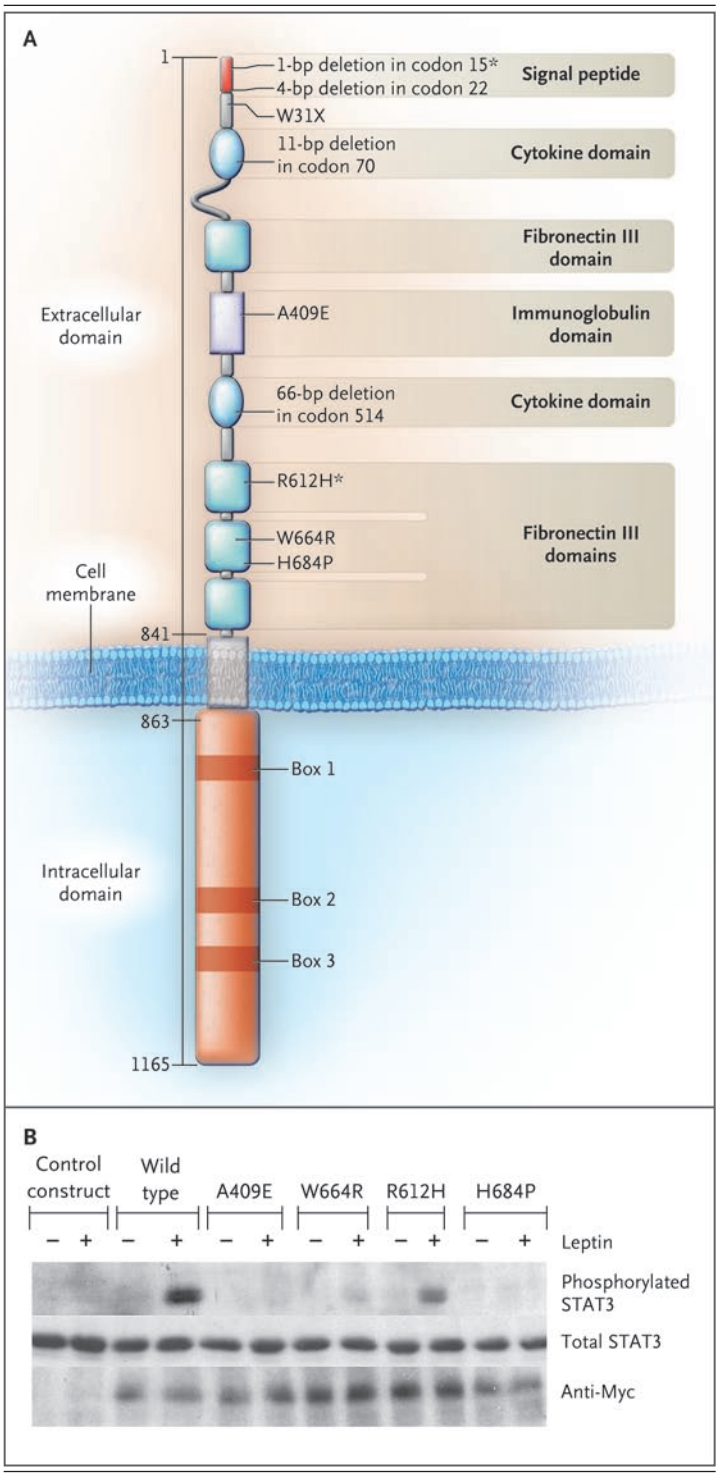


Figure 1. Mutations in the Leptin-Receptor Gene (Panel A) and in Vitro Function of the Leptin-Receptor Mutant Constructs (Panel B).

Panel A shows the positions of the identified mutations in the leptin receptor. We observed homozygous mutations in seven severely obese probands; in an eighth (Subject 1 in Family 8, a compound heterozygote), we observed a nonsense mutation and a missense mutation (asterisks). Amino acid numbers are shown on the left-hand side. Panel B shows the induction of the phosphorylation of signal transducer and activator of transcription 3 (STAT3) by wild-type and mutant leptin receptors in the absence (minus signs) and presence (plus signs) of recombinant human leptin. The control construct was an empty pcDNA3 vector.

dicted to result in the loss of all leptin-receptor isoforms. We examined the signaling properties of receptors with missense mutations (Fig. 1B). Three of the missense mutations (A409E, W664R, and H684P) resulted in a complete loss of signaling, as measured by leptin-stimulated phosphorylation of signal transducer and activator of transcription 3 (STAT3). The other missense mutation, R612H, encodes receptors with some residual ability to phosphorylate STAT3 in response to leptin (Fig. 1B).

CLINICAL PHENOTYPE OF LEPTIN-RECEPTOR DEFICIENCY

We studied the clinical phenotype of leptin-receptor deficiency in 10 subjects (6 probands and 4 affected relatives) with early-onset obesity who were homozygous for complete loss-of-function mutations in *LEPR*. All 10 had become obese in early childhood; at the time of study, 4 were adults and 6 were children. We compared their phenotype with that of five subjects with congenital leptin deficiency and with that of five subjects who were homozygous for complete loss-of-function mutations in MC4R, whom we had studied previously using identical protocols[8,9] (and unpublished data).

Body Composition and Energy Balance

In all cases, the body weight of subjects with *LEPR* mutations deviated from predicted percentiles within the first year of life (data not shown). The mean BMI standard-deviation score for these subjects was 5.1±1.6, as compared with 6.8±2.1 for subjects with congenital leptin deficiency ($P=0.005$) and 5.0±1.5 for MC4R-deficient subjects. The mean percentage of body fat among homozygous carriers of *LEPR* mutations was high (52.8±3.2; normal range, 15 to 25) and was similar to that in the subjects with congenital leptin deficiency (mean, 58.0±3.5) but higher than that in the subjects lacking MC4R (45.2±3.3; $P<0.001$) (Fig. 3B). The percentage of lean mass was lower in subjects with

LEPR mutations than in equally obese subjects without such mutations, although the actual amount of lean mass in kilograms for each subject was within the age-related normal range[21,22] (data not shown).

All subjects had a history of increased food-seeking behavior in childhood, which continued into later life in the adult subjects. During an ad libitum test meal, the probands with *LEPR* mutations consumed almost three times the amount of energy that control subjects consumed (Fig. 3C). This increased intake was similar to that of subjects lacking MC4R but considerably less than that of subjects with congenital leptin deficiency. The basal metabolic rate was greater in leptin-receptor–deficient, obese subjects than in subjects of normal weight, as one would expect. However, the basal metabolic rate is usually adjusted for lean mass (in kilograms) to allow for differences in body weight (and thus lean mass) among subjects. The adjusted basal metabolic rate per kilogram of lean mass in the subjects with *LEPR* mutations was similar to that predicted for persons with this body composition on the basis of accepted age- and sex-specific calculations[23] (see Fig. 1A in the Supplementary Appendix).

Metabolic and Endocrine Function

Most of the subjects with *LEPR* mutations had normal glucose concentrations (Table 2), although the two oldest adults (Subjects 1 and 2 in Family 4) had type 2 diabetes, which was managed with oral hypoglycemic medication. All subjects had hyperinsulinemia (Table 2) to a degree consistent with the degree of obesity (see Fig. 1B in the Supplementary Appendix).

In childhood, linear growth was normal, and the standard-deviation scores for height of children with *LEPR* mutations (Table 2) were similar to those of equally obese children without *LEPR* mutations.[8] Serum levels of IGF-1 were appropriate for the age of the children (Table 2), and growth hormone was secreted in a pulsatile fashion (data not shown). However, final height was reduced in adults with *LEPR* mutations, owing to the lack of a pubertal growth spurt. This is reflected in the short statures of the 15-year-old male subject with a standard-deviation score for height of –1.7 and the four female subjects with a score of –2.0 (Table 2).

All four adults (all of whom were female) had clinical evidence of hypogonadism, with lack of a pubertal growth spurt and reduced expression of secondary sexual characteristics; three had no menses until after 20 years of age. The 18-year-old female (Subject 1 in Family 2) and the 15-year-old male (Subject 2 in Family 2), both of whom were clinically prepubertal, had low sex-steroid levels and low follicle-stimulating hormone and luteinizing hormone levels, indicative of hypogonadotropic hypogonadism. In addition, Subject 2 in Family 1 had a complete loss of luteinizing hormone pulsatility (see Fig. 1C in the Supplementary Appendix). Notably, the females who were 31, 41, and 55 years of age had irregular menses after the age of 20 years and had estradiol, luteinizing hormone, and follicle-stimulating hormone levels that were consistent with their age. Free thyroxine and thyrotropin levels were within the normal range in all subjects (Table 2).

Serum leptin levels in subjects with *LEPR* mutations (Table 2) were similar to those in equally obese subjects with a normal leptin-receptor gene sequence (Fig. 3D). Serum leptin levels correlated with the fat mass in subjects with *LEPR* mutations and in age- and BMI-matched subjects (see Fig. 1D in the Supplementary Appendix).

Immune Function

All the children with *LEPR* mutations had more frequent childhood infections (predominantly of the upper respiratory tract) than did their siblings with wild-type *LEPR*. The premature deaths of two obese children in these families were associated with acute respiratory tract infections. Since our group previously found that leptin-deficient subjects have a marked reduction in the CD4+ T-cell count and reduced T-cell proliferation,[9] we analyzed the immunophenotype and T-cell responses in subjects with *LEPR* mutations and compared these data with those of age-matched control subjects. We observed a modest reduction in the absolute CD4+ T-cell counts in the leptin-receptor–deficient subjects (mean, 988±186 cells per cubic millimeter; 42±13%), as compared with those in control subjects (mean, 1100±892 cells per cubic millimeter; 44±7%), although the CD4+:CD8+ ratios were similar (2±2 and 2±1). However, leptin-receptor–deficient subjects had a significant compensatory increase in the CD19+ cell (B-cell) count (mean, 460±238 cells per cubic millimeter; 18±6%) as compared with control subjects (mean, 280±328 cells per cubic millimeter; 11±3%) ($P=0.006$). T cells from subjects with *LEPR* mutations showed reduced pro-

liferative responses to a variety of polyclonal stimuli specific to T cells, such as muromonab-CD3 (OKT3), phytohemagglutinin, phorbol myristate acetate (PMA) or ionomycin (Iono), and the recall antigen purified protein derivative (PPD) (see Fig. 1E in the Supplementary Appendix). The cytokine pattern of the subjects with leptin-receptor deficiency was less impaired than that of the leptin-deficient subjects, with only a modest reduction in secretion of the proinflammatory cytokine interferon-γ as compared with age-matched controls — especially during OKT3 and PMA/Iono stimulation — and increased secretion of the inhibitory cytokine interleukin-10 during stimulation with purified protein derivative (see Fig. 1F and 1G in the Supplementary Appendix), but no significant change in interleukin-4 secretion (see Fig. 1H in the Supplementary Appendix).

Figure 2 (facing page). Pedigrees of Consanguineous Families and Nonconsanguineous Families with Mutations in the Leptin-Receptor Gene That Segregate with Severe Obesity.

The squares represent male family members, and the circles female family members; open symbols represent unaffected family members, and solid symbols family members with obesity (in adults, defined as a BMI [the weight in kilograms divided by the square of the height in meters] of 30 or more; in children, defined as a BMI above the 99th age-adjusted percentile). A slash through the symbol denotes a subject who has died. Below each symbol, age is given, followed by the BMI value, the BMI standard-deviation score, and the genotype, with N denoting the normal (wild-type) allele and M the mutant allele. In Family 8, M_F denotes the frame-shift mutation, and M_M the missense mutation. For subjects in whom it was available, the percentage of body fat, measured by dual-energy x-ray absorptiometry, is listed below the genotype.

Heterozygote Phenotype

We assessed the level of obesity in the 22 family members who were heterozygous and the 6 who were homozygous for wild-type *LEPR*. Heterozygous subjects were not severely obese, and their mean BMI standard-deviation score (0.6±0.8) was similar to that of their relatives who were homozygous for wild-type *LEPR* (0.6±1.0). We compared the measured percentage of body fat and that predicted according to height and weight; the absolute difference was significantly greater in the 14 of the 22 heterozygotes for whom fat-mass data were available than in their relatives with wild-type *LEPR*[6] (mean, 8.2% vs. 2.1%; P=0.009).

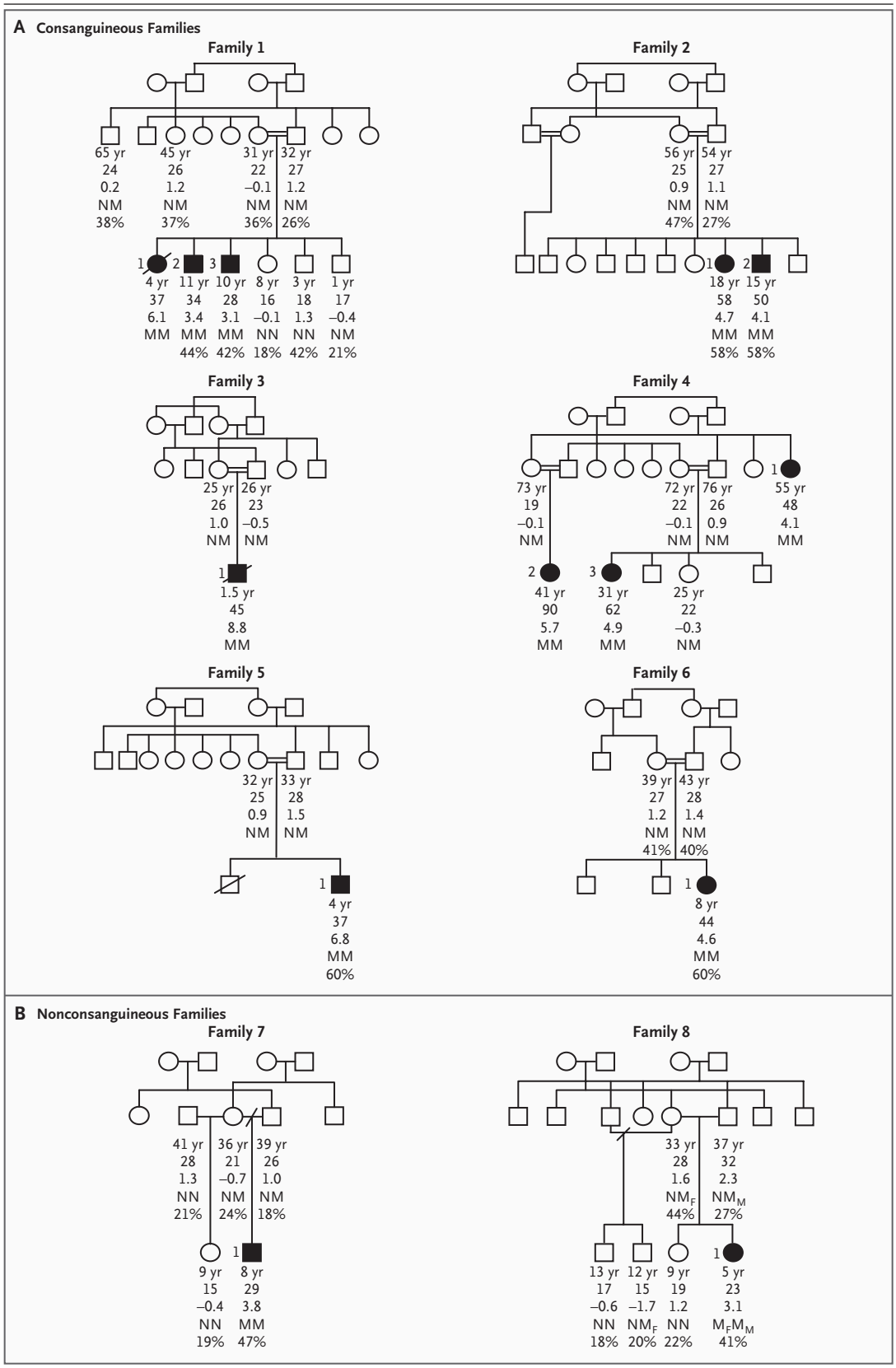
DISCUSSION

The prevalence of pathogenic *LEPR* mutations in our subjects with hyperphagia and severe early-onset obesity was 3%. The prevalence of *LEPR* mutations in this highly selected cohort is unlikely to reflect that in unrelated populations of obese subjects or in populations in which the age at the onset of obesity is more heterogeneous.[24,25] Six of the probands were from consanguineous families, but two probands (including the compound heterozygote) were whites in the United Kingdom whose parents were not known to be related. Although the prevalence of *LEPR* mutations is likely to be higher in ethnic groups in which consanguinity is common, *LEPR* deficiency should be considered in all patients with hyperphagic obesity of early onset.

None of the subjects with *LEPR* mutations characterized in this study, including those with nonsense mutations that were predicted to result in the loss of all isoforms, had disproportionately elevated serum leptin levels. Thus, serum leptin levels are not a generally useful marker of leptin-receptor deficiency — contrary to a previous suggestion.[13]

Congenital leptin-receptor deficiency is characterized by severe, early-onset obesity associated with selective deposition of fat mass, as seen in subjects with leptin deficiency.[9] All of our subjects had hyperphagia from an early age, and we demonstrated that the ad libitum energy intake was greatly increased in children with leptin-receptor deficiency, with no evidence of a major deficit in basal energy expenditure.

Children with leptin-receptor deficiency had normal linear growth during childhood and had normal IGF-1 levels. However, because of the lack of a pubertal growth spurt, the final height of adult subjects was reduced. In the one previously described family, short stature and abnormal serum growth hormone levels and IGF-binding protein 3 levels were noted in childhood.[5] However, assessment of the growth hormone–IGF axis is difficult in obese children and adults, since obesity is itself associated with abnormal results of basal and of dynamic tests of this axis.[26,27] We conclude that impaired linear growth does not appear to be a common characteristic of patients with this disease.



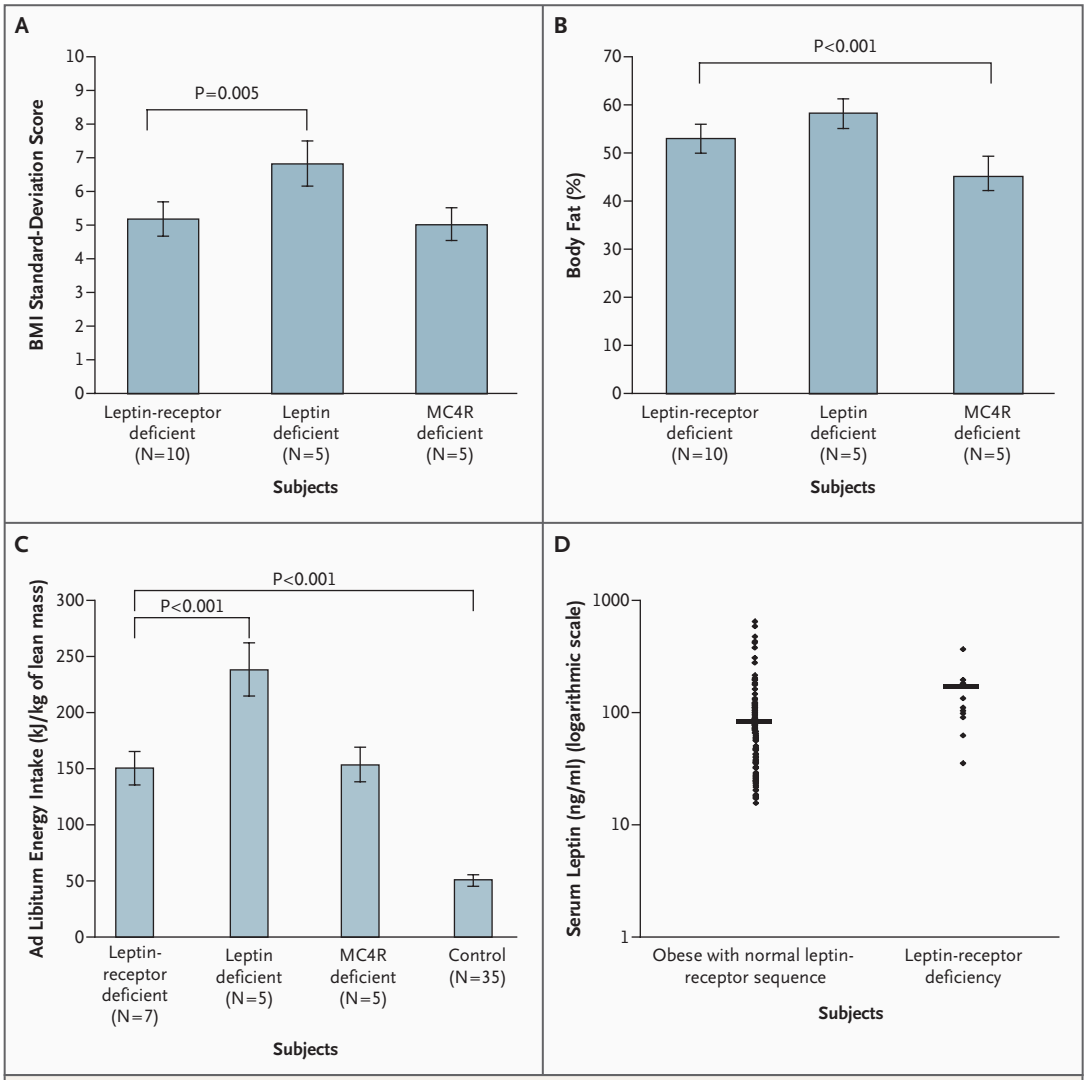


Figure 3. Body-Mass Index (BMI) (Panel A), Percentage of Body Fat (Panel B), Ad Libitum Energy Intake (Panel C), and Serum Leptin Levels (Panel D) in Leptin-Receptor Deficiency.

Panel A shows the BMI standard-deviation score.[14] Panel B shows the percentage of body fat as measured by dual-energy x-ray absorptiometry. Panel C shows the ad libitum energy intake at a test meal (adjusted for kilograms of lean mass).[8] In Panel D, the horizontal line indicates the geometric mean. The data in Panels A, B, and C are means ±SD. Subjects 1, 2, and 3 in Family 4 were not able to travel to the United Kingdom, so data for 7 (rather than 10) subjects who were homozygous for *LEPR* mutations are presented in Panels B and C. The BMI is calculated as the weight in kilograms divided by the square of the height in meters. MC4R denotes melanocortin 4 receptor.

Adults with leptin-receptor deficiency have hypogonadotropic hypogonadism and do not undergo puberty. Irregular menses developed in the third and fourth decades in the three oldest women in our study, as reported previously for one woman with leptin deficiency.[28] It is plausible that the excess mass of adipose tissue leads to the production of sufficient estrogen (owing to the action of aromatase) to result in uterine development and irregular menses in the absence of fully developed secondary sexual characteristics. However, this may not be the only explanation, since luteinizing hormone and follicle-stimulating hormone levels in these three subjects were within the normal range for the follicular phase of the menstrual cycle, suggesting that even in the absence of leptin

Table 2. Metabolic and Endocrine Features of Leptin-Receptor Deficiency in Subjects Homozygous for *LEPR* Mutations.*

Variable	Subject										Normal Range†
Family no.	5	6	7	1	1	2	2	4	4	4	
Subject no.	1	1	1	3	2	2	1	3	2	1	
Age (yr)	4	8	8	10	11	15	18	31	41	55	
Sex	M	F	M	M	M	M	F	F	F	F	
Glucose (mg/dl)	76	76	81	88	83	94	72	99	157	130	75–115
Insulin (μU/ml)	4	14	17	28	81	26	29	15	Diabetes mellitus	Diabetes mellitus	5–20
Standard-deviation score for height	1.8	4.2	2.1	1.3	1.8	–1.7	–2.0	–2.0	–2.0	—	
IGF-1 (U/ml)	4	29.1	27.3	19.5	16.5	8.4	8.0	8.5	5.7	—	Age-specific
FSH (IU/L)	0.3	0.3	0.2	0.2	1.3	3.7	1.8	4.2	7.7	13.7	Age-specific
Luteinizing hormone (IU/L)	0.1	0.1	0.2	0.2	0.2	1.8	0.9	4.8	6.8	7.6	Age-specific
Estradiol (pg/ml)	—	18.3	—	—	—	—	17.2	54.8	50.4	13.6	Age-specific
Testosterone (ng/ml)	—		0.058	0.058	0.058	0.26	—	—	—	—	Age-specific
Thyrotropin (mU/L)	3.3	1.6	4.1	0.8	2.1	0.9	0.9	2.9	4.2	3.8	0.6–4.6
Free thyroxine (ng/dl)	1.1	1.0	0.9	1.1	1.1	1.1	1.1	0.9	1.2	1.0	0.7–1.7
Serum leptin (ng/ml)	36	194	14	97	110	178	365	180	133	90	

* To convert values for glucose to millimoles per liter, multiply by 0.05551; to convert values for insulin to picomoles per liter, multiply by 6.95; to convert values for estradiol to picomoles per liter, multiply by 3.671; to convert values for testosterone to nanomoles per liter, multiply by 3.467; and to convert values for thyroxine to picomoles per liter, multiply by 12.87.

† The age-specific normal ranges for insulin-like growth factor 1 (IGF-1) are 2.5 to 20.0 U per liter for 0.0 to 6.9 years, 4.5 to 37.5 for 7.0 to 9.9 years, 7.0 to 50.0 for 10.0 to 10.9 years, 8.5 to 60.0 for 11.0 to 11.9 years, 10.0 to 75.0 for 12.0 to 12.9 years, and 7.0 to 50.0 for 13.0 years or older; for follicle-stimulating hormone (FSH), less than 0.2 U per liter for clinically prepubertal subjects, 2.9 to 8.4 for women in the follicular phase of the menstrual cycle, 21.0 to 140.0 for postmenopausal women, and 1.0 to 10.0 for men; for luteinizing hormone (LH), less than 0.2 U per liter for clinically prepubertal subjects, 1.3 to 8.4 for women in the follicular phase, 16.0 to 75.0 for postmenopausal women, and 1.5 to 6.3 for men; for estradiol, less than 22 pg per milliliter for clinically prepubertal persons, 27 to 204 for women in the follicular phase, and less than 30 for postmenopausal women; and for testosterone, less than 14.3 ng per deciliter for clinically prepubertal persons and 229.0 to 1086.0 for men.

activity, some activation of the hypothalamic–pituitary–gonadal axis is possible, albeit temporally delayed.

Subjects with *LEPR* mutations tended to have a lower CD4+ T-cell count and a significantly greater compensatory B-cell count than age-matched control subjects — findings that are consistent with the known effects of leptin on immune function.[29] Lymphocytes in the affected subjects showed decreased proliferation and altered cytokine release in response to nonspecific and antigen-specific stimuli. In two families, very obese children died after an infection in the first decade of life. It is likely that this immune dysfunction, perhaps together with impaired respiratory reserve as a result of severe obesity, contributed to these early deaths.

Heterozygotes who were leptin-receptor–deficient but not obese had an increased fat mass, a finding consistent with our observation that heterozygote carriers of a leptin mutation had 23% more fat than was predicted with anthropometric methods.[17] These findings are consistent with those of Chung et al., who found an increase in the fat mass of mice that were heterozygous for deletion of leptin or of the leptin receptor ($ob^{+/-}$ or $db^{+/-}$).[30]

Our data suggest that several phenotypic features seen in subjects with leptin-receptor deficiency are not as severe as those in subjects with leptin deficiency.[9,11] This is surprising, given the fact that the *LEPR* protein product is the only known receptor for leptin and given the phenotypic similarity between mice lacking leptin (*ob*/*ob*) and mice lacking the leptin receptor (*db*/*db*) that share the same genetic background.[31] The differ-

ences seen between the two groups of subjects may relate to the fact that our leptin-receptor–deficient subjects were, on average, older than the leptin-deficient subjects we studied previously.[9] Also, the leptin-deficient subjects were of Pakistani origin, whereas the leptin-receptor–deficient subjects were from various ethnic groups. However, the differences between these two groups are striking in magnitude and consistency and raise the possibility that in humans, the canonical leptin receptor may not be the only receptor that mediates the actions of leptin, at least when serum leptin levels are high.

Congenital leptin-receptor deficiency cannot be ruled out by measuring serum leptin levels, and this diagnosis should be considered in all patients with severe obesity and hyperphagia in the absence of developmental delay and dysmorphic features. This diagnosis has implications for the care of these patients, both in terms of genetic counseling of the affected families and in terms of future prospects for treatment, since these patients would be predicted to have a favorable response to drugs targeted at pathways downstream of the leptin receptor.

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Immunity
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A Key Role of Leptin in the Control of Regulatory T Cell Proliferation

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SUMMARY

We report here that leptin can act as a negative signal for the proliferation of human naturally occurring Foxp3$^+$CD4$^+$CD25$^+$ regulatory T (T_{reg}) cells. Freshly isolated T_{reg} cells produced leptin and expressed high amounts of leptin receptor (ObR). In vitro neutralization with leptin monoclonal antibody (mAb), during anti-CD3 and anti-CD28 stimulation, resulted in T_{reg} cell proliferation, which was interleukin-2 (IL-2) dependent. T_{reg} cells that proliferated in the presence of leptin mAb had increased expression of Foxp3 and remained suppressive. The phenomena appeared secondary to leptin signaling via ObR and, importantly, leptin neutralization reversed the anergic state of the T_{reg} cells, as indicated by downmodulation of the cyclin-dependent kinase inhibitor p27 ($p27^{kip1}$) and the phosphorylation of the extracellular-related kinases 1 (ERK1) and ERK2. Together with the finding of enhanced proliferation of T_{reg} cells observed in leptin- and ObR-deficient mice, these results suggest a potential for therapeutic interventions in immune and autoimmune diseases.

INTRODUCTION

Thymus-derived, naturally occurring, regulatory T (T_{reg}) cells are a subset of T lymphocytes that constitutes about 5%–10% of peripheral CD4$^+$ T cells. T_{reg} cells constitutively express the high-affinity interleukin-2 (IL-2) receptor α chain CD25 and can inhibit effector T cell responses in vitro and in vivo (Shevach, 2002; Sakaguchi, 2004; Suri-Payer et al., 1998; Thornton and Shevach, 1998; Ng et al., 2001). T_{reg} cells express the forkhead family transcription factor Foxp3, a key control gene for their development and function (Shevach, 2002; Sakaguchi, 2004). Given the importance of T_{reg} cells in the mechanisms of immune regulation and their protective role in several autoimmune conditions, there has been wide interest in finding strategies that expand T_{reg} cell numbers in the periphery (Kretschmer et al., 2005; Tang et al., 2004; Jiang et al., 2003). However, technical difficulties including hyporesponsiveness of freshly isolated T_{reg} cells to T cell receptor (TCR) stimulation in culture has hampered this process (Thornton and Shevach, 1998; La Cava et al., 2004). Nonetheless, murine and human T_{reg} cell numbers have been expanded in vitro up to 200-fold in the presence of high doses of IL-2 (Tang et al., 2004; Jiang et al., 2003). These proliferated T_{reg} cells continue to express cell-surface molecules and intracellular markers consistent with a regulatory phenotype and effectively suppress in vitro proliferative responses to either anti-CD3 or allogeneic stimuli, in a fashion similar to naturally occurring T_{reg} cells (Tang et al., 2004; Jiang et al., 2003).

Leptin is a cytokine-like hormone structurally similar to IL-6 and is involved in the control of food intake, metabolism, and T cell function (Friedman and Halaas, 1998; La Cava and Matarese, 2004). We report here that freshly isolated human T_{reg} cells constitutively expressed high amounts of both leptin and the leptin receptor (ObR) and that the leptin pathway can act as a negative signal for the proliferation of T_{reg} cells. These findings may partly explain why chronic leptin- and leptin-receptor deficiency associate with increased susceptibility to infection and resistance to autoimmunity (La Cava and Matarese, 2004; Ikejima et al., 2005; Farooqi et al., 2002; Matarese et al., 2002) and the increased risk of infection and reduced incidence of autoimmunity in individuals with low leptin (Matarese et al., 2002). These results may also suggest new possibilities for leptin-based manipulation of the T_{reg} cells.

RESULTS

Human T_{reg} Cells Express Higher Amounts of ObR than Do CD4$^+$CD25$^-$ Effector T Cells

Previous studies have shown that leptin receptor (ObR) is expressed on CD4$^+$ T cells and that it is able to switch immune responses toward a T helper 1 (Th1) phenotype (La Cava and Matarese, 2004). We sought to analyze the expression of ObR on T_{reg} cells and to correlate its

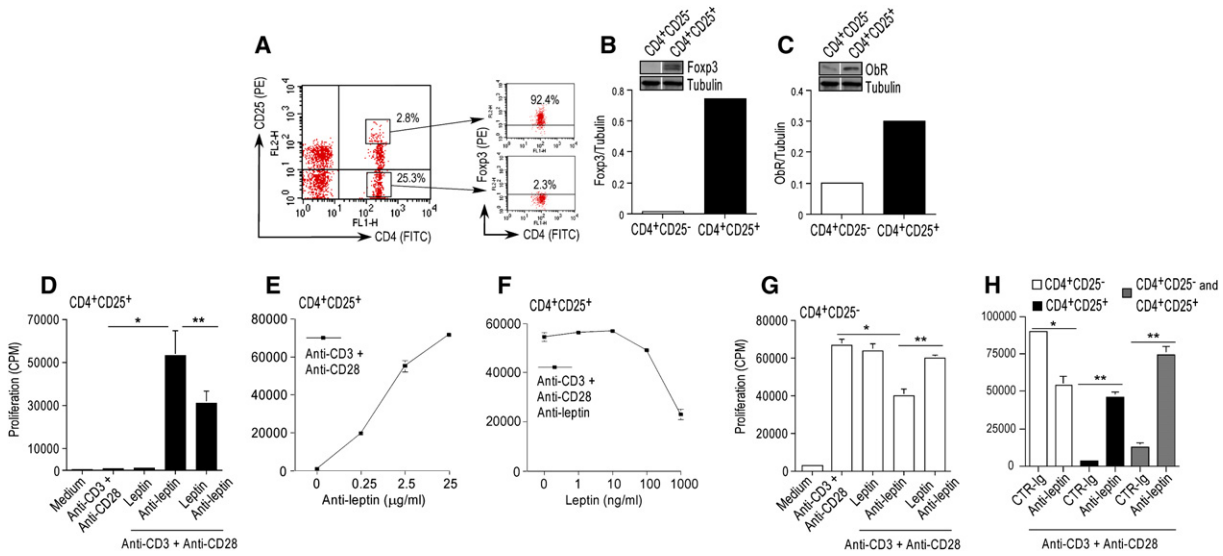

Figure 1. Human T_{reg} Cells Express High ObR, and Leptin Neutralization Reverses Their Hyporesponsiveness

(A) Representative flow cytometry plot of human T cells stained for CD4, CD25, and Foxp3.

(B and C) Immunoblot analysis of sorted $CD4^+$ T cells on the basis of their CD25 expression. Graphs show quantitation of Foxp3 and ObR with respect to tubulin. One representative out of five independent experiments is shown.

(D) Proliferation of $CD4^+CD25^+$ T_{reg} cells treated with recombinant human leptin (250 ng/ml) in the presence or absence of leptin mAb (10 μg/ml). The data are shown as mean ± SD (n = 5, *p < 0.0001; **p < 0.01).

(E) Dose dependency of T_{reg} cell proliferation induced by leptin mAb. Proliferation was measured after treatment with indicated doses of leptin mAb. The data are shown as mean ± SD (n = 5).

(F) Proliferation of T_{reg} cells induced by a fixed dose of leptin mAb in the presence of increasing concentration of recombinant leptin. The data are shown as mean ± SD (n = 5).

(G) Proliferation of $CD4^+CD25^-$ effector T cells treated with recombinant human leptin (250 ng/ml) in the presence or absence of leptin mAb (10 μg/ml). The data are shown as mean ± SD (n = 5, *p < 0.01; **p < 0.01).

(H) Proliferation of $CD4^+CD25^-$ effector T cells, T_{reg} cells, and of both cell types in coculture in the presence or absence of leptin mAb (10 μg/ml). The data are shown as mean ± SD (n = 5, *p < 0.01; **p < 0.0001).

expression with Foxp3. $CD4^+CD25^+$ T_{reg} cells and $CD4^+CD25^-$ effector T cells purified from human healthy donors (Figure 1A, left) were analyzed for Foxp3 expression by FACS analysis and immunoblot (Figure 1A, right, and Figure 1B, respectively). As expected, T_{reg} cells showed high amounts of Foxp3 protein whereas $CD4^+CD25^-$ did not show detectable amount of the protein in cell extracts (Figures 1A and 1B). Instead, ObR was expressed on both freshly isolated cellular subsets, although at significantly higher amounts ($p < 0.001$) in T_{reg} cells as compared to the $CD4^+CD25^-$ T cell effectors (Figure 1C).

Leptin Neutralization Results in the Proliferation of Human T_{reg} Cells Stimulated with Anti-CD3 and Anti-CD28

Human T_{reg} cells were hyporesponsive to anti-CD3 and anti-CD28 stimulation (Figure 1D), in agreement with previous findings (Ng et al., 2001). Addition of exogenous recombinant leptin to the cultures did not alter T_{reg} cells hyporesponsiveness (Figure 1D). However, addition of neutralizing leptin monoclonal antibody (mAb) reversed the hyporesponsiveness and promoted T_{reg} cell proliferation (Figure 1D) in a dose-dependent fashion (Figure 1E). Analogous results were observed with different leptin or ObR-blocking antibodies (see Figure S1 in the Supplemental Data available online). Confirming specificity, addition of exogenous recombinant leptin to anti-CD3- and anti-CD28-stimulated T_{reg} cells antagonized the proliferation induced by leptin mAb (Figure 1D). Moreover, dose-dependent increase of the proliferation of stimulated T_{reg} cells in the presence of leptin mAb (Figure 1E) was reversed by addition of increasing doses of recombinant leptin (Figure 1F). Interestingly, leptin mAb inhibited the proliferation of purified effector $CD4^+CD25^-$ T cells, a phenomenon that was reversed by addition of exogenous leptin (Figure 1G). Thus, neutralization of leptin had opposite effects on effector $CD4^+CD25^-$ T cells and T_{reg} cells—it inhibited proliferation on the former lymphocyte subset (Figure 1G), whereas it promoted expansion of the latter subpopulation (Figure 1D). This effect was also evident morphologically, as formation of cell clumps in the cultures of T_{reg} cells stimulated with anti-CD3 and anti-CD28 and leptin mAb, but not in the cultures of T_{reg} cells stimulated with anti-CD3 and anti-CD28 in the absence of leptin mAb (data not shown). Finally, in coculture experiments, T_{reg} cells efficiently suppressed the proliferation of $CD4^+CD25^-$ T cells, and leptin neutralization reversed the suppression by T_{reg} cells (Figure 1H).

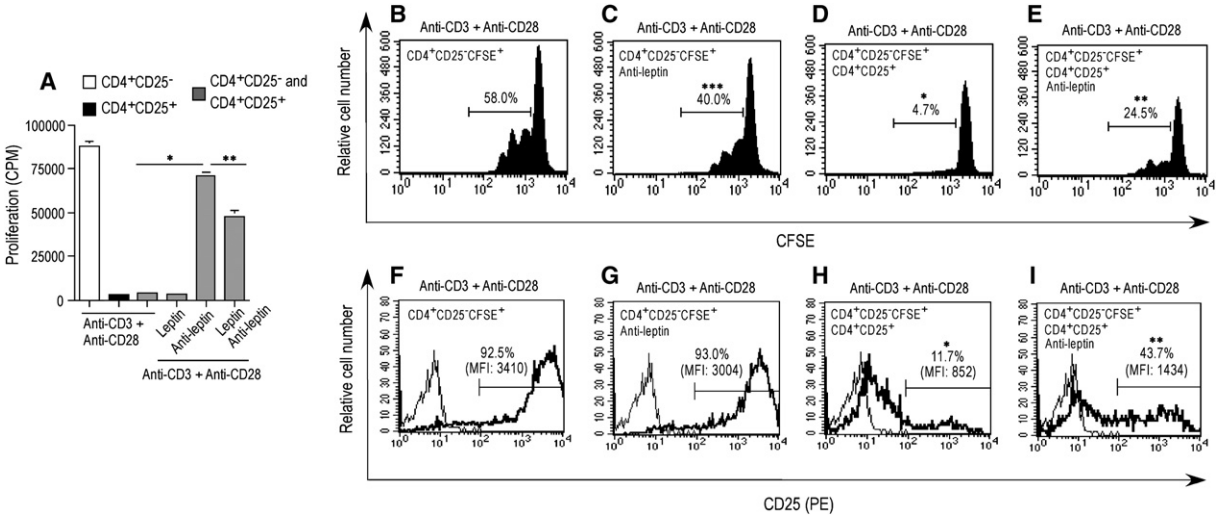

Figure 2. Human T_{reg} Cells Exhibit Partial Suppressive Capacity upon Leptin-mAb-Induced Proliferation

(A) Proliferation of $CD4^+CD25^-$ effector T cells, T_{reg} cells, and of both cell types in coculture treated with recombinant human leptin (250 ng/ml) in the presence or absence of leptin mAb (10 μg/ml). The data are shown as mean ± SD (n = 3, *p < 0.0001; **p < 0.01).

(B–I) Proliferative response (B–E) and CD25 expression analysis (F–I) of CFSE-labeled-$CD4^+CD25^-$ effector T cells alone or in coculture with untreated or leptin-mAb-treated unlabeled T_{reg} cells. The thin line represents the isotype-matched negative control and the thick line represents the CD25 staining. One representative out of three independent experiments is shown (*p < 0.0001; **p < 0.01; ***p < 0.05, as compared with $CD4^+CD25^-CFSE^+$).

Proliferated T_{reg} Cells Have Partial Suppressive Capacity that Attains Full Activity when They Enter the Resting Phase

The suppressive capability of T_{reg} cells in the presence of leptin mAb was tested in coculture experiments of T_{reg} cells together with $CD4^+CD25^-$ T cells. Addition of exogenous leptin did not affect suppression of T_{reg} cells on $CD4^+CD25^-$ T cells (Figure 2A). In contrast, suppression of proliferation was apparently abrogated in the presence of leptin mAb (Figure 2A, gray columns). Concomitant addition of leptin together with the leptin mAb partly reduced the anti-leptin-induced reversal of suppression, confirming specificity for leptin neutralization in the coculture experiments (Figure 2A, gray columns).

To understand whether T_{reg} cells during anti-leptin-induced proliferation could exert suppressive capacity, we performed coculture experiments with CFSE-labeled $CD4^+CD25^-$ T cells in the presence of unlabeled T_{reg} cells (Figures 2B–2E). As expected, T_{reg} cells suppressed the expansion of $CD4^+CD25^-$ effectors (Figure 2D), and suppression was partially maintained in the presence of leptin mAb (Figure 2E)—albeit at a lower degree when compared with untreated T_{reg} cells (Figure 2D). This suggested that T_{reg} cells during anti-leptin-induced proliferation are partially functional in terms of suppressive capability. Moreover, the coculture experiments of T_{reg} cells with $CD4^+CD25^-$ T cells, in the presence of leptin mAb, indicated that the reversal of suppression (Figure 2A, gray columns) was apparent only because $CD4^+CD25^-$ effectors were inhibited in part in their proliferation, as indicated by the CFSE dilution (Figure 2E). Thus, the high amount of [^{3}H]thymidine incorporation in coculture of T_{reg} cells with $CD4^+CD25^-$ T cells in the presence of leptin mAb (Figures 1H and 2A) has to be ascribed to both T_{reg} cell proliferation and partly to $CD4^+CD25^-$ T cells. These phenomena were confirmed in terms of expression of the activation marker CD25 on CFSE-labeled $CD4^+CD25^-$ effector T cells. The anti-CD3 and anti-CD28 activation induced a substantial upregulation of CD25 on the cell surface (Figure 2F) and was significantly inhibited in the presence of T_{reg} cells (Figure 2H). A significant reduction of CD25 expression on effector T cells was observed in the presence of anti-leptin-expanding T_{reg} cells (even if at lower extent when compared with untreated T_{reg} cells) (Figure 2I). In any case, the suppressive capacity of anti-leptin-expanding T_{reg} cells was diminished by about 40%, and a certain number of $CD4^+CD25^-$ T cells was still able to proliferate and divide (Figure 2E).

Next, we performed two-step experiments to address whether anti-leptin-proliferated T_{reg} cells in the resting phase could retain their suppressive capacity over time. 8 days after the first expansion, stimulated T_{reg} cells were still suppressive for $CD4^+CD25^-$ effector T cells in in vitro coculture experiments (Figures S2A–S2C). Although effector $CD4^+CD25^-$ T cells cultured in the presence of leptin mAb proliferated upon restimulation and did not suppress allogeneic $CD4^+CD25^-$ T cells expansion in coculture experiments (Figure S2B), the anti-leptin-expanded T_{reg} cells maintained their hyporesponsiveness after restimulation and were capable of suppressing proliferation of allogeneic $CD4^+CD25^-$ effector T cells (Figure S2C). These data suggested that the anti-leptin-proliferated T_{reg} cells, once in the resting phase, maintain their suppressive capacity over time.

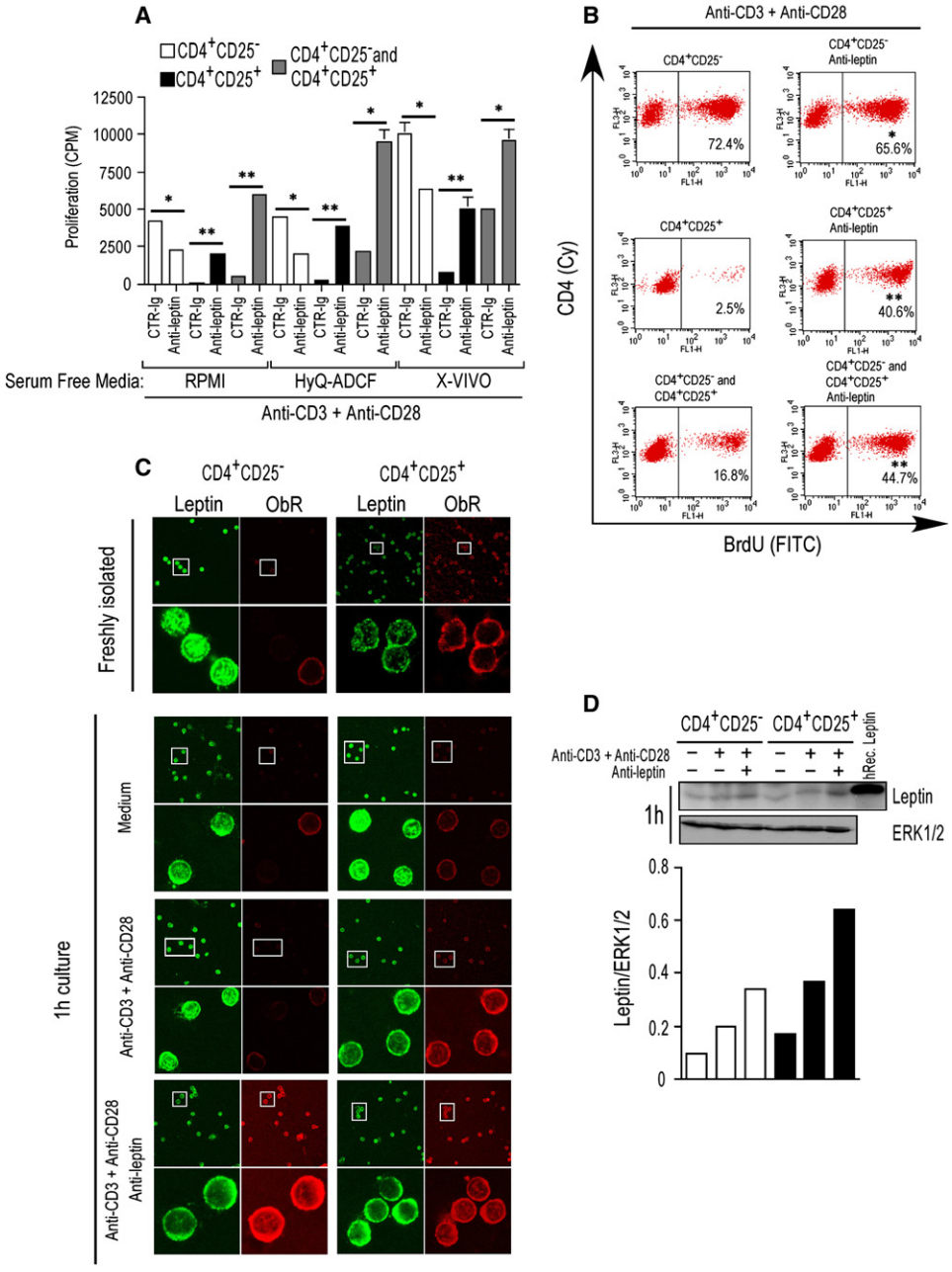

Figure 3. T_{reg} Cells Produce Leptin and Express High Amounts of ObR

(A) Proliferation of $CD4^+CD25^-$ effector T cells, T_{reg} cells, and of both cell types in coculture in three different serum- and leptin-free media in the presence or absence of leptin mAb. The data are shown as mean ± SD (n = 6, *p < 0.01; **p < 0.001).

(B) Flow cytometry plot of BrdU incorporation of $CD4^+CD25^-$ effector T cells, T_{reg} cells, and of both cell types in coculture in serum-free medium (X-VIVO), in the presence or absence of leptin mAb during anti-CD3 and anti-CD28 stimulation. One representative out of three independent experiments is shown (*p < 0.05; **p < 0.001).

(C) Confocal microscopy of freshly isolated and 1 hr-stimulated T_{reg} cells and $CD4^+CD25^-$ effectors stained for leptin (in green) and leptin receptor (ObR) (in red). One representative out of three independent experiments is shown.

(D) Immunoblot for leptin on cell lysates from T_{reg} cells and $CD4^+CD25^-$ effectors. The graph shows quantitation of leptin with respect to total ERK1/2. One representative out of three independent experiments is shown.

Leptin Production from Human T_{reg} Cells

Leptin is present in media supplemented with human serum, such as the medium used in our experiments (see Experimental Procedures). To test whether human T_{reg} cells could expand in the absence of leptin, we stimulated T_{reg} cells with anti-CD3 and anti-CD28 in three different types of serum- and leptin-free media (Figure 3A). Under these conditions, T_{reg} cells maintained hyporesponsiveness

even in the absence of exogenous leptin. Surprisingly, addition of leptin mAb to the cultures resulted in T_{reg} cell proliferation (Figure 3A). This finding was also confirmed by BrdU incorporation in serum-free medium cultures (Figure 3B). This finding suggests the possibility that leptin may be produced by T_{reg} cells in a fashion similar to the production of leptin by $CD4^+$ T cells and monocytes in multiple sclerosis (Sanna et al., 2003; Matarese et al., 2005). To test this possibility, we examined leptin and ObR expression on T_{reg} cells and $CD4^+CD25^-$ T cells. Both freshly isolated T_{reg} cells and $CD4^+CD25^-$ T cells stained positive for leptin and ObR, with different intensity patterns (Figure 3C). Moreover, after 1 hr culture, T_{reg} cells showed higher leptin production than did $CD4^+CD25^-$ T cells, and this tendency was maintained during anti-CD3 and anti-CD28 stimulation, both in the presence and in the absence of leptin mAb (Figure 3C). Parallel quantitation by immunoblotting analysis for leptin on cell lysates confirmed the difference (Figure 3D). Indeed, the presence of a basal production of leptin increased significantly after anti-CD3 and anti-CD28 stimulation (Figure 3D), and T_{reg} cells always produced more leptin than did $CD4^+CD25^-$ T cells (Figure 3D). Interestingly, leptin neutralization induced a compensatory leptin production and ObR upregulation in both T_{reg} cells and $CD4^+CD25^-$ T cells, and again, to a higher amount in the T_{reg} cells (Figures 3C and 3D). These results were confirmed at 12 hr by confocal microscopy and immunoblotting studies (data not shown). Additionally, to address the capacity of T_{reg} cells and $CD4^+CD25^-$ T cells to secrete leptin, we performed a human leptin-specific ELISA (see Experimental Procedures) and confocal microscope analysis at different time points (12 hr and 36 hr) on culture supernatants (Figure S3). At 12 hr, the secretion of leptin was similar in both T_{reg} cells and $CD4^+CD25^-$ T cells, whereas at 36 hr the amount of leptin secreted was significantly higher in the T_{reg} cells, either unstimulated or treated with anti-CD3 and anti-CD28 plus leptin mAb (Figure S3). The finding was confirmed by the observation of a reduced content of leptin in T_{reg} cells at 36 hr (Figure S3) in confocal microscopy and immunoblotting studies on cell lysates (data not shown). Finally, these results on leptin secretion were also confirmed by immunoblotting for leptin on culture supernatants (data not shown). Thus, these results suggest the presence of an autocrine loop of leptin secretion by T_{reg} cells controlling their hyporesponsiveness.

Leptin-mAb-Induced Proliferation of T_{reg} Cells Is IL-2 Dependent, and IL-2-Supported Expansion of T_{reg} Cells Is Not Affected by Leptin

To test whether the leptin-mAb-induced proliferation of T_{reg} cells was IL-2 dependent, we evaluated the effects of IL-2 neutralization on the T_{reg} cell proliferative responses and IL-2 production. Addition of human IL-2-neutralizing mAb enhanced the inhibitory effects of leptin mAb treatment on the $CD4^+CD25^-$ T cell proliferation and IL-2 secretion (Figures 4A and 4B, white bars). IL-2 mAb reduced both T_{reg} cell proliferation and IL-2 production induced by the neutralization of leptin (Figures 4A and 4B, black bars). Moreover, the apparent reversal of suppression in coculture experiments resulting from anti-leptin-induced T_{reg} cells proliferation was inhibited by anti-IL-2 (Figures 4A and 4B, gray bars). IL-2 secretion was evaluated via CTLL-2 proliferation (Figure 4B). These results were also confirmed by intracellular staining for IL-2 in FACS analysis and ELISA (Figure S4).

Next we compared IL-2-induced proliferation versus anti-leptin-induced proliferation on T_{reg} cells. Addition of exogenous IL-2 reversed T_{reg} cells unresponsiveness upon anti-CD3 and anti-CD28 stimulation (Figure 4C, left), as expected. Of interest, IL-2-induced proliferation was less than that observed upon leptin blockade (Figure 4C, left). To also address whether anti-leptin treatment affected the ability of T_{reg} cells to proliferate in the presence of IL-2 but in the absence of TCR stimulation, we measured the proliferation of unstimulated T_{reg} cells cultured with leptin mAb or IL-2 and anti-leptin plus IL-2. No proliferation was observed in the absence of TCR engagement in all the above conditions (Figure 4C, left). Finally, we tested whether leptin could inhibit IL-2-induced expansion of T_{reg} cells. The anti-proliferative effect of increasing doses of recombinant leptin on T_{reg} cell proliferation induced by IL-2 was not significant (Figure 4C, right), suggesting that IL-2 signaling may be independent and dominant of the leptin-mediated inhibitory effects on the cell proliferation.

T_{reg} Cells Expanded with Leptin mAb Have Increased Expression of Foxp3

We next addressed whether leptin neutralization could affect the amount of Foxp3 in T_{reg} cells. Foxp3 expression was evaluated after leptin neutralization at 1 hr and 12 hr during anti-CD3 and anti-CD28 stimulation. As shown in Figure 4D, Foxp3 expression was increased at early time points after anti-CD3 and anti-CD28 stimulation, and leptin mAb treatment did not alter Foxp3 protein amounts. Conversely, at 12 hr, T_{reg} cells that proliferated after leptin mAb showed increased Foxp3 amounts when compared to those activated with anti-CD3 and anti-CD28 alone (Figure 4E). As expected, Foxp3 was undetectable in $CD4^+CD25^-$ T effectors after 1 hr of stimulation in all the experimental conditions (Figure 4D), whereas at 12 hr, there was a little expression of Foxp3 after anti-CD3 and anti-CD28 stimulation either in the presence or in the absence of leptin mAb that did not reach statistical significance (Figure 4E). All these data were confirmed by real-time PCR (data not shown).

The Effect of Leptin Neutralization on ObR and STAT3 Activation Pathway

The molecular effects of leptin mAb were studied at early (1 hr) or late (12 hr) time points on highly purified human T_{reg} cells or effector $CD4^+CD25^-$ T cells, activated or not with anti-CD3 anti-CD28.

We first analyzed the leptin-ObR-STAT3 biochemical pathway: 1 hr and 12 hr cultures confirmed that in unstimulated cells, the ObR was expressed at higher amounts in T_{reg} cells than in $CD4^+CD25^-$ T cells (Figures 3C, 5A,

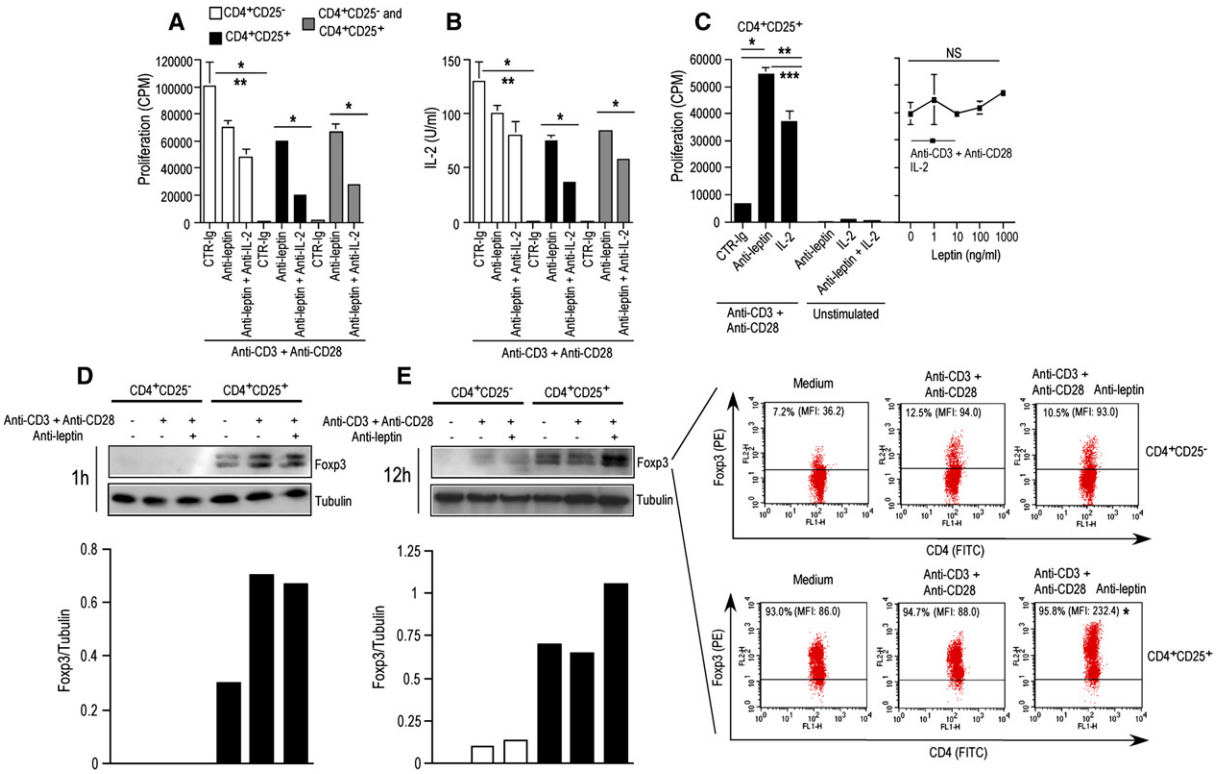

Figure 4. Leptin-mAb-Induced Proliferation of Human T_{reg} Cells Is IL-2 Dependent, and Foxp3 Expression Is Increased during Proliferation

(A) Proliferation of $CD4^+CD25^-$ effector T cells, T_{reg} cells, and of both cell types in coculture in the presence or absence of leptin mAb and IL-2-neutralizing mAb. The data are shown as mean ± SD (n = 5, *p < 0.01, **p < 0.05, white bars; *p < 0.001, black bars; *p < 0.01, gray bars).

(B) IL-2 secretion of $CD4^+CD25^-$ effector T cells, T_{reg} cells, and of both cell types in coculture in the presence or absence of leptin mAb and IL-2-neutralizing mAb. The data are shown as mean ± SD (n = 5, *p < 0.01 and **p < 0.05, white bars; *p < 0.001, black bars; *p < 0.01, gray columns).

(C) Proliferation of T_{reg} cells in the presence of either leptin mAb or recombinant IL-2 (left), stimulated or not with anti-CD3 and anti-CD28. The data are shown as mean ± SD (n = 5, *p < 0.0001; **p < 0.01; ***p < 0.05). Addition of scalar doses of recombinant leptin to proliferating T_{reg} cells (right) stimulated with anti-CD3 and anti-CD28 in the presence of recombinant IL-2. The data are shown as mean ± SD (n = 5, NS, not significant).

(D) Immunoblot analysis of $CD4^+CD25^-$ effector T cells and T_{reg} cells in the presence or absence of leptin mAb, at 1 hr stimulation with anti-CD3 and anti-CD28. The graph shows quantitation of Foxp3 with respect to tubulin. One representative out of five independent experiments is shown.

(E) Immunoblot analysis and flow cytometry plot of $CD4^+CD25^-$ effector T cells and T_{reg} cells in the presence or absence of leptin mAb, at 12 hr stimulation with anti-CD3 and anti-CD28. The graph shows quantitation of Foxp3 with respect to tubulin. One representative out of five independent experiments is shown (*p < 0.01, as compared with anti-CD3 and anti-CD28).

and 5B). In vitro stimulation with anti-CD3 and anti-CD28 induced an upregulation of the ObR expression on both cell types and was more evident at 1 hr than at 12 hr (Figures 3C, 5A, and 5B). Leptin neutralization on both T_{reg} cells and $CD4^+CD25^-$ T cells further upregulated the expression of ObR, particularly at 1 hr, suggesting that leptin blockade might induce a compensatory upregulation of its receptor.

As readout of ObR activity, we measured phospho-STAT3 (P-STAT3) amounts, because STAT3 is known to participate in the intracellular signaling pathways of ObR (reviewed in La Cava and Matarese, 2004). Activation of $CD4^+CD25^-$ T cells induced strong STAT3 phosphorylation at 1 hr and 12 hr and anti-leptin treatment reduced the P-STAT3 amounts, particularly at 1 hr (Figures 5A and 5B). Conversely, stimulation of T_{reg} cells was not associated with a marked increase of P-STAT3, whereas anti-leptin induced amounts of P-STAT3 at 1 hr and 12 hr comparable to those observed in proliferating $CD4^+CD25^-$ T cells (Figures 5A and 5B). Of note, at 1 hr, $CD4^+CD25^-$ T cells expressed both STAT3 spliced isoforms (Maritano et al., 2004)—STAT3α and STAT3β—whereas T_{reg} cells mainly expressed the STAT3α isoform. This phenomenon was not as evident after 12 hr when the two spliced isoforms were similar in both cell subsets, suggesting that the tissue-culture conditions could induce STAT3β in T_{reg} cells, independently of the different type of stimulation. Finally, in unstimulated or anti-CD3- and anti-CD28-stimulated T_{reg} cells, at both 1 hr and 12 hr, the amounts of P-STAT3 were higher than those observed in unstimulated $CD4^+CD25^-$ cells, suggesting an increased "basal" leptin-mediated signaling in T_{reg} cells. This result could be secondary to higher basal expression of ObR on T_{reg} cells (Figures 1C, 3C, 5A, and 5B). No induction of P-STAT3 was observed in T_{reg} cells treated with leptin mAb alone without TCR stimulation (Figure S5).

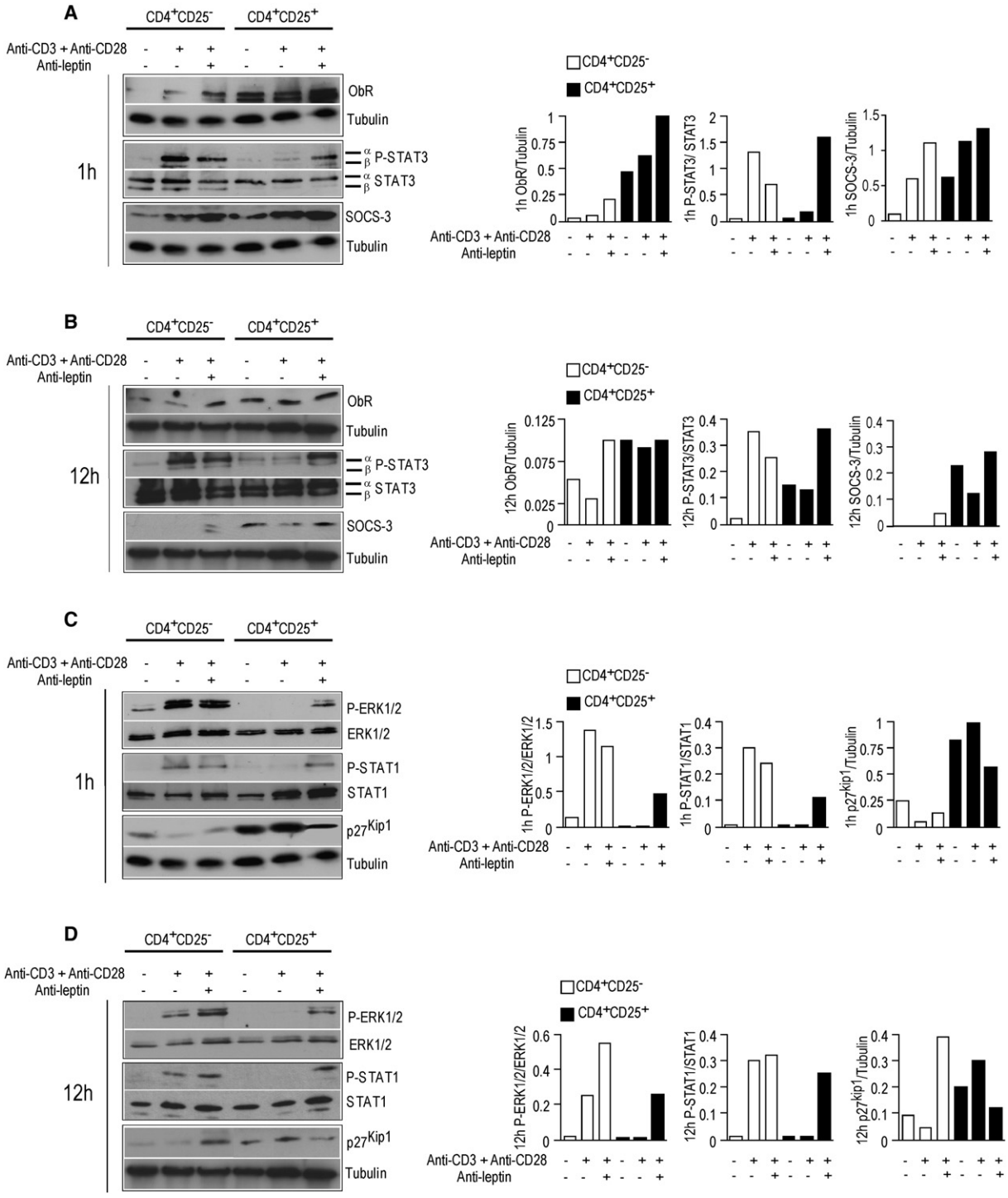

Figure 5. Molecular Effects of Leptin Neutralization on Human T_{reg} Cells

(A and B) Immunoblot for ObR, STAT3, and SOCS3 on $CD4^+CD25^-$ T cells and T_{reg} cells in the presence or absence of leptin mAb stimulated with anti-CD3 and anti-CD28 at 1 hr and 12 hr, respectively. Graphs show quantitation of each specific protein. One representative out of five independent experiments is shown.

(C and D) Immunoblot for ERK1/2, STAT1, and $p27^{kip1}$. Graphs show quantitation of each specific protein. One representative out of five independent experiments is shown.

We also investigated in our system the expression of suppressor of cytokine signaling 3 (SOCS3), a key negative regulator of cytokine signaling, including leptin (Kinjyo et al., 2006; La Cava and Matarese, 2004). SOCS3 in anti-CD3- and anti-CD28-stimulated $CD4^+CD25^-$ T cells increased markedly and was further increased by leptin neutralization at 1 hr (Figure 5A). This finding inversely correlated with the corresponding P-STAT3 expression. At a later time point (12 hr), SOCS3 was undetectable and could be found at low amounts upon leptin neutralization (Figure 5B). At 1 hr, SOCS3 in T_{reg} cells was markedly higher than in $CD4^+CD25^-$ cells in all the experimental conditions. Specifically, SOCS3 expression was high in unstimulated T_{reg} cells and increased after anti-CD3 and anti-CD28 stimulation despite little STAT3 activation. Treatment with anti-leptin, which induced P-STAT3, did not alter the SOCS3 amounts (Figure 5A). At 12 hr, basal SOCS3 expression was higher in the T_{reg} cells than in $CD4^+CD25^-$ cells and was markedly reduced after anti-CD3 and anti-CD28 stimulation. In conclusion, leptin mAb treatment—which associates with STAT3 activation in T_{reg} cells—also induced SOCS3 expression (Figure 5B).

The Effect of Leptin Neutralization on Biochemical Pathways Involved in T Cell Activation and Anergy

To evaluate whether leptin neutralization could affect T cell activation and anergy, we studied tyrosine-phosphorylation of the extracellular signal-regulated kinases 1 and ERK2 (ERK1/2). Leptin neutralization in the presence of anti-CD3 and anti-CD28 in $CD4^+CD25^-$ T cells increased ERK1/2 phosphorylation as compared to anti-CD3 and anti-CD28 stimulation alone, particularly at 12 hr (Figures 5C and 5D). Treatment of T_{reg} cells with anti-CD3 and anti-CD28 did not induce phospho-ERK1/2 (P-ERK1/2), confirming their anergic state. However, leptin mAb induced high amounts of ERK1/2 tyrosine-phosphorylation at both 1 hr and 12 hr, concomitantly with the induction of T_{reg} cell proliferation and a reversal of their hyporesponsiveness (Figures 5C and 5D).

Moreover, we investigated whether leptin neutralization could alter STAT1, another transcription factor whose activity is required for the development and function of T_{reg} cells (Nishibori et al., 2004). We found that leptin neutralization did not affect STAT1 phosphorylation (P-STAT1) in $CD4^+CD25^-$ effectors at both time points. In contrast, P-STAT1 was markedly induced in the leptin-neutralized T_{reg} cells (Figures 5C and 5D).

Last, we studied the modulation of the cyclin-dependent kinase inhibitor p27 ($p27^{kip1}$), a molecule involved in the control of cell cycle, T cell anergy and known to block the cell-cycle progression in T_{reg} cells (Li et al., 2005; Wells et al., 2001). $p27^{kip1}$ was downmodulated by anti-CD3 and anti-CD28 stimulation in $CD4^+CD25^-$ effectors whereas anti-leptin did not downmodulate $p27^{kip1}$ expression at 1 hr and markedly increased its expression at 12 hr (Figures 5C and 5D), explaining, at least in part, the inhibition of $CD4^+CD25^-$ T cells proliferation induced by leptin neutralization. In contrast, T_{reg} cells showed elevated amounts of $p27^{kip1}$ before and after anti-CD3 and anti-CD28 stimulation, confirming their anergic state associated with cell-cycle arrest. Leptin mAb treatment induced degradation of this molecule at both 1 hr and 12 hr, which could explain reversal of their anergic state and subsequent proliferation (Figures 5C and 5D).

Leptin Deficiency Promotes Proliferation of T_{reg} Cells in Mice

T_{reg} cells, despite their in vitro hyporesponsiveness, can expand in vivo in normal, nonlymphopenic hosts (Trenado et al., 2003; Gavin et al., 2002). We used nonirradiated, nonlymphopenic recipient mice to avoid a homeostatic expansion of the T_{reg} cells that would occur in lymphopenic hosts (Trenado et al., 2003; Gavin et al., 2002). The in vivo proliferative capacity of T_{reg} cells in anti-leptin-treated wild-type (WT) mice versus control-Ig-treated mice was tested with CFSE-labeled-$CD4^+$ T cells from normal WT mice and by measuring the CFSE dilution in the $CD4^+Foxp3^+$ cells (Figure 6A). Mouse T_{reg} cells expanded in vivo more robustly and earlier (day 4 and day 7 after transfer) when adoptively transferred into leptin-neutralized WT mice (Figure 6A). This result was confirmed by adoptive transfer of CFSE-labeled-$CD4^+$ T cells from normal WT mice into leptin-deficient *ob/ob* mice, in which the T_{reg} cells expanded more robustly and earlier (day 4 and day 7 after transfer) when compared with cells transferred into normal WT mice (Figure 6B). We also confirmed our data in a transgenic antigen-specific system utilizing donor mice carrying the (TcrAND)53Hed transgene (AND-TCR Tg mice) expressing the clonotypic $V\alpha11.1/V\beta3$ TCR chains, specific for the carboxy-terminal fragment of pigeon cytochrome c (PCC) (Kaye et al., 1989). Assessment 4 and 7 days after adoptive transfer revealed that transferred CFSE-labeled clonotypic AND-TCR T_{reg} cells proliferated more vigorously in *ob/ob* mice as compared with normal WT controls (Figure 6C). This phenomenon was reversed by recombinant leptin administration to leptin-deficient *ob/ob* mice (Figure 6C). To avoid interference of $CD4^+$ T cell lymphopenia on the T_{reg} cell expansion in vivo, in the adoptive transfer experiments in *ob/ob* mice, we utilized 6-week-old mice (in which the leptin deficiency has not yet determined significant reduction of the $CD4^+$ T cell number). The in vivo results were also confirmed by CFSE dilution experiments gating on the $CD4^+CD25^+$ T_{reg} cells in both leptin-neutralized WT and leptin-deficient *ob/ob* mice, respectively (Figures S6A and S6C, respectively). Foxp3 expression was increased in the T_{reg} cells of the leptin-neutralized WT mice (Figure S6B). Finally, to confirm our data, we tested the ability of T_{reg} cells to expand in the presence of an anti-leptin receptor (anti-ObR)-blocking antibody. The data showed a marked increase in the percentage of $CD4^+Foxp3^+$ T_{reg} cells in treated WT mice (data not shown).

Proliferative Potential and Functional Capacity of T_{reg} Cells from Leptin Receptor-Deficient Mice

We studied the in vitro proliferation and suppressive capacity of $CD4^+CD25^-$ effectors and T_{reg} cells from congenitally leptin-receptor-deficient *db/db* mice and normal

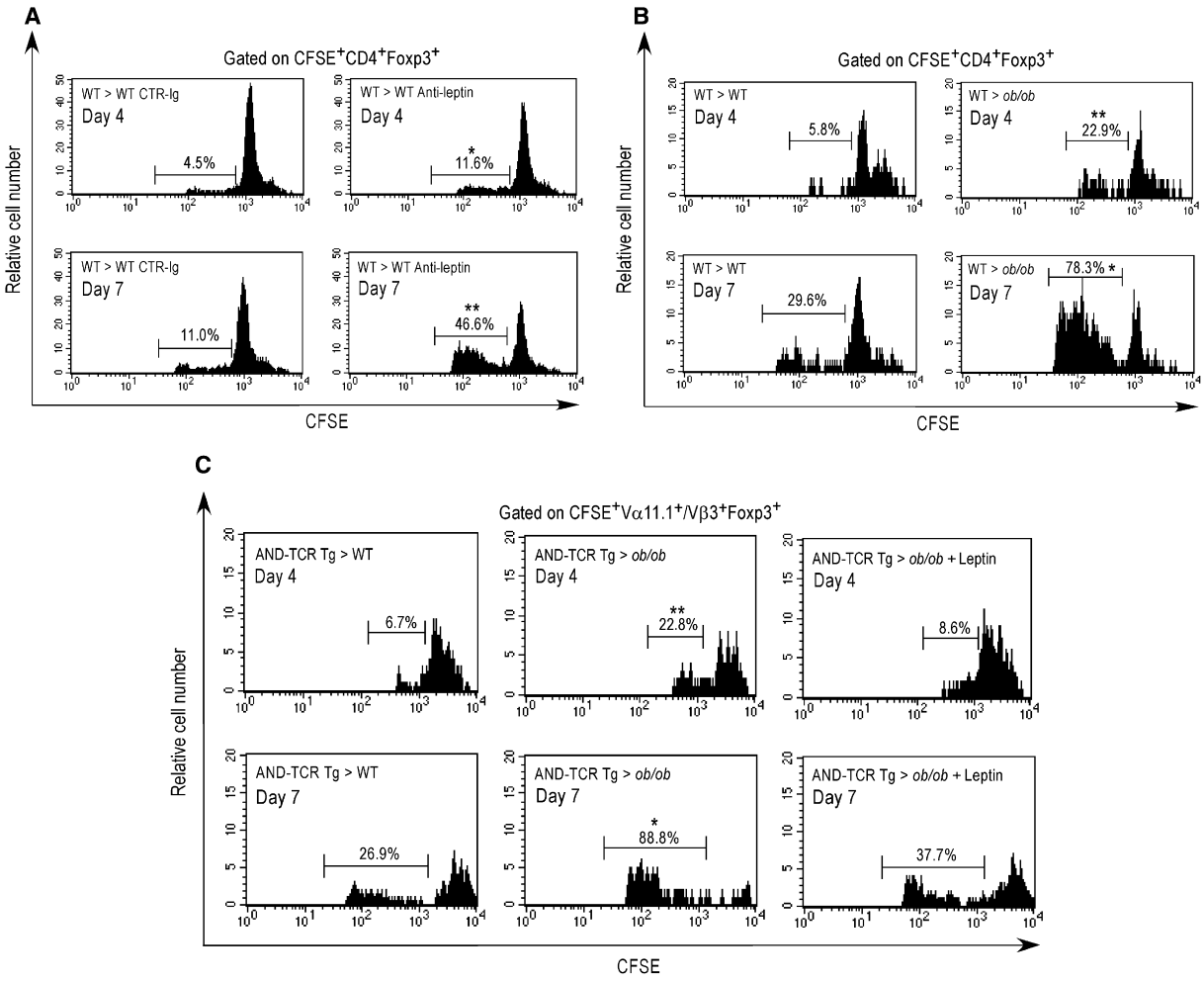

Figure 6. In Vivo Leptin Neutralization or Congenital Leptin Deficiency Associate with Proliferation of T_{reg} Cells

(A) Proliferation measured as CFSE dilution of CFSE-labeled T_{reg} cells obtained from WT mice and transferred into control (CTR)-Ig or mouse leptin neutralizing Ab-treated WT mice. The histogram shows the percent of $CFSE^+$ T_{reg} cells (gated on $CD4^+Foxp3^+$ cells) that had divided 4 and 7 days after transfer. One representative out of three independent experiments with 3 mice per group is shown (*$p < 0.01$; **$p < 0.001$).

(B) CFSE dilution profile of CFSE-labeled T_{reg} cells (gated on $CD4^+Foxp3^+$ cells) obtained from WT mice and transferred into WT or leptin-deficient *ob/ob* mice, 4 and 7 days after transfer. One representative out of three independent experiments with 3 mice per group is shown (*$p < 0.01$; **$p < 0.001$).

(C) CFSE dilution profile of PCC-specific CFSE-labeled AND-TCR T_{reg} cells (gated on $V\alpha11.1^+/V\beta3^+Foxp3^+$ cells) adoptively transferred into WT, *ob/ob*, and *ob/ob* treated with recombinant leptin, 4 and 7 days after transfer. One representative out of two independent experiments with 4 mice per group is shown (**$p < 0.001$; *$p < 0.01$).

db/+ heterozygous controls (Figures 7A–7C). Stimulation with anti-CD3 and anti-CD28 of $CD4^+CD25^-$ effector T cells from *db/db* mice was less effective in inducing proliferation than stimulation on $CD4^+CD25^-$ from *db*/+ mice (Figure 7A). Conversely, in vitro stimulation of T_{reg} cells from *db/db* mice induced significantly higher proliferation than that of T_{reg} cells from *db*/+ heterozygous controls (Figure 7B). These data suggested that the absence of the ObR impaired the expansion of $CD4^+CD25^-$ effectors and enhanced the proliferative potential of T_{reg} cells. The ObR deficiency seemed to affect the proliferative potential of the T_{reg} cells rather than their qualitative or functional activity, because T_{reg} cells from *db/db* mice suppressed the proliferation of $CD4^+CD25^-$ T cells in a fashion similar to that of T_{reg} cells from *db*/+ control mice (Figure 7C), confirming the finding that addition of exogenous leptin did not alter the suppressive capacity of T_{reg} cells in vitro (Figure 2A).

Subsequent studies were performed analyzing antigen-specific responses in an autoimmune disease model: the in vitro proliferative capacity of T_{reg} cells against the pancreatic autoantigen glutamic acid decarboxylase (GAD)65 was evaluated in autoimmune diabetes nonobese diabetic (NOD/LtJ) mice versus leptin-receptor mutant NOD-$Lepr^{db5J}$/LtJ mice (Figure 7D; Lee et al., 2005). These recently isolated ObR mutant mice display obesity, hyperphagia, and resistance to the development of pancreatic β-islets infiltration (Lee et al., 2005). We found that after 5 days of splenocyte cultures, T_{reg} cells from NOD-$Lepr^{db5J}$/LtJ proliferated more robustly against mouse

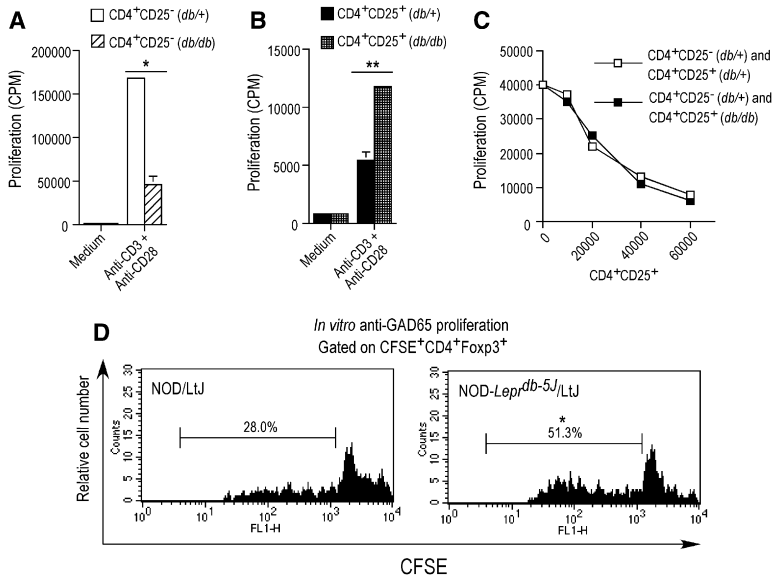

Figure 7. ObR Deficiency Increases T_{reg} Cells Proliferative Potential, Does Not Alter Their Suppressive Capacity, and Impairs $CD4^+CD25^-$ Proliferation

(A) Proliferation of $CD4^+CD25^-$ effector T cells from *db*/+ and leptin receptor-deficient *db*/*db* mice stimulated with anti-CD3 and anti-CD28. The data are shown as mean ± SD (n = 5, *p < 0.001).

(B) Proliferation of T_{reg} cells from *db*/+ and *db*/*db* mice stimulated with anti-CD3 and anti-CD28. The data are shown as mean ± SD (n= 5, **p < 0.01).

(C) Proliferation of $CD4^+CD25^-$ effector T cells from *db*/+ mice in the presence of increasing number of either *db*/+ or *db*/*db* T_{reg} cells, stimulated with anti-CD3 and anti-CD28. The data are shown as mean ± SD (n = 5).

(D) CFSE dilution of T_{reg} cells (gated on $CD4^+Foxp3^+$ cells) from leptin receptor mutant NOD-*Lepr*$^{db-5J}$/LtJ mice stimulated with mouse recombinant GAD65, after 5 days culture. One representative out of five independent experiments is shown (*p > 0.01).

GAD65 than T_{reg} cells from NOD/LtJ females, suggesting an increased autoantigen-specific proliferative potential of the T_{reg} cells from mice with impaired ObR signaling (Figure 7D). In contrast, $Foxp3^-$ T cells from NOD-*Lepr*^{db5J}/LtJ proliferated significantly less than that of NOD/LtJ controls (data not shown), suggesting that an absence of ObR impaired the expansion of $Foxp3^-$ effectors and enhanced the proliferative potential of T_{reg} cells.

Leptin-Deficient Mice Have Increased Numbers of T_{reg} Cells that Can Be Reduced by Administration of Leptin

Mice with genetic deficiency of leptin (*ob*/*ob*) or leptin-receptor (*db*/*db*) have reduced susceptibility to autoimmunity (Matarese et al., 2001; Sanna et al., 2003; Siegmund et al., 2004; Faggioni et al., 2000; Lee et al., 2005). T_{reg} cells play a central role in regulating autoimmunity (Shevach, 2002; Sakaguchi, 2005), so we tested whether genetic deficiency of leptin associated with effects on T_{reg} cells. A significant increase of the percentage of peripheral T_{reg} cells was observed in *ob*/*ob* as compared to WT (Figures S7A and S7B, respectively). Administration of leptin reduced the elevated number of T_{reg} cells in the *ob*/*ob* mice to a number comparable to that found in the WT mice (Figures S7A and S7B).

DISCUSSION

In this study we establish a unique link between T_{reg} cells and leptin by showing that leptin can modulate the hyporesponsiveness and proliferation of T_{reg} cells both in vitro and in vivo. Freshly isolated human T_{reg} cells express high amounts of ObR and produce substantial amounts of leptin that are responsible for an autocrine inhibitory loop that constrains the expansion of T_{reg} cells. Although leptin neutralization inhibits the proliferation of effector $CD4^+CD25^-$ T cells, this condition leads to an expansion of the T_{reg} cells, which maintain their suppressive phenotype in the resting phase. The opposite effects of leptin blockade on $CD4^+CD25^-$ T cells and T_{reg} cells associate with a differential expression of intracellular leptin and cell-surface ObR in the two cell subsets.

It must be noted that in our experimental system, the culture media supplemented with human serum contained low—albeit significant—concentrations of human leptin (0.5–1 ng/ml in the 5% human serum [HS]/95% RPMI). The contribution of HS-derived leptin versus T_{reg} cell-derived leptin was analyzed in cultures with three different serum-free media—all tested for the absence of leptin. The anti-leptin-induced expansion of the T_{reg} cells in serum-free conditions indicated that the T_{reg} cell-derived leptin was sufficient to act as negative signal for the expansion of the T_{reg} cells. It is possible to speculate that a leptin-mediated negative autocrine loop may operate on T_{reg} cells, because both freshly isolated and cultured T_{reg} cells stain positive for and secrete leptin. On the other hand, the leptin present in the culture medium and secreted by the $CD4^+CD25^-$ T cells could act as a positive signal for T cell proliferation. These differential effects induced by leptin could rely upon the different ObR levels and leptin secretory capacity, which would ultimately affect the intracellular signaling differentially.

Our T_{reg} cells were cultured in medium supplemented with 5% HS because fetal calf serum (FCS) is rich in bovine leptin (10–20 ng/ml in RPMI 10% FCS) that is not neutralized by human leptin mAb (data not shown). We also found that 10% FCS abrogated the effects induced by human leptin mAb on human T_{reg} cells (data not shown). Therefore, it was crucial, for efficient leptin neutralization, to avoid exposure to FCS for the ObR-expressing T_{reg} cells. The same issue has to be taken into account had the T_{reg} cells been isolated by positive selection and thus undergone repeated steps of FCS exposure compromising leptin neutralization. Moreover, we also noted that

T_{reg} cells isolated by positive selection with anti-CD25 mAb (either by FACS or magnetic bead cell sorting) had a reduced capacity to proliferate in vitro after leptin neutralization when the anti-CD25 mAb was not removed from the cell surface after isolation. The reasons for an interference of the anti-CD25 mAb with leptin neutralization process and T_{reg} cell expansion are currently being investigated and might include an interference with IL-2 signaling, a functional inactivation of the T_{reg} cells by the anti-CD25 mAb recently demonstrated (Kohm et al., 2006), and an influence on the kinetics and the degree of leptin secretion by the T_{reg} cells. In any case, detachment of the anti-CD25 mAb after cell isolation completely eliminates possible problems of proliferation for the anti-leptin-treated stimulated T_{reg} cells (see Experimental Procedures for details).

Previous experimental evidence suggests that the growth, homeostasis, and function of the T_{reg} cells is dependent on IL-2 in vitro and in vivo (Setoguchi et al., 2005; Fontenot et al., 2005). Our data also indicated that leptin neutralization could induce IL-2 secretion by T_{reg} cells and that leptin mAb-induced-proliferation was IL-2 dependent. When comparing the efficiency of leptin neutralization and IL-2 to expand T_{reg} cells in vitro, we observed that leptin mAb had a better efficiency than recombinant IL-2 (as indicated by T_{reg} cell proliferation and number after 8 days culture). Conversely, addition of increasing doses of recombinant leptin to the cell cultures did not affect the IL-2-mediated proliferation of the T_{reg} cells, suggesting that the control of leptin on the expansion of the T_{reg} cells was independent and not overcome by IL-2 signaling. McHugh et al. (2002) demonstrated that cultures of T_{reg} cells with neutralizing glucocorticoid-induced TNF receptor (GITR) mAb allowed the T_{reg} cells to respond to exogenous IL-2 in the absence of TCR stimulation. We also tried to induce T_{reg} cell proliferation in the absence of TCR stimulation by adding high concentration of IL-2 and anti-leptin, but we found lack of proliferation, indicating that anti-leptin-induced proliferation of the T_{reg} cells needs concomitant TCR engagement. This evidence was also confirmed at biochemical level; anti-leptin alone in the absence of TCR stimulation did not induce significant P-STAT3 levels in T_{reg} cells.

Foxp3—the master gene for T_{reg} cell development and function (Sakaguchi, 2005)—was induced in leptin-neutralized T_{reg} cells at late time points (12 hr), supporting the evidence that removal of leptin from culture medium not only expanded the T_{reg} cells but also allowed the maintenance of their phenotype. The phenomenon might possibly be ascribed to the increased IL-2 secretion induced by leptin neutralization, because others have shown that IL-2 is capable of upregulating Foxp3 expression in human T_{reg} cells (Zorn et al., 2006).

At the molecular level, T_{reg} cells expressed high levels of ObR and of $p27^{kip1}$ but no phosphorylation of ERK1/2 or STAT1 and little phosphorylation of STAT3. In contrast, leptin neutralization upon anti-CD3 and anti-CD28 stimulation and T_{reg} cell expansion was associated with a rapid degradation of $p27^{kip1}$ as well as a marked phosphorylation of ERK1/2, STAT1, and STAT3 (phosphorylation of STAT3 in T_{reg} cells could be partly explained by both the induction of the ERK1/2, which is known to phosphorylate STAT3 independently of ObR [Quadros et al., 2004; Barboza et al., 2004], as well as by the secretion of STAT3-activating cytokines, i.e., IL-2 and IL-6 [Doganci et al., 2005]). Recently, Zorn and collegues (Zorn et al., 2006) showed that IL-2 upregulates Foxp3 expression in human T_{reg} cells through a STAT3-dependent mechanism, confirming that induction of Foxp3 (in our case, during leptin neutralization) may occur via a STAT3-dependent mechanism. Intriguingly, $CD4^+CD25^-$ T cells, after leptin neutralization, showed an increase of $p27^{kip1}$ associated with sustained ERK1/2 phosphorilation—a phenotype often observed in anergic T cells (Wells et al., 2001; Chen et al., 1999; Waiczies et al., 2005) and in effector T cells from mice treated with anti-leptin antibodies during autoimmune encephalomyelitis (De Rosa et al., 2006). The fact that leptin neutralization reduced the levels of STAT3 phosphorylation and had little effect on STAT1 needs an explanation. This partial effect on the ObR-STAT3-mediated signaling could be ascribed to the fact that also other cytokines activate STAT3 (Doganci et al., 2005). As such, removal of leptin could only inhibit in part STAT3 phosphorylation. In this context, it is interesting to note that SOCS3, a key negative regulator of STAT3-activating cytokines (Kinjyo et al., 2006), also was differentially expressed in the T_{reg} cells and in the $CD4^+CD25^-$ T cells. The regulation of STAT3 signaling by SOCS3 in $CD4^+CD25^-$ effectors was biphasic, with a first peak at 1 hr and a rapid degradation at 12 hr, as previously described (Wormald et al., 2006). Leptin neutralization increased SOCS3 amounts concomitantly with a reduced phosphorylation of STAT3. This is also consistent with the SOCS3 inhibition of IL-2 production and T cell proliferation that we observed in our work (Matsumoto et al., 2003). In T_{reg} cells, SOCS3 was highly expressed in basal conditions and markedly induced during acute stimulation, in agreement with the anergic state of the T_{reg} cells and their reduced IL-2 secretion.

Our results indicate that leptin neutralization can "unlock" the hyporesponsiveness of T_{regs} via a rapid degradation of the cell-cycle inhibitor $p27^{kip1}$, associated with the phosphorylation of ERK1/2. These biochemical events would allow the T_{reg} cells to enter the G1/S phase of the cell cycle, induce IL-2 gene transcription, and, consequently, reverse their anergic state (Li et al., 2005). Our data also indicate that these changes associate with the activation of STAT1 and STAT3, two transcription factors related with cytokine signaling and proliferation of the T_{reg} cells (Doganci et al., 2005; Nishibori et al., 2004).

Incidentally, this study also describes a novel strategy to expand human peripheral T_{reg} cells, via leptin neutralization, although this approach needs to be further investigated. Current strategies to expand T_{reg} cells employ ex vivo addition of cytokines to cultured cells during TCR stimulation (Tang et al., 2004; Jiang et al., 2003). Because neutralization of the leptin present in human serum of tissue cultures and of leptin produced by T_{reg} cells may be sufficient to determine an expansion of the T_{reg} cells, it

might be possible that leptin neutralization, in addition with exogenous IL-2, may improve the protocols of expansion for T_{reg} cells. We are currently investigating this possibility.

In vivo studies in leptin-deficient *ob/ob* mice show higher percentage and absolute number of circulating T_{reg} cells. They can expand up to 90% in vivo in normal nonlymphopenic hosts by day 28 after transfer (Trenado et al., 2003). Adoptive transfer experiments of CFSE-labeled T_{reg} cells from WT mice into leptin-deficient *ob/ob* mice showed a significant in vivo expansion of T_{reg} cells. It is also noteworthy that chronic leptin deficiency allows higher expansion of T_{reg} cells when compared with acute leptin neutralization induced by leptin Ab (the percentage of proliferating T_{reg} cells in *ob/ob* mice was always higher than that observed in leptin-neutralized WT mice). Importantly, in vivo leptin neutralization also determined an increased Foxp3 expression in the T_{reg} cells, suggesting maintenance of their suppressive phenotype after expansion in vivo. Taken together, our data suggest that the presence of ObR may be crucial in the control of the expansion of T_{reg} cells because stimulated T_{reg} cells from *db/db* mice proliferated better than T_{reg} cells from controls in vitro, whereas the suppressive capability of T_{reg} cells from *db/db* mice was similar to that of control mice. Confirmation of the crucial function of ObR came in an antigen-specific system of autoimmune diabetes in the NOD/LtJ mouse with the finding of increased anti-GAD65 proliferative response of T_{reg} cells from ObR mutants NOD-*Lepr*^{db5J}/LtJ mice.

The fact that leptin can act as a negative signal for the proliferation of T_{reg} cells envisions new possibilities of anti-leptin-based approaches for the immunotherapy of conditions characterized by low numbers of T_{reg} cells. Leptin might act as an endogenous "sensing" factor linking the environment (availability of nutrients) to circulating T_{reg} cell number. Because nutritional deprivation increases susceptibility to infection and associates with amelioration of clinical manifestations of autoimmunity (Kuchroo and Nicholson, 2003; Payne, 2001), it will be important to address how this relates to the influence of leptin on T_{reg} cells and whether anti-leptin-based intervention can be applied to tune cognate T cell responses in immune regulation.

EXPERIMENTAL PROCEDURES

Purification, Cultures, and Assays with T Cells

Human $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were purified from human healthy donors PBL either by magnetic cell separation with the Dynal $CD4^+CD25^+$ T_{reg} Kit (Dynal-Biotech, Oslo, Norway) or by flow cytometry cell sorting (MoFlo high-performance cell sorter, Dako, Glostrup, Denmark) and were rapidly cleaned with the Detach reagent (Dynal-Biotech) from surface-bound CD25 mAb. Both magnetic beads-based and flow cytometry-based purification techniques yielded a highly expressing $CD25^+$ population (95%–98% pure by FACS analysis), 90% of which expressed Foxp3. The T_{reg} cells:effector ratio in the suppression experiments was 1:1. Cells were cultured (5×10^4 cells/well) in round-bottom 96-well plates (Becton-Dikinson Falcon, Franklin Lakes, NJ) with RPMI medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies Inc., Gaithersburg, MD) and 5% AB human serum (Sigma-Aldrich, St. Louis, MO). Cells were stimulated for 3 days in the presence of anti-CD3/CD28 Dynabeads (0.1 bead/cell) (Dynal-Biotech). On the last day, [^{3}H]thymidine (0.5 μCi/well) (Amersham-Pharmacia Biotech, Cologno Monzese, Italy) was added to the cultures and cells harvested after 12 hr. Radioactivity was measured with a β-cell-plate scintillation counter (Wallac, Gaithersburg, MD).

For suppression experiments in the mouse, T_{reg} cells were isolated with the Regulatory T Cell Isolation Kit (Miltenyi Biotec, Gladbach, Germany) and stimulated with Dynabeads mouse anti-CD3/CD28 (0.5 bead/cell; 5×10^4 cells/well). The T_{reg} cells:effector ratio in the suppression experiments was 1:2. Purified cells (98% pure by FACS analysis) were cultured in round-bottom 96-well plates (Becton-Dikinson Falcon) with RPMI medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies) and 5% FCS or 5% mouse serum (Hyclone-Pierce, Rockford, IL). Cells were stimulated for 3 days, labeled with [^{3}H]thymidine (0.5 μCi/well) for the last 16 hr of culture, and harvested similarly to what done for human T cell cultures.

Incorporation of Bromodeoxyuridine (BrdU) and FACS analysis were performed in T cell cultures in serum-free medium (X-VIVO) with the BrdU Flow Kit from BD-Pharmingen in accordance to the manufacturer's instructions.

IL-2 Measurement

IL-2 measurement was done with the CTLL-2 cell line (kindly provided by N. Davey, Imperial College London, UK) according to a previously described method (Ng et al., 2001). Cells were maintained in culture in supplemented RPMI medium with 10% FCS (Hyclone-Pierce) and human recombinant (rh)-IL-2 (10 U/ml, Boehringer Mannheim, Mannheim, Germany). They were subcultured every 2 to 3 days. Cells were rested in medium without IL-2 overnight prior to use in the assays.

Reagents, Leptin-Neutralizing Antibodies, and Leptin Measurement

For in vitro blocking experiments, human leptin-neutralizing mAb (R&D Systems, Minneapolis, MN) was used at a final concentration of 0.25 to 25 μg/ml; controls were irrelevant isotype-matched antibodies (Biovendor Laboratory Medicine Inc., Brno, Czech Republic). Human recombinant leptin was purchased from R&D Systems. Human IL-2-neutralizing mAb (MQ1-17H12) was from BD Pharmingen and was utilized at final concentration of 5–10 μg/ml. FITC-anti-human-CD4 and PE-anti-human-CD25 were from BD Pharmingen; the anti-human Foxp3 staining set was from eBiosciences (San Diego, CA). Recombinant mouse GAD65 was purified from GAD65-producing cells that were kindly provided by R. Tisch (University of North Carolina, Chapel Hill, NC); recombinant GAD65 was tested for purity by SDS-PAGE and silver staining before the experimental use. The endotoxin content in the preparations was below the detection limit (about 10 pg of endotoxin/μg of protein) of the Limulus amebocyte lysate (LAL) method. Human leptin-specific ELISA was purchased from R&D Systems and measurements were performed according to the manufacturer's instructions (Matarese et al., 2005). Serum-free media were RPMI (Life Technologies), HyQ-ADCF (Animal Derived Component Free, from Hyclone-Pierce), and X-VIVO (BioWittaker).

Mice and In Vivo Experiments

6-week-old female leptin-deficient C57BL6/J-*ob/ob* (*ob/ob*), C57BL6/J, leptin-receptor deficient C57BL/Ks-*db/db* (*db/db*), and C57BL/Ks-*db*/+ lean controls (*db*/+) mice were purchased from Harlan Italy s.r.l. (Corezzana, Italy); B10.Cg.Tg(TcrAND)53Hed/J (AND-TCR Tg) PCC-specific transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME); NOD/LtJ and NOD-*Lepr*^{db5J}/LtJ mutants were kindly provided by E. Leiter from The Jackson Laboratory. Experiments were conducted in accordance with the animal welfare guidelines under an approved protocol of the Istituto Superiore di Sanità, Roma, Italy. Mice were age-matched for individual experiments and housed with a 12 hr light/dark cycle in the animal facility at the

Università di Napoli "Federico II." *ob/ob* mice were injected intraperitoneally twice daily for 10 days with mouse recombinant leptin (R&D Systems) dissolved in 200 μl of PBS at a dose of 1 μg/g of body weight.

The mouse leptin-blocking antibody was produced in our laboratory after immunization of C57BL/6J mice with mouse recombinant leptin (R&D Systems) emulsified in complete Freund's adjuvant (CFA) CFA (Difco Laboratories, Detroit, MI); mouse leptin-specific antibodies (of the IgM class) were affinity purified with recombinant mouse leptin (R&D Systems) bound to AminoLink Plus Immobilization Gel (AminoLink Plus Immobilization Kit from Pierce, Rockford, IL) from serum and ascites of immunized mice. Affinity-purified IgM were used as control (BD Pharmingen). WT mice were treated for 3 days either with 100 μg of control mouse IgM or with mouse leptin Abs intraperitoneally in a total volume of 100 μl of PBS.

Adoptive transfer experiments were performed by labeling highly purified (98% pure by FACS analysis) $CD4^+$ T cells obtained from C57BL6/J WT mice (cells were purified with the mouse $CD4^+$ negative isolation kit from Dynal) with the fluorescent dye CFSE (5-, 6-carboxyfluorescein diacetate succinimidyl ester) from Molecular Probes (Eugene, OR) used at 1 μg/ml. In brief, 10^7 CFSE-labeled $CD4^+$ T cells were adoptively transferred into mice intravenously. 4 and 7 days later, spleen cells were harvested from mice and stained with PE-anti-Foxp3 (eBioscience) and Cy-anti-CD4 (BD PharMingen). In experiments of adoptive transfer performed with CFSE-labeled AND-TCR Tg $CD4^+$ T cells (98% pure), after 4 and 7 days, spleen cells were harvested from recipient mice and stained with anti-clonotypic PE-anti-Vα11.1 (RR8.1), biotynilated-anti-Vβ3 (KJ25) (both from BD PharMingen), and APC-anti-Foxp3 (eBioscience). Flow cytometric analysis of CFSE dilution was performed by gating on $CFSE^+CD4^+Foxp3^+$ cells (in non-Tg mice) (Figures 6A and 6B) and on $CFSE^+Vα11.1^+/Vβ3^+Foxp3^+$ cells (in AND-TCR Tg mice) (Figure 6C) with a FACScalibur (Becton-Dickinson, San Diego, CA) and analyzed by Cell Quest software (Becton-Dickinson).

Western Blots, Biochemical Analyses, and Confocal Microscopy

Total cell lysates were obtained in 50 mM HEPES (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1.0% Triton X-100, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin. 50 μg of total proteins were subjected to SDS-PAGE under reducing conditions. After electrophoresis, proteins were transferred onto a nitrocellulose filter membrane (Protan, Schleicher & Schuell) with a Trans-Blot Cell (Bio-Rad) and transfer buffer containing 25 mM Tris, 192 mM glycine, 20% methanol. Membranes were placed in 5% nonfat milk in phosphate-buffered saline, 0.5% Tween 20 (PBST) at 4°C for 2 hr to block the nonspecific binding sites. Filters were incubated with specific antibodies before being washed three times in PBST and then incubated with a peroxidase-conjugated secondary antibody (Amersham Biosciences). After washing with PBST, peroxidase activity was detected with the ECL system (Amersham Biosciences) or Femto (Pierce). The antibodies used were the following: anti-$p27^{Kip-1}$, anti-STAT3 and anti-phospho-STAT3 (Y705), anti-STAT1 and anti-phospho-STAT1 (Y701) (Cell Signaling Technology, Beverly, MA); anti-leptin, anti-ObR, anti-ERK 1/2, and anti-phospho-ERK 1/2 (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA); anti-Foxp3 (eBioscience); anti-SOCS3 (Serotec Ltd, Kidlington, UK). The filters were also probed with a tubulin antibody (Sigma) to normalize the amount of loaded protein. Only for the human leptin western blots, performed on a 15% SDS-PAGE gel to better visualize 16 kDa leptin, normalization was performed against total ERK1/2. All filters were quantified by densitometric analysis of the bands utilizing the program ScionImage 1.63 for Mac (Scion Corporation, Frederick, MD).

Confocal microscopy was performed in parallel with proliferation and biochemistry at 1 hr and 36 hr on 98% pure T_{reg} cells and $CD4^+CD25^-$ cells. Cells were washed in PBS and 10^4 cells were seeded on multitest slide (ICN Biomedicals Inc., Aurora, OH), air-dried and fixed for 1 min in methanol, washed in PBS, permealized in PBS containing 0.2% Triton X-100 for 3 min, and incubated in blocking solution (PBS 1% BSA) for 1 hr. Cells were then washed three times in PBS and incubated overnight at 4°C with primary antibodies (rabbit polyclonal anti-leptin and mouse anti-ObR mAb, both from Santa Cruz Biotechnology Inc.) diluted 1:100 in blocking solution, washed in PBS, and incubated with the secondary antibody (goat anti-rabbit 488 and goat anti-mouse 543, both from Molecular Probes Inc.) for 1 hr at room temperature diluted 1:100 in blocking solution, washed in PBS, and finally mounted in PBS/Glycerol 1:1. Immunofluorescence analysis was performed with a confocal laser scanner microscope Zeiss LSM 510. The wavelength of the Argon ion laser was set at 488 nm; that of the HeNe laser was set at 543 nm. Fluorescence emission was revealed by BP 505–530 band pass filter for Alexa Fluor 488 and by BP 560-615 band pass filter for Alexa Fluor 543. Double-staining immunofluorescence images were acquired simultaneously in the green and red channels at a resolution of 1024 × 1024 pixels.

Statistical Analysis

Analyses were performed with the Mann-Whitney U-test for unrelated two-group analyses and the Kruskal-Wallis ANOVA test for three or more group analyses with the StatView software (Abacus Concepts Inc., Cary, NC). Results are expressed as mean ± SD. p values < 0.05 were considered statistically significant.

Supplemental Data

Seven Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/26/2/241/DC1/.

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Leptin neutralization interferes with pathogenic T cell autoreactivity in autoimmune encephalomyelitis

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Recent evidence has indicated that leptin, an adipocyte-secreted hormone belonging to the helical cytokine family, significantly influences immune and autoimmune responses. We investigate here the mechanisms by which in vivo abrogation of leptin effects protects SJL/J mice from proteolipid protein peptide $PLP_{139–151}$-induced EAE, an animal model of MS. Blockade of leptin with anti-leptin Abs or with a soluble mouse leptin receptor chimera (ObR:Fc), either before or after onset of EAE, improved clinical score, slowed disease progression, reduced disease relapses, inhibited $PLP_{139–151}$-specific T cell proliferation, and switched cytokine secretion toward a Th2/regulatory profile. This was also confirmed by induction of forkhead box p3 (Foxp3) expression in $CD4^+$ T cells in leptin-neutralized mice. Importantly, anti-leptin treatment induced a failure to downmodulate the cyclin-dependent kinase inhibitor p27 ($p27^{Kip-1}$) in autoreactive $CD4^+$ T cells. These effects were associated with increased tyrosine phosphorylation of both ERK1/2 and STAT6. Taken together, our data provide what we believe is a new molecular basis for leptin antagonism in EAE and envision novel strategies of leptin-based molecular targeting in the disease.

Introduction

Leptin is a cytokine-like hormone that links nutritional status with the immune system (1, 2). It is mainly produced by adipose tissue in proportion to body mass index and at lower levels by organs such as the stomach and placenta (1, 2). Leptin regulates body weight through inhibition of food intake and stimulation of energy expenditure. Moreover, leptin affects both innate and adaptive immunity (2, 3). On innate immunity, leptin modulates the activity of neutrophils, increases the phagocytosis of monocytes/macrophages, and enhances the secretion of inflammatory mediators of the acute phase response (2, 3). On adaptive immunity, leptin promotes proliferation and IL-2 secretion by naive T cells, whereas on memory T cells it promotes the switch toward Th1 immune response by increasing IFN-γ and TNF-α secretion (2, 3). Another important role of leptin in adaptive immunity is highlighted by the observation that leptin-deficient *ob/ob* mice are resistant to induction of EAE, an animal model of MS (2, 4).

EAE can be induced in susceptible strains of mice by immunization with self antigens derived from myelin. The disease is characterized by chronic or relapsing/remitting paralysis due to autoreactive Th1 $CD4^+$ cells that infiltrate the brain and spinal cord and damage the self antigen myelin (5, 6). A direct role of $CD4^+$ T cells in the pathogenesis of EAE has been demonstrated in adoptive transfer studies, where myelin-reactive Th1 $CD4^+$ T cells induced the disease after transfer (5, 6).

We have recently reported that leptin administration to susceptible mice worsens EAE by increasing secretion of proinflammatory cytokines (4). In a similar fashion, leptin replacement in leptin-deficient *ob/ob* mice restores susceptibility to both active and passive EAE (4). Since we and others have also described that leptin is expressed in active inflammatory lesions of the CNS during acute EAE and MS (7–9), we investigated the molecular effects of leptin neutralization either with anti-mouse leptin Abs or with soluble mouse leptin receptor chimera-Fc (ObR:Fc), on induction and progression of EAE.

Results

Leptin blockade improves clinical score and delays disease progression in actively induced EAE. We evaluated the ability of either anti-leptin neutralizing Abs or ObR:Fc to affect induction and progression of EAE after immunization with proteolipid protein 139–151 ($PLP_{139–151}$) myelin peptide. Treatment with anti-leptin Abs or ObR:Fc was initiated prior to immunization (from day –1 to day 1 relative to immunization) or during the acute phase of the disease (days 8–11), for 3 and 4 consecutive days, respectively (Figure 1, A and B, and Table 1; see Methods for details). In both cases, leptin neutralization led to a statistically significant reduction in EAE clinical score and percentage of initial body weight loss as well as a reduced number of inflammatory infiltrates (Figure 1, A and B, and Table 1). These effects led to significant clinical improvement and delayed disease progression during the following 40 days of observation (Figure 1, A and B, and Table 1), indicating that leptin blockade inhibited both development and progression of EAE. Moreover, observation of animals over a longer period of time (90–120 days) revealed a significant reduction in relapse rate (Table 1), suggesting that this protection was long lasting.

Nonstandard abbreviations used: DTH, delayed-type hypersensitivity; Foxp3, forkhead box p3; ObR:Fc, leptin receptor chimera-Fc; PLP, proteolipid protein; $p27^{Kip-1}$, cyclin-dependent kinase inhibitor p27; VLA-4, very late antigen-4.

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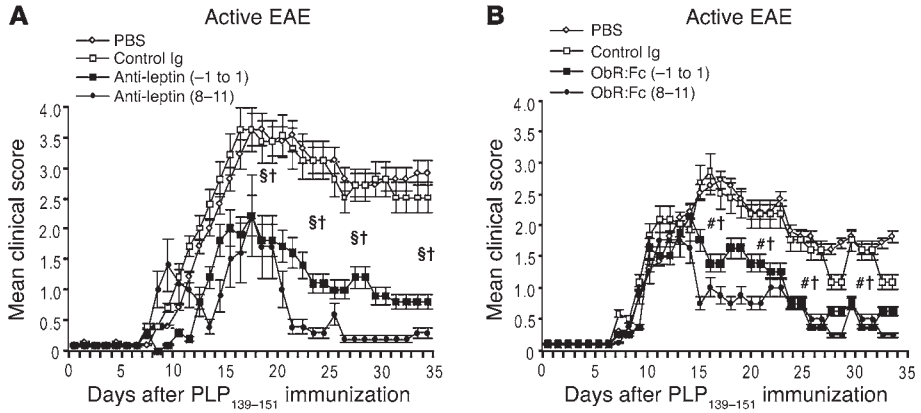

Figure 1
Leptin blockade during actively induced EAE with either anti-leptin Abs or ObR:Fc reduces the clinical severity of EAE. (**A**) Mean clinical score of SJL/J female mice treated with anti-mouse leptin Abs injected either from day –1 to day 1 or on days 8–11 (100 μg/d i.p.). Leptin blockade significantly delayed disease onset and reduced clinical score and body weight loss (see Table 1). Data are from 1 representative experiment of 3. (**B**) Mean clinical score of SJL/J female mice treated with mouse ObR:Fc chimera injected either from day –1 to day 1 or on days 8–11 (100 μg/d i.p.). Data are from 1 representative experiment of 3. §$P = 0.01$, day –1 to day 1, #$P = 0.02$, day –1 to day 1, and †$P = 0.04$, days 8–11, versus PBS or control Ig.

Leptin blockade improves clinical score and delays disease progression in passively induced EAE. We next tested the ability of either anti-leptin Abs or ObR:Fc to modify the onset and progression of adoptively transferred EAE (Figure 2, A and B, and Table 1). Treatment with Abs was repeated for 3–4 consecutive days, before (from day –1 to day 1) or after (days 8–11) the adoptive transfer of 2×10^7 purified $PLP_{139–151}$-specific $CD4^+$ T cells (see Methods). Mice treated with control Igs all developed severe EAE (Figure 2A and Table 1). In contrast, mice treated with anti-leptin Abs from day –1 to day 1 displayed a milder disease (Figure 2A and Table 1), while those treated on days 8–11 were fully protected (Figure 2A and Table 1). Treatment with ObR:Fc from day –1 to day 1 also led to a reduction of EAE clinical signs and delayed progression of the disease with significantly more efficiency than did anti-leptin Ab treatment from day –1 to day 1 (Figure 2, A and B, and Table 1). A significant reduction in the relapse rate was also found in animals observed for 90–120 days (Table 1).

Leptin blockade is accompanied by inhibition of delayed-type hypersensitivity and reduces autoreactive T cell proliferation to $PLP_{139–151}$ peptide during EAE. To determine the nature of the in vivo T cell response against $PLP_{139–151}$ peptide in leptin-neutralized mice, delayed-type hypersensitivity (DTH) reactions were performed in all groups of mice. On day 35 of observation, mice were challenged with 25 μg $PLP_{139–151}$ peptide injected intradermally in the footpad. The degree of local footpad swelling was measured as readout for the DTH reaction (see Methods). Typical DTH kinetics were observed with footpad swelling peaking between 24–48 hours and subsiding after 72–96 hours. DTH responses to the $PLP_{139–151}$ peptide priming epitope were

Table 1
Effect of treatment with either anti-leptin Abs or ObR:Fc chimera on neurological impairment during active and passive EAE in SJL/J female mice

Group	Incidence, no./total (%)	Day of onset (range)	Peak clinical score	Average CDI[A]	Body wt at disease peak (% of original)	No. inflammatory foci	Relapse rate after treatment[B]
PBS (active EAE)	18/18 (100.0)	9.0 ± 0.5 (8–9)	3.6 ± 0.9	70.9 ± 13.0	71.5 ± 0.8	45.5 ± 10.0	2.00
Control IgM	18/18 (100.0)	8.1 ± 0.5 (8–9)	3.5 ± 1.0	70.3 ± 8.5	74.7 ± 0.5	40.1 ± 5.0	1.94
Anti-leptin (–1 to 1)	18/18 (100.0)	11.8 ± 1.0 (10–13)	2.0 ± 0.5[C]	30.7 ± 6.0[C]	83.8 ± 0.6[C]	15.5 ± 10.0[C]	0.55
Anti-leptin (8–11)	18/18 (100.0)	12. 1 ± 3.0 (8–16)	1.6 ± 0.8[D]	20.1 ± 10.7[D]	95.1 ± 0.6[D]	9.0 ± 4.0[D]	0.33
PBS (active EAE)	18/18 (100.0)	8.0 ± 0.5 (8–9)	2.7 ± 0.8	51.7 ± 8.0	77.0 ± 0.5	31.0 ± 10.0	1.55
Control IgG1	18/18 (100.0)	8.1 ± 0.4 (8–9)	2.6 ± 0.5	49.5 ± 7.0	77.5 ± 0.5	31.9 ± 12.0	1.66
ObR:Fc (–1 to 1)	18/18 (100.0)	9.4 ± 1.0 (8–12)	1.2 ± 0.4[C]	28.5 ± 10.0[C]	87.5 ± 0.4[C]	15.0 ± 7.0[C]	0.55
ObR:Fc (8–11)	17/18 (94.4)	9.0 ± 0.5 (8–10)	0.9 ± 0.5[D]	22.1 ± 12.0[D]	101.4 ± 0.1[D]	11.0 ± 5.0[D]	0.55
PBS (passive EAE)	18/18 (100.0)	7.1 ± 1.0 (6–8)	2.8 ± 1.0	39.0 ± 5.0	75.1 ± 1.0	27.0 ± 5.0	1.38
Control IgM	18/18 (100.0)	7.0 ± 0.7 (6–8)	2.6 ± 0.8	36.6 ± 3.0	79.0 ± 0.8	27.5 ± 6.0	1.44
Anti-leptin (–1 to 1)	18/18 (100.0)	8.8 ± 2.0 (6–11)	1.6 ± 0.8[C]	25.9 ± 6.0[C]	81.5 ± 1.2[C]	10.5 ± 9.0[C]	0.61
Anti-leptin (8–11)	14/18 (77.7)	17.8 ± 1.1 (17–20)	0.3 ± 0.4[D]	6.6 ± 2.0[D]	106.0 ± 2.0[D]	0.2 ± 0.5[D]	0.05
PBS (passive EAE)	18/18 (100.0)	7.2 ± 1.2 (7–8)	3.1 ± 1.0	44.5 ± 3.0	78.0 ± 1.0	35.5 ± 6.0	1.27
Control IgG1	18/18 (100.0)	7.5 ± 0.7 (7–8)	3.0 ± 0.9	41.5 ± 2.5	78.0 ± 2.0	34.3 ± 10.5	1.33
ObR:Fc (–1 to 1)	14/18 (77.7)	9.5 ± 0.7 (8–10)	0.5 ± 0.0[D]	8.6 ± 1.0[D]	101.4 ± 1.2[D]	0.5 ± 0.1[D]	0.00
ObR:Fc (8–11)	13/18 (72.2)	10.0 ± 0.7 (8–10)	0.5 ± 0.0[D]	10.5 ± 1.0[D]	102.9 ± 2.0[D]	0.5 ± 0.4[D]	0.00

Data were cumulated and averaged from 3 independent experiments, each performed with 6 mice per group, and are presented as mean ± SD. See Methods for details of isotype controls utilized. CDI, cumulative disease index. [A]The sum of daily scores was determined for each mouse of the indicated group and averaged. [B]Calculated as total number of relapses per group divided by the total number of mice in that group (mice were observed up to 90–120 days after disease induction to asses the relapse rate). [C]$P = 0.001$ and [D]$P = 0.0001$ versus respective PBS and control Ig groups.

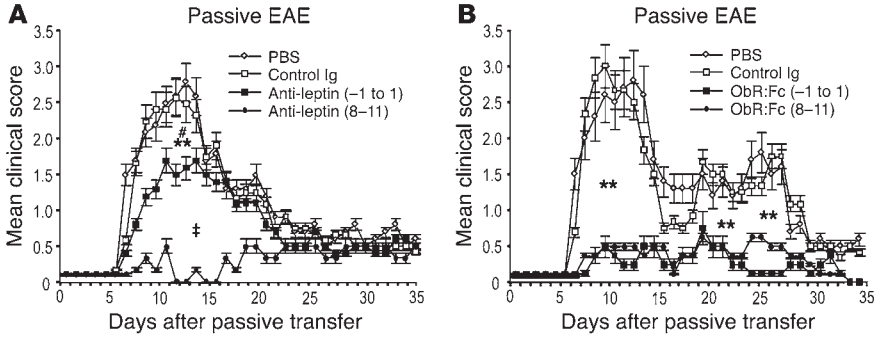

Figure 2
Leptin blockade during adoptively transferred EAE with either anti-leptin Abs or ObR:Fc chimera reduces the clinical severity of EAE. (**A**) Mean clinical score of SJL/J female mice treated with anti-mouse leptin Abs injected either from day –1 to day 1 or on days 8–11 (100 μg/d i.p.). Leptin blockade significantly reduced clinical score and body weight loss (see Table 1). Data are from 1 representative experiment of 3. $^{\#}P = 0.02$, day –1 to day 1, and $^{**}P = 0.001$, days 8–11, versus PBS or control Ig; $^{\ddagger}P = 0.02$, day –1 to day 1 versus days 8–11. (**B**) Mean clinical score of SJL/J female mice treated with mouse ObR:Fc chimera injected either from day –1 to day 1 or on days 8–11 (100 μg/d i.p.). Data are from 1 representative experiment of 3. $^{**}P = 0.001$, day –1 to day 1 and days 8–11 versus control Ig.

significantly reduced in mice treated with anti-leptin Abs and ObR:Fc, whereas the controls exhibited a marked DTH reaction (Figure 3A and Figure 4A).

We also examined whether leptin blockade could affect proliferation of $PLP_{139–151}$-specific T cells in vitro. T cell response to $PLP_{139–151}$ peptide was tested on draining lymph nodes taken from all groups of treated mice 15 days after immunization and cultured in the presence of increasing concentrations of $PLP_{139–151}$. T cells derived from either anti-leptin Abs– or ObR:Fc-treated mice showed a significant reduction in proliferation (Figure 3B and Figure 4B, respectively), whereas no difference was observed when T cells were stimulated in parallel with a polyclonal stimulator such as the anti-CD3ε mAb (2C11 hybridoma, Figure 3C and Figure 4C, respectively; see Methods). Of note, inhibition of in vitro anti-$PLP_{139–151}$ proliferation was more efficient in mice treated with the ObR:Fc chimera (Figure 3B and Figure 4B).

Leptin blockade in EAE is associated with a switch of the cytokine profile toward a Th2/regulatory phenotype and upregulation of forkhead box p3 expression. We next asked whether EAE protection was associated with an immune response characterized by considerable cytokine switching. The production of IFN-γ was significantly reduced by both leptin-neutralizing treatments in the presence of increasing concentrations of $PLP_{139–151}$ as well as during polyclonal anti-CD3ε stimulation (Figure 5, A and B, and Figure 6, A and B). In addition, a significant increase in the Th2/regulatory-type cytokines such as IL-4 (Figure 5, C and D, and Figure 6, C and D) and IL-10 (Figure, 5, E and F, and Figure 6, E and F) was observed during both anti-$PLP_{139–151}$–specific proliferation and polyclonal stimulation with anti-CD3ε. In addition, to determine whether treatment with ObR:Fc induced forkhead box p3 (Foxp3) expression in $CD4^+$ T cells, we isolated these cells from mice with EAE and measured Foxp3 expression by Western blot. Leptin neutralization induced significant increase of Foxp3 levels (Figure 7, A and B), suggesting induction of regulatory T cell markers in leptin-neutralized mice.

Leptin neutralization reduces the expression of ICAM-1 and OX-40 and upregulates very late antigen-4 on $CD4^+$ T cells during EAE. We then investigated in more detail the cellular events leading to an improvement of clinical symptoms and progression of EAE. Cytofluorimetric analysis of $CD4^+$ T cells from mice treated with anti-leptin Abs or ObR:Fc obtained on day 15 after immunization with $PLP_{139–151}$ revealed a significant reduction of ICAM-1 and

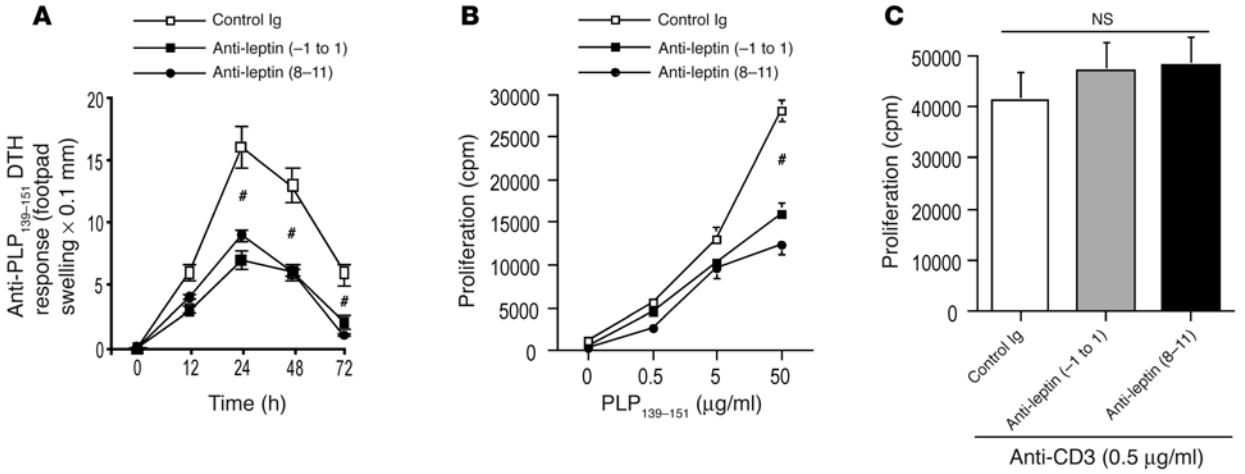

Figure 3
In vivo leptin neutralization with anti-mouse leptin Abs in SJL/J mice inhibits DTH response and induces T cell hyporesponsiveness to $PLP_{139–151}$ myelin peptide. (**A**) DTH reaction in leptin-neutralized and control Ig–treated mice measured as footpad swelling. Data are from 1 representative experiment of 2. (**B**) Proliferative response of lymph node–derived T cells against $PLP_{139–151}$ was impaired after treatment with anti-mouse leptin-neutralizing Abs compared with the control Ig–treated group. Data are from 1 representative experiment of 3. (**C**) Anti-mouse leptin treatment did not affect polyclonal T cell proliferation induced with anti-CD3ε stimulation. $^{\#}P = 0.02$, day –1 to day 1 and days 8–11 versus control Ig.

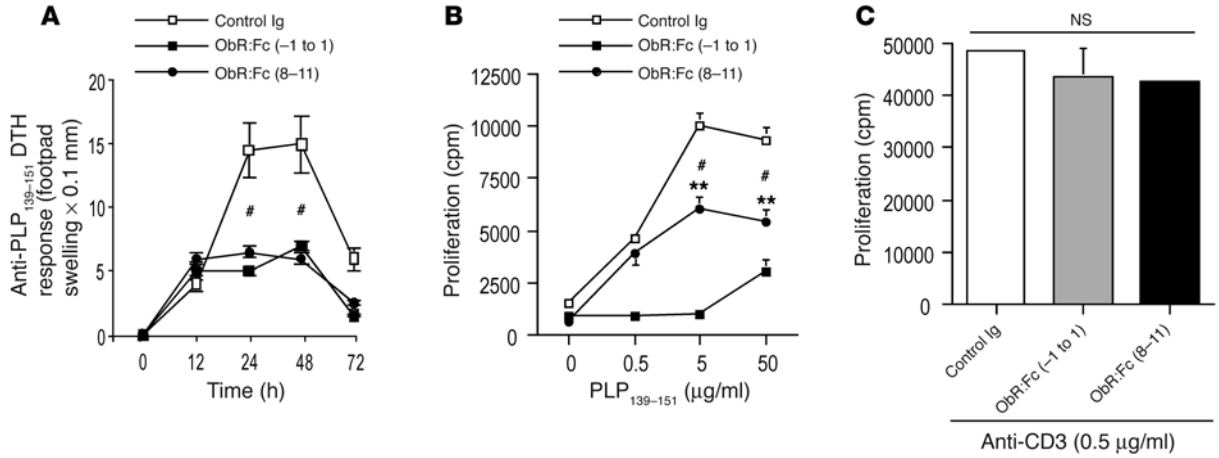

Figure 4
In vivo leptin neutralization with mouse ObR:Fc chimera in SJL/J mice inhibits DTH response and induces T cell hyporesponsiveness to $PLP_{139–151}$ myelin peptide. (**A**) DTH reaction in ObR:Fc leptin-neutralized and control Ig–treated mice measured as footpad swelling. Data are from 1 representative experiment of 2. $^{\#}P = 0.02$, day –1 to day 1 and days 8–11 versus control Ig. (**B**) Proliferative response of lymph node–derived T cells against $PLP_{139–151}$ was impaired after treatment with mouse ObR:Fc chimera compared with the control Ig–treated group. Data are from 1 representative experiment of 3. $^{**}P = 0.001$, day –1 to day 1, and $^{\#}P = 0.02$, days 8–11, versus control Ig. (**C**) ObR:Fc treatment did not affect polyclonal T cell proliferation induced with anti-CD3ε stimulation.

OX-40 (Figure 8, A and B, respectively), both classically involved in the pathogenesis of EAE (10–12). Conversely, the overall expression of the α4β1 integrin, very late antigen-4 (VLA-4) (13), was increased, with particular upregulation of the population at high fluorescence intensity (Figure 8C). The effects of leptin blockade on lymphocytes could not be ascribed to intrinsic toxicity of the molecule, as the circulating number and apoptosis (measured by annexin V staining) of $CD4^+$, $CD8^+$, B, γδ, NK, and monocytes in the spleen and lymph nodes of treated mice were not different from controls (data not shown).

Ex vivo $CD4^+$ T cells from leptin-neutralized mice fail to downregulate the anergy factor cyclin-dependent kinase inhibitor p27 and induce the ERK1/2 and STAT6 pathways. To evaluate whether leptin neutralization affects the molecular machinery of autoreactive $CD4^+$ T cells, we analyzed a series of biochemical markers specific to T cell activation, anergy, and Th2/regulatory-type cytokine secretion (14–18). More specifically, we studied ex vivo purified $CD4^+$ autoreactive T cells from spleens and lymph nodes of ObR:Fc-treated mice on day 15 of disease, previously immunized with $PLP_{139–151}$ peptide (see Methods). In $CD4^+$ T cells ObR:Fc treatment led to a failure to downmodulate the cyclin-dependent kinase inhibitor p27 ($p27^{Kip-1}$; Figure 9A) associated with increased tyrosine phosphorylation of ERK1/2 (Figure 9B). Moreover, we observed an upregulation of STAT6 tyrosine phosphorylation levels (Y641, known to be associated with Th2/regulatory-type cytokine secretion; ref. 18) in ObR:Fc-treated mice compared with control mice (Figure 9C).

Discussion

Previous studies by our group and others have shown the relevance of leptin in the pathogenesis of EAE (4, 7–9). In particular, it was previously reported that *ob/ob* mice are resistant to induction of the disease (4), whereas in wild-type, EAE-susceptible controls, a surge of serum leptin precedes acute EAE (7). In this work, we show for the first time to our knowledge that in vivo neutralization of leptin is effective at blocking initiation, progression, and clinical relapses of EAE, an animal model of MS (Figures 1 and 2 and Table 1).

We and others have previously reported that in the CNS of EAE mice, both infiltrating T cells and neurons express leptin during the acute phase of the disease, and the degree of leptin expression within the lesions correlates with CNS inflammatory score and disease severity (7, 9). Because of the possibility of an autocrine loop sustaining autoreactive Th1 lymphocytes in EAE (7, 8), we investigated the DTH response as well as T cell proliferation and cytokine secretion in response to $PLP_{139–151}$ in treated mice versus controls. Anti-leptin–treated animals showed reduced DTH and T cell proliferative responses to $PLP_{139–151}$ peptide associated with a Th2/regulatory-type cytokine shift (Figures 3–6). This evidence was also supported by increased expression levels of the regulatory T cell master gene Foxp3 in $CD4^+$ T cells from mice with EAE (Figure 7).

It is interesting to note that in vivo leptin neutralization differentially affected proliferative responses and regulatory cytokine switch during polyclonal anti-CD3 stimulation (Figures 3–6). Indeed, while we observed a reduction of proliferation and a Th2/regulatory cytokine switch toward $PLP_{139–151}$, anti-CD3 polyclonal stimulation was only affected in terms of cytokine secretion and not of proliferative response at the relatively low doses of leptin blockers utilized in vivo. The evidence that leptin itself differentially influences polyclonal versus antigen-specific proliferation and cytokine secretion may account for these apparently contrasting effects (19). On the other hand, it is also possible to speculate that leptin neutralization–induced perturbations of the cytokine milieu during antigen-specific stimulation may preferentially modulate cytokine profile rather than proliferative responses induced by anti-CD3.

Leptin blockade also affected expression of ICAM-1, OX-40, and VLA-4 on $CD4^+$ T cells (Figure 8). In particular, reduced expression of ICAM-1 was consistent with our previous findings showing that leptin treatment increases surface expression of this adhesion molecule on T cells (20). This finding suggested the possibility that neutralization of leptin directly affects the cognate interaction leading to reactive and/or autoreactive T cell activation. More-

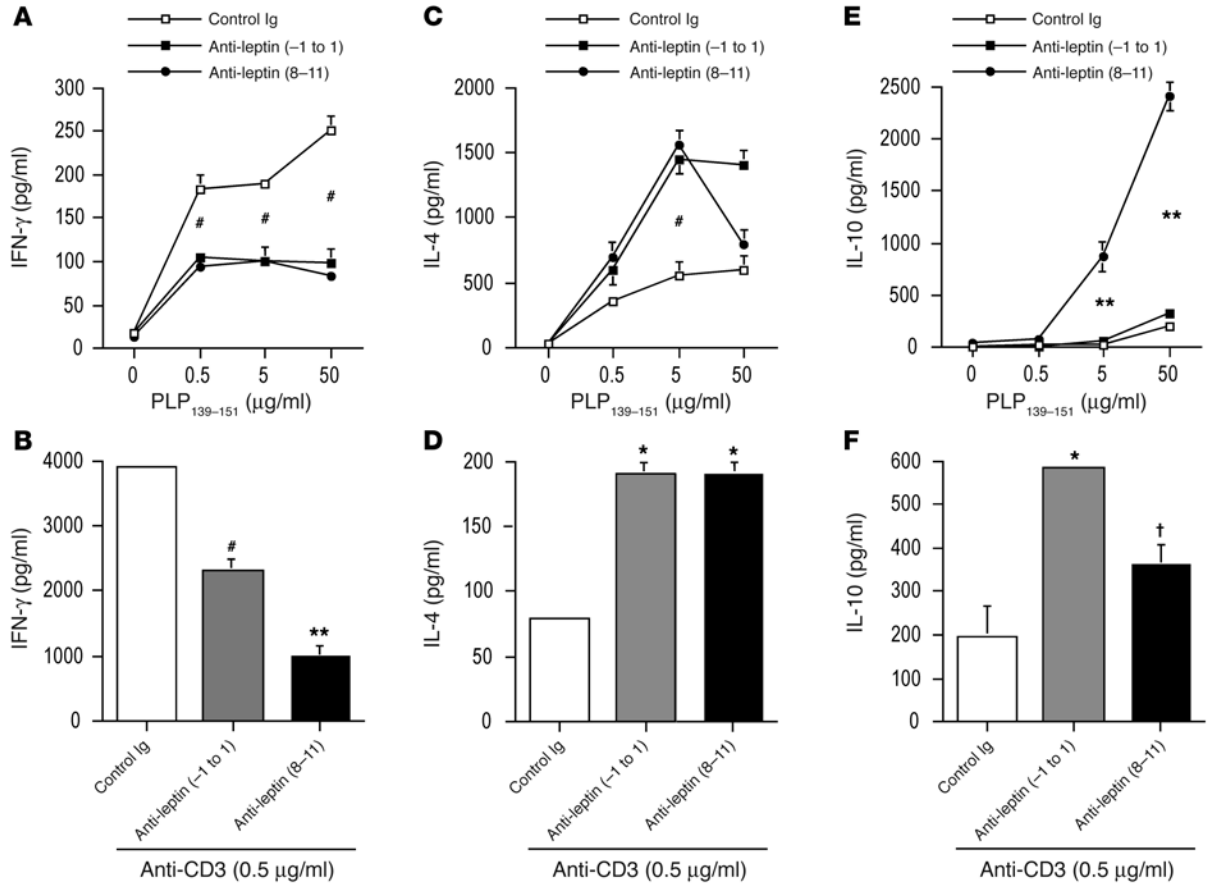

Figure 5

In vivo leptin neutralization with anti-mouse leptin Abs inhibits IFN-γ production and induces the secretion of IL-4 and IL-10 regulatory cytokines. (**A** and **B**) IFN-γ secretion of lymph node–derived T cells was inhibited by anti-leptin treatment when T cells were stimulated with the myelin antigen $PLP_{139–151}$ (**A**) and by anti-CD3ε (**B**). (**C** and **D**) IL-4 secretion of lymph node–derived T cells was enhanced by anti-leptin treatment when T cells were stimulated with the myelin antigen $PLP_{139–151}$ (**C**) and by anti-CD3ε (**D**). (**E** and **F**) IL-10 secretion of lymph node–derived T cells was markedly increased by anti-leptin treatment when T cells were stimulated with the myelin antigen $PLP_{139–151}$ (**E**) and by anti-CD3ε (**F**). (**A**, **C**, and **E**) $^{\#}P = 0.02$, day –1 to day 1 and days 8–11, and $^{**}P = 0.001$, days 8–11, versus control Ig. Data are from 1 representative experiment of 3. (**B**, **D**, and **F**) $^{\#}P = 0.02$, $^{**}P = 0.001$, $^{*}P = 0.002$, $^{\dagger}P = 0.04$ versus control Ig.

over, marked reduction of OX-40 was also observed after leptin blockade. Since OX-40 is an important costimulatory molecule with prosurvival activity for $CD4^+$ T cells, and signaling through this molecule breaks peripheral T cell tolerance (11, 21), our data suggest that leptin may affect expression of key molecules on T lymphocytes involved in the mechanisms of immune tolerance. Surprisingly, we also observed that leptin neutralization induced increased expression of VLA-4, the α4β1 integrin shown to play an integral part in the homing and migration of cells that induce EAE (13). However, experimental evidence has shown that administration of anti–VLA-4 ameliorated EAE only if it was initiated before disease onset, whereas treatment during acute disease exacerbated EAE and enhanced the accumulation of T cells in the CNS (13). Therefore, we are tempted to hypothesize that the induction of VLA-4 on $CD4^+$ T cells after leptin neutralization could be associated in part with an increased cell capability to migrate into the CNS and produce regulatory cytokines able to downmodulate EAE. Of note, these data are in agreement with other findings showing that adhesion molecules are increased on regulatory T cells in experiments of protection from EAE (22, 23).

To further address, at the biochemical level, whether in vivo leptin neutralization interferes with the signalling capacity of autoreactive T cells, we analyzed several molecular pathways associated with T cell anergy/activation and cytokine switch (14–18). We found that $CD4^+$ T cells from mice treated with leptin antagonists showed hyporesponsiveness to $PLP_{139–151}$ peptide, which was indicated by accumulation of $p27^{Kip-1}$. This negative cell cycle regulator plays a central role in blocking clonal expansion of T cells and is therefore critical for anergy induced by blockade of costimulatory pathways (14, 17, 24). We also found that the hyporesponsive state induced by leptin antagonism was associated with marked increase of ERK1/2 phosphorylation, confirming involvement of ERK1/2 in the improvement of EAE (25). It is interesting to observe that our findings with leptin antagonism seem to involve pathways affected by statins, cholesterol-lowering drugs that have recently been shown to reduce production of leptin by adipocytes (26), promote Th2 responses, and improve EAE (27) by disabling downregulation of $p27^{Kip-1}$ and upregulating phosphorylation of ERK1/2 (28). Finally, we also observed at the biochemical level the induction of phosphorylation of the

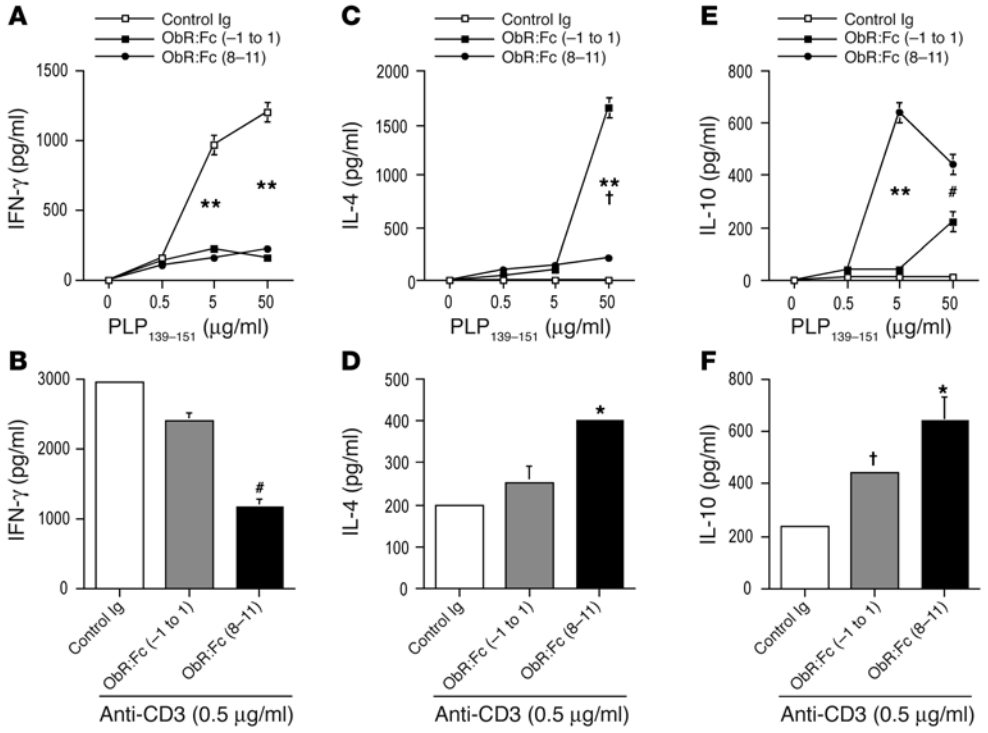

Figure 6
In vivo leptin neutralization with ObR:Fc inhibits IFN-γ production and induces the secretion of IL-4 and IL-10 regulatory cytokines. (**A** and **B**) IFN-γ secretion of lymph node–derived T cells was inhibited by ObR:Fc treatment when T cells were stimulated with the myelin antigen $PLP_{139–151}$ (**A**) and by anti-CD3ε (**B**). (**C** and **D**) IL-4 secretion of lymph node–derived T cells was enhanced by ObR:Fc treatment when T cells were stimulated with the myelin antigen $PLP_{139–151}$ (**C**) and by anti-CD3ε (**D**). (**E** and **F**) IL-10 secretion of lymph node–derived T cells was markedly increased by ObR:Fc treatment when T cells were stimulated with the myelin antigen $PLP_{139–151}$ (**E**) and by anti-CD3ε (**F**). Data are from 1 representative experiment of 3. (**A**) $^{**}P = 0.001$, day –1 to day 1 and days 8–11 versus control Ig. (**C**) $^{**}P = 0.001$, day –1 to day 1, and $^{\dagger}P = 0.04$, days 8–11, versus control Ig. (**E**) $^{**}P = 0.001$, days 8–11, and $^{\#}P = 0.02$, day –1 to day 1, versus control Ig. (**B**, **D**, and **F**) $^{\#}P = 0.02$, $^{*}P = 0.002$, $^{\dagger}P = 0.04$ versus control Ig.

STAT6 transcription factor, well known to be able to induce the transcription of IL-4 and associated with a classical Th2/regulatory-type cytokine response during EAE (18).

In conclusion, we report that leptin neutralization was able to improve clinical onset, progression, and clinical relapses of both actively induced and passively transferred EAE. This effect was associated with marked inhibition of DTH reaction against $PLP_{139–151}$ peptide, $CD4^+$ T cell hyporesponsiveness, and increased IL-4 and IL-10 production against myelin antigens. Foxp3 expression was also induced on $CD4^+$ T cells in leptin-neutralized mice, suggesting the induction of a regulatory phenotype. At the biochemical level, T cell hyporesponsiveness might be explained by the failure to downmodulate the anergy factor $p27^{Kip-1}$ and by the increase in the tyrosine phosphorylation levels of ERK1/2 and STAT6. Taken together, our results provide a framework for leptin-based intervention in EAE and identify molecules with possible therapeutic potential for the disease.

Methods

Mice. Female SJL/J mice ($H\text{-}2^s$), 6–8 weeks old, were obtained from Harlan-Italy. All animal experiments were approved by the Istituto Superiore di Sanità (Rome, Italy). All mice were age matched for individual experiments and were group housed (2–6 mice per standard cage according to the different experimental protocol) with a 12-hour light/dark cycle. Paralyzed mice were afforded easier access to food and water to prevent dehydration.

Reagents and Abs. We used the immunodominant mouse $PLP_{139–151}$ peptide (HSLGKWLGHPDKF) in this study. It was synthesized by INBIOS s.r.l.; purity was assessed by HPLC (>97% pure), and amino acid composition was verified by mass spectrometry. $PLP_{139–151}$ peptide batches for in vivo and in vitro assays were all from 1 preparation initially solubilized in LPS-free saline solution at 4 mg/ml concentration and stored at –80°C.

ObR:Fc was from R&D Systems. It is constituted by the extracellular domain of mouse leptin receptor (ObR) fused to the Fc region of human IgG_1. The endotoxin level for all experimental Abs was ≤0.1 ng/μg protein. The mouse anti-leptin polyclonal Ab was produced in our laboratory after immunization of C57BL/6J mice with mouse recombinant leptin (R&D Systems) emulsified in CFA (Difco; BD Diagnostics — Diagnostic Systems); anti-mouse leptin-specific Abs (of the IgM class) were affinity purified from serum and ascites of immunized mice, with recombinant mouse leptin bound to AminoLink Plus Immobilization Gel (AminoLink Plus Immobilization kit; Pierce Biotechnology Inc.). For treatment with anti-leptin Abs, affinity-purified IgM was used as a control (BD Biosciences — Pharmingen); for ObR:Fc fusion protein treatment, affinity-purified IgG_1 was used as a control (BD Biosciences — Pharmingen).

EAE induction and clinical and histological assessment. For active induction of EAE, mice ($n = 6$ per group) were immunized s.c. in the flank with 100 μl CFA (Difco; BD Diagnostics — Diagnostic Systems) emulsified with 100 μg $PLP_{139–151}$ peptide on day 0 and with 200 ng pertussis toxin (Sigma-Aldrich s.r.l.) i.p. on days 0 and 1. Control mice ($n = 5$ per group) were injected with CFA emulsified with PBS plus pertussis toxin according to the same schedule (data not shown). For adoptively transferred EAE, 9–10 female donor SJL/J mice (6–8 weeks old) were primed s.c. with 200 μg $PLP_{139–151}$ peptide in CFA distributed over 4 sites. After 9–10 days, draining lymph nodes (axillary and inguinal) and spleens were harvested, homogenized into single-cell suspension, and cultured separately in vitro in 24-well plates (8×10^6 cells/well, Falcon; BD) in the presence of 25 μg/ml $PLP_{139–151}$ peptide. After 4 days in culture, nonadherent cells were harvested and centrifuged over Ficoll gradient (Pharmacia), and $CD4^+$ T cells were purified by passing the cells over the $CD4^+$ subset columns (R&D Systems). Recipient syngeneic naive female SJL/J mice received, in a 200-μl PBS i.v. injection, 2×10^7 highly purified $PLP_{139–151}$-specific $CD4^+$ T cells. Mice also received 200 ng pertussis toxin immediately after cell transfer as well as 1 day later.

Individual mice were observed daily for clinical signs of disease for up to 40 days after immunization and after adoptive transfer. Mice were weighed and scored daily in a blinded fashion according to clinical severity of symptoms

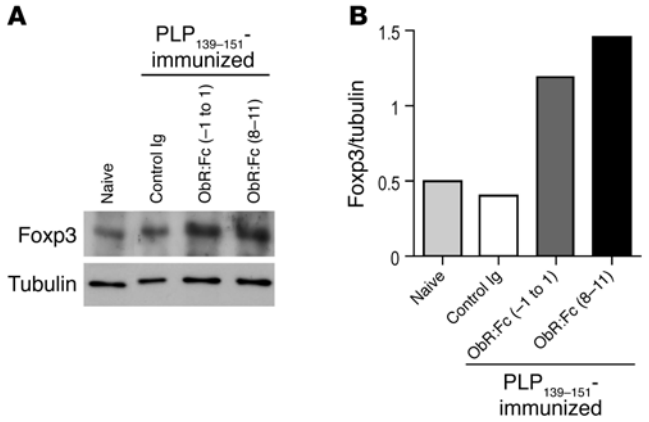

Figure 7
Increased expression of Foxp3 in CD4+ T cells induced by leptin neutralization in mice with EAE. (**A**) Western blot analysis for Foxp3 on purified CD4+ T cells obtained from SJL/J mice immunized with $PLP_{139–151}$ revealed significant increase of the expression of this molecule after leptin neutralization with ObR:Fc. (**B**) Results are presented as Foxp3 protein level normalized to tubulin expression. Data are from 1 representative experiment of 3.

on a scale of 0 to 6, with 0.5 points for intermediate clinical findings: grade 0, no abnormality; grade 0.5, partial loss of tail tonicity, assessed by inability to curl the distal end of the tail; grade 1, reduced tail tone or slightly clumsy gait; grade 2, tail atony, moderately clumsy gait, impaired righting ability, or any combination; grade 3, hind limb weakness or partial paralysis; grade 4, complete hind limb paralysis or fore limb weakness; grade 5, tetraplegia or moribund state; grade 6, death. The data were plotted as daily mean clinical score for all animals in a particular treatment group. Scores of asymptomatic mice (i.e., scores of 0) were included in the calculation of the daily mean clinical score for each group. A relapse was defined as a sustained (more than 2 days) increase in clinical score by at least 1 full grade after the animal had improved previously by at least 1 full grade and stabilized for at least 2 days. The data are plotted as the relapse rate of all the animals of that group (total number of relapses per group divided by total number of mice in the group; see Table 1). The brains and spinal cords were dissected between 15 and 20 days after immunization, according to the clinical stage of disease, and fixed in 10% formalin. Paraffin-embedded sections of 5 μm thickness were cut and stained with H&E (Sigma-Aldrich). Sections from 4–10 segments per mouse were examined blindly for the number of inflammatory foci by using a previously published scoring system (7, 8).

In vivo Ab treatment. Mice were treated 3 or 4 times with 100 μg control mouse IgM or control mouse IgG_1 or with anti-mouse leptin-specific blockers (either anti-leptin Abs or ObR:Fc chimera, respectively) i.p. in a total volume of 100 μl of PBS. Treatment was initiated with $PLP_{139–151}$ peptide or the adoptive transfer of pathogenic T cells for 3 consecutive days (days –1, 0, and 1) or during the acute phase of the disease for 4 consecutive days (days 8–11).

Induction of DTH (footpad-swelling assay). DTH responses to $PLP_{139–151}$ peptide during induction of disease were also quantitated using a time-dependent (12–72 hours) footpad-swelling assay. Briefly, mice previously sensitized with $PLP_{139–151}$ in CFA were challenged by s.c. injection of 25 μg $PLP_{139–151}$ (in 50 μl PBS) into the right hind footpad. PBS alone was injected into the left footpad to serve as a control for measurements. As a negative control, we used mice sensitized with CFA alone. Footpad thickness was measured 12, 24, 48, and 72 hours after challenge by an experimenter blinded to sample identity using a caliper-type engineer's micrometer. The footpad swelling response was calculated as the thickness of the right footpad (receiving antigen) minus the baseline thickness of the left footpad (receiving PBS).

Proliferation assays and cytokine analysis. Spleen and lymph node cells were obtained from mice 15 days after $PLP_{139–151}$ sensitization, dissociated into single-cell suspension, and cultured for proliferation assays in flat-bottomed, 96-well microtiter plates (Falcon; BD) at a density of 5×10^5 viable cells per well in a total volume of 200 μl RPMI-1640 medium (Invitrogen

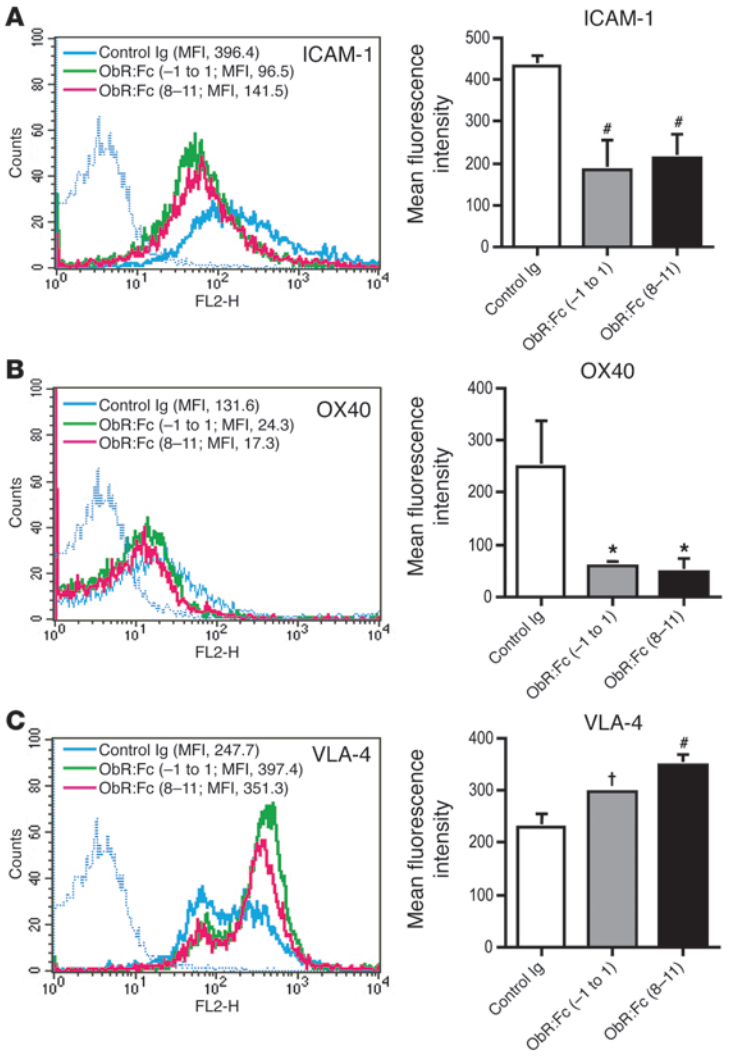

Figure 8
Leptin neutralization suppresses ICAM-1 and OX-40 expression on CD4+ cells but upregulates VLA-4 in mice with EAE. (**A**) Flow cytometric analysis of cell-surface ICAM-1 molecules on CD4+ T cells from ObR:Fc-treated mice and controls (left) and mean fluorescence intensity (MFI) from 3 independent experiments (right). (**B**) OX-40 surface expression was also reduced by ObR:Fc treatment. (**C**) VLA-4 expression was enhanced by ObR:Fc treatment, particularly on CD4+ T cells at high intensity. $^{\#}P = 0.02$, $^{*}P = 0.002$, $^{\dagger}P = 0.04$ versus control Ig.

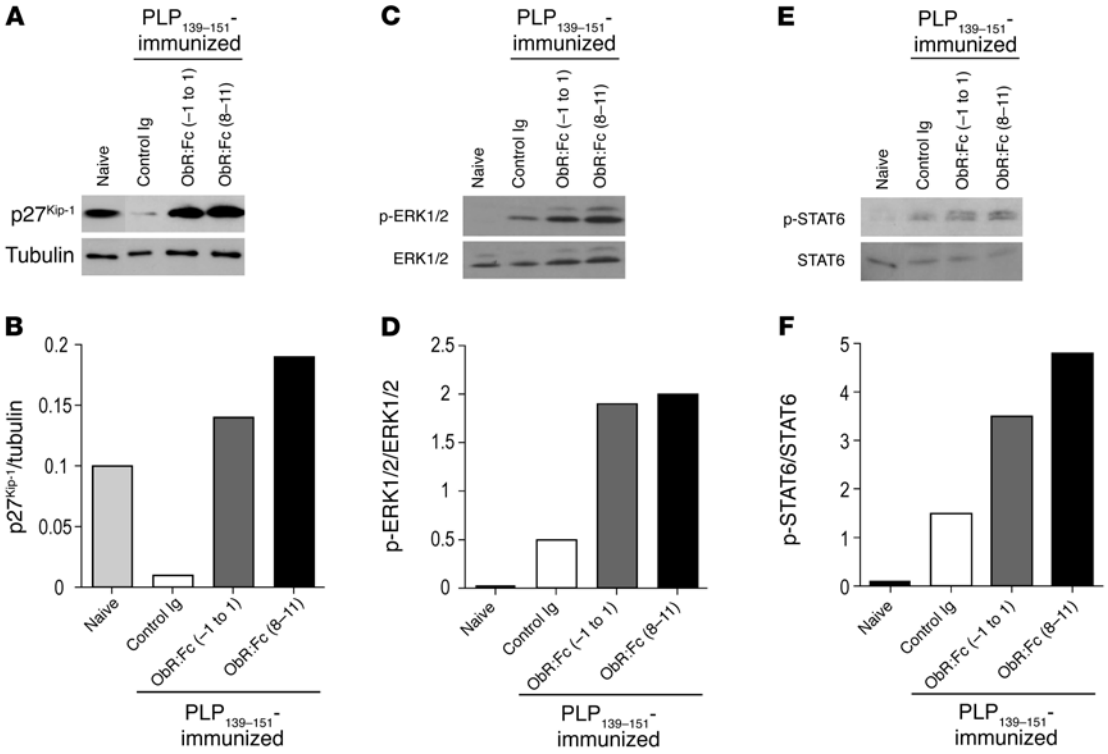

Figure 9

Leptin neutralization determines the failure to downmodulate the anergy factor $p27^{Kip-1}$ and is associated with sustained phosphorylation of ERK1/2 and STAT6. (**A** and **B**) Western blot analysis for $p27^{Kip-1}$ and tubulin on purified $CD4^+$ T cells obtained from SJL/J mice immunized with $PLP_{139–151}$. Ex vivo analysis revealed high levels of $p27^{Kip-1}$ in resting $CD4^+$ T cells from naive mice; this phenomenon was accompanied by a strong downmodulation of $p27^{Kip-1}$ in control Ig mice that developed classical EAE. Conversely, ObR:Fc treatment either from day –1 to day 1 or on days 8–11 caused a failure to downmodulate $p27^{Kip-1}$, resulting in massive $p27^{Kip-1}$ accumulation. (**C** and **D**) Ex vivo analysis revealed very low levels of phosphorylation of ERK1/2 in resting $CD4^+$ T cells from naive mice. Conversely, control Ig–treated mice with EAE showed an increase in ERK1/2 phosphorylation. Leptin neutralization either from day –1 to day 1 or on days 8–11 induced sustained phosphorylation of the ERK1/2 molecule compared with control Ig–treated mice. (**E** and **F**) Ex vivo analysis revealed low levels of STAT6 phosphorylation in resting $CD4^+$ T cells from naive mice; conversely, control Ig–treated mice with EAE showed a modest increase in STAT6 phosphorylation. Leptin neutralization also induced marked phosphorylation of STAT6. For each panel, 1 representative experiment of 5 is shown.

Corp.) supplemented with 2% FCS (Invitrogen Corp.), 2 mM L-glutamine (Invitrogen Corp.), 0.1 mM nonessential amino acids (Invitrogen Corp.), 1 mM sodium pyruvate (Invitrogen Corp.), 50 μM 2-mercaptoethanol (Sigma-Aldrich), and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Corp.). Cells were cultured at 37°C in 100% humidity and 5% CO_2 in the presence or absence of varying concentrations of $PLP_{139–151}$ peptide (0–50 μg/ml). As a control for proliferation, anti-CD3 Ab stimulation (2C11, 0.5 μg/ml final concentration; BD Biosciences – Pharmingen) was also performed. After 48–60 hours' culture, cell supernatants (100 μl) were removed from single wells and frozen at –80°C for cytokine assay. IFN-γ, IL-4, and IL-10 were measured by ELISA developed in our laboratory using cytokine-specific capture and detection Abs (Abs R4-6A2 and XMG1.2 for detection of IFN-γ; Abs BVD4-1D11 and BVD6-24G2 for the detection of IL-4; and Abs JES5-2A5 and SXC-1 for the detection of IL-10) according to the manufacturer's instructions (BD Biosciences — Pharmingen). Standard curves for each assay were generated using recombinant mouse cytokines (IFN-γ, IL-4, and IL-10; BD Biosciences — Pharmingen), and the concentration of the cytokines in the cell supernatants was determined by extrapolation from the appropriate standard curve. The lower limits of detection for each assay were 2 pg/ml for IFN-γ, 0.6 pg/ml for IL-4, and 3 pg/ml for IL-10. The remaining cells were incubated for an additional 16 hours, pulsed with 0.5 μCi/well of [^{3}H] thymidine (Amersham Pharmacia Biotech), harvested on glass-fiber filters using a Tomtec (Orange) 96-well cell harvester, and counted in a 1205 Betaplate liquid scintillation counter (Wallac). Results obtained from triplicate cultures are expressed as mean cpm ± SD.

Flow cytometry analysis. FITC-conjugated mAbs to CD11c, CD44, CD25, CD4, and Pan B (B220); PE-conjugated mAbs to CD14, CTLA-4 (CD152), CD40, VLA-4 (CD49d), ICAM-1 (CD54), OX-40, CD8, γδ, and NK; and allophycocyanin-conjugated mAbs to CD45RB and CD4 were all purchased from BD Biosciences — Pharmingen. Isolated spleens and lymph nodes were prepared for flow cytometry by incubating cells with the appropriate Abs or control isotype–matched Abs followed by PBS washes. Data collection and analyses were performed on a FACScalibur flow cytometer (BD Biosciences — Immunocytometry Systems).

Western blot and biochemical analyses. Total cell lysates were obtained in 50 mM HEPES (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1.0% Triton X-100, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and 2 μg/ml each of aprotinin, leupeptin, and pepstatin. Total proteins (50 μg) from each lysate were subjected to SDS-PAGE under reducing conditions. After electrophoresis, proteins were transferred onto a nitrocellulose filter membrane (Protan; Schleicher & Schuell BioScience) using a Trans-Blot Cell (Bio-Rad Laboratories) and transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. Membranes were placed in 5% nonfat milk in PBS plus 0.5% Tween 20 (PBST) at 4°C for 2 hours to block the nonspecific bind-

ing sites. Filters were incubated with specific Abs before being washed 3 times in PBST and then incubated with a peroxidase-conjugated secondary Ab (Amersham Biosciences). After further washing with PBST, peroxidase activity was detected by using the ECL system (Amersham Biosciences). The Abs used were the following: anti-$p27^{Kip-1}$, anti–STAT6, and anti–phosphorylated STAT6 (Y641; all from Cell Signaling Technology Inc.); anti-ERK1/2 and anti–phosphorylated ERK1/2 (Santa Cruz Biotechnology Inc.); and anti-Foxp3 (eBioscience). The filters were also probed with an anti-tubulin Ab (Sigma-Aldrich) to normalize the amount of loaded protein. All filters were quantified by densitometric analysis of the bands utilizing the program ScionImage (version 1.63 for Mac; Scion Corp. Inc.)

Statistics. Analyses were performed using Mann-Whitney *U* test (for unpaired 2-group analysis) and Kruskal-Wallis ANOVA test (for analysis of 3 or more groups). Results are expressed as mean ± SD; *P* values less than 0.05 were considered statistically significant.

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Regulatory CD4 T Cells: Sensing the Environment

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Abstract

The immunosuppressive properties of naturally occurring regulatory T cells (Tregs) have classically been linked to an intrinsic state of hyporesponsiveness, yet paradoxically Tregs are phenotypically in an activated state and have intact proliferative capacity. In consideration of several recent biochemical reports on the intracellular signalling pathways operating in activated $CD4^+CD25^+$ Tregs, we argue that the responsiveness of Tregs depends highly on the local microenvironment. In particular, what influences Tregs to remain anergic or to proliferate arises from their ability to probe the extracellular milieu to respond to external stimuli for the modulation of intracellular signalling events leading to very different quantitative and qualitative functional outcomes.

Regulatory T cells: history of a renaissance

A central problem in immunology has been to detail how peripheral tolerance is maintained. The phenomenon of T cell-mediated suppression of autoimmunity was initially described in the late 1960s (though it was not appreciated as such at the time) (1), and the concept expanded further in the 1970s (2, 3) before a demise of this field of investigation. New impetus came in the mid-1990s after to the description of Sakaguchi and colleagues of the surface expression of CD25 (the IL-2 receptor α chain) as a marker for the identification of a subset of suppressor T cells, the $CD4^+CD25^+$ regulatory T cells (Tregs) (4). Depletion of the $CD25^+$ cells from splenocytes and transfer of the resulting $CD25^-$ T cells to immunocompromised hosts induced autoimmunity, whereas co-transfer of $CD4^+CD25^+$ cells inhibited the induction of autoimmunity (4, 5).

Although several additional immune cell subsets with suppressive capacity have been identified so far, the $CD4^+CD25^+$ Tregs that suppress proliferation and release of pro-inflammatory cytokines from effector cells (6, 7) and/or directly inhibit antigen presenting cells (8, 9) represent by far the most studied immunoregulatory cell type.

In mice kept under specific pathogen free laboratory conditions, the $CD4^+$ T cells that co-express CD25 represent a rather homogeneous immune regulatory cell population, while in humans there is a considerable number of activated, non-regulatory $CD4^+$ T cells that express CD25 (10, 11). Typically, Tregs are more abundant in mice (3 to 10%, depending on the strain) than in humans, in which they only constitute about 1-2% of the $CD4^+$ T cells with highest surface expression of CD25 (12). More recently, they have been also further characterized and identified by their nuclear expression of the forkhead winged-helix transcription factor, Foxp3 (forkhead box p3), whose expression has been proposed to be the crucial factor in the induction of the Tregs population (13). Tregs are classically hyporesponsive to antigenic stimulation

in vitro, a condition reversible upon exposure to interleukin (IL)-2 or strong costimulation (14-18). Because of this property, IL-2 has been used to expand Tregs for adoptive immune therapy and the suppression of autoimmune responses (19, 20). In that regard, it must be noted that significant heterogeneity exists among the Tregs. In addition to the naturally occurring, thymus-derived $Foxp3^+$ Tregs which have a contact-dependent, cytokine-independent mechanism of action, other subsets of $CD4^+$ regulatory T cells with a very similar phenotype and mechanism of action are generated in the periphery (21). Of the peripheral (adaptive) Tregs, two subsets have cytokine-dependent mechanisms of action. One, called Tr1 cells, produce predominantly IL-10 (22), and another, called Th3 cells, produce predominantly TGF-β (23). Each of these Tregs subsets can also express CD25, at least transiently. Horwitz *et al.* have proposed a useful nomenclature in which the natural Tregs would be called "Trn" cells, to differentiate them from the Tregs cells induced in the periphery, and the Th3 cells would be renamed "Tr2" cells (24). Notably, Trn Tregs can induce conventional $CD4^+$ cells to become suppressor cells through a phenomenon called "infectious tolerance" (25), and that Trn-like Tregs generated *ex vivo* can educate $CD4^+CD25^-$ cells to become suppressors (26).

Another way to discriminate between $CD25^+$ Tregs with immunosuppressive properties and recently activated $CD25^+$ T cells relates to a downregulated phenotypic expression of the IL-7 receptor (CD127) (27, 28).

Given all these premises, the best marker for their suppressive capacity remains the expression of the nuclear transcription factor Foxp3 (13), both in mice and in humans (29).

Anergy of regulatory T cells: is it the whole story?

As mentioned above, one central aspect of the biology of essentially all Tregs is their anergic state *in vitro*. Tregs and effector T cells respond differently to TCR stimulation: the former without proliferation or cytokine production; the latter activating specific intracellular pathways that result in the induction of effector

functions including the production of IL-2. These events can be linked in part to a difference in the biochemistry of the two cell types, i.e. Tregs have defects in calcium mobilization as compared to effector T cells (30), and a reduced CD3ζ phosphorylation and ZAP70 recruitment following TCR engagement (31). On the other hand, Tregs can proliferate *in vitro* in response to T cell receptor (TCR) ligation after addition of exogenous IL-2, and the reduced TCR-activated signalling pathways in these cells can be overcome by providing a second messenger such as diacylglycerol, or bypassing the TCR by using phorbol myristate acetate (PMA) and ionomycin (32). These data indicate that Tregs are not unable to signal, but rather have altered signalling which makes them respond to stimuli differently than activated effector T cells. From a biochemical viewpoint, we could consider this aspect of Tregs cell biology similar to that of anergic T cells that have an abortive activation of the *ras* pathway, of the mitogen-activated protein kinase 1 and 2 (MAPK1 and 2), and of the extracellular signal-regulated kinase 1 and 2 (ERK1/2), with concomitant blockade of cell-cycle progression secondary to decreased cyclin E and cyclin A and increased cyclin-dependent kinase inhibitor of the cell-cycle p27kip1 (33).

Regarding the presentation of antigen to Tregs, it appears that dendritic cells can have multiple effects (likely related to stage of maturation, subset of cells, or local microenvironment) on these cells. Dendritic cells can play a critical role in the induction and maintenance of immune tolerance via controlling the differentiation, expansion, and/or immunosuppressive ability of Tregs (34-36), and they could form with the Tregs a regulatory loop in the periphery in which tolerogenic dendritic cells would induce the generation of Tregs and, in turn, Tregs would program the generation of tolerogenic dendritic cells (37). At the same time, dendritic cells could also in some cases block the immunosuppressive capacity of the Tregs (38). In particular, Tregs could modulate phenotype and downregulate the function of dendritic cells (8), as indicated by the finding that Tregs can inhibit development of colitis dependent on accumulation of activated $CD134L^+$ dendritic cells in mesenteric lymph nodes (9). One mechanism possibly involved in this

could be CTLA-4 expressed on the Tregs, which upregulates indoleamine 2,3-dioxygenase (IDO) expression initiating tryptophan catabolism in the dendritic cells (39).

Anergy and proliferation of Tregs

Despite the hyporesponsiveness to antigenic stimulation *in vitro*, Tregs have an intact proliferative potential (30). For example, Tregs unable to flux Ca^{++} after TCR engagement *in vitro* proliferate in response to lymphopenia, and such homeostatic proliferation does not abolish their anergic state but associates with augmented suppressive function (30). Studies of adoptive transfer of Tregs labelled with the fluorescent dye 5, 6-carboxy fluorescein succinimidyl ester (CFSE) have confirmed that Tregs can expand *in vivo*, in both lymphopenic and non-lymphopenic hosts (40, 41). Recently, using the deuterium labelling technique for cell cycling *in vivo*, it was shown that human $CD4^+CD25^+Foxp3^+CD45RO^+$ Tregs proliferate *in vivo* with a doubling time of 8 days, a time considerably shorter than the 24 days of the memory $CD4^+CD25^-Foxp3^-CD45RO^+$ cells or the 199 days of naïve $CD4^+CD25^-Foxp3^-CD45RA^+$ T cells (40). Importantly, proliferating $CD4^+CD25^+Foxp3^+CD45RO^+$ Tregs were found to be highly susceptible to apoptosis, suggesting that ongoing homeostasis helps to control the rapid expansion and/or turnover of these cells *in vivo* (40). Thus, Tregs have the dichotomous capacity to both be anergic *in vitro* and to proliferate *in vivo*, but does this relate to their function?

Anergy and suppression of Tregs

Anergy and suppression of Tregs appear intimately linked, as Tregs operate *in vitro* as hyporesponsive cells capable of actively suppressing the proliferation and release of proinflammatory cytokines from target cells (32, 42). While acknowledging the possibility of resistance of effector immune cells to Tregs-mediated suppression, we will only consider here that in the Tregs the two phenomena of anergy and suppression

123 can be uncoupled, and high doses of anti-CD28 or exogenous IL-2 can abrogate suppression in co-cultures
124 (43).
125 Since Tregs have the capacity to proliferate, one can wonder how the proliferative state of the Tregs can
126 affect their suppressive capacity. Several studies have indicated that *in vitro* expanded adaptive Tregs
127 maintain or even increase their suppressive capacity on target cells (21, 43, 44). Considering that Tregs
128 have a phenotypic profile of highly active cells — and are in a very active metabolic state at the intracellular
129 level – it is likely that they respond promptly to suitable signals coming from the environment to suppress
130 depending on the environmental conditions. These signals would influence a numeric expansion of
131 functional suppressor cells, an acquisition of a suppressive phenotype and/or an upregulated intracellular
132 expression of Foxp3 in individual cells. To better clarify this point it has to be considered that while anergic
133 T cells have suppressive activity *in vitro* (e.g. the so-called 'Civil Service Model' of Waldmann and
134 colleagues) (45), adoptive transfer of anergic cells into animals is not necessarily linked to suppression -
135 unless the anergic state associates with a Tregs cell phenotype. Also, freshly isolated natural Tregs are in
136 an activated state but have relatively modest suppressive capacity as compared to adaptive Tregs that
137 have received additional activation. These differences in the suppressive capabilities of the Tregs may
138 depend, at least in part, on the expression of Foxp3, which promotes the suppression of the Tregs and
139 stabilizes to a large extent anergy and dependence on IL-2, concomitantly repressing the intracellular
140 expression of the cyclic nucleotide phosphodiesterase 3B (*Pde3b*), which is important in permitting
141 homoeostasis and gene expression in the Tregs (46).
142
143 ## Tregs, metabolism, and the environment
144 The identification of the intracellular pathways that can modulate the suppressor program in the Tregs has
145 raised great interest. Antigen stimulation of immune cells activates the transcription factor NFAT, which
146 regulates the state of activation and anergy of T cells. NFAT can promote immune regulation in the Tregs

147 by switching transcriptional partners (47). This aspect is important in that it indicates that Tregs have the
148 capability to "sense" specific signals and subsequently respond to them (Table 1 and Figure 1). A notable
149 example in this regard comes from the work on murine $CD4^{+}CD25^{+}CD127^{low}$ suppressive Tregs, in which
150 stimulation of the TCR upregulates the expression of CD44 (48). CD44 is a cell surface molecule that binds
151 hyaluronan (HA), an extracellular matrix component. High molecular weight (HMW) HA has anti-
152 inflammatory properties (49). However, the activity of hyaluronidases - triggered e.g. by infection or injury -
153 generates low molecular weight (LMW) HA that promotes inflammatory responses (50, 51). Recent work
154 has shown that the state of HA in the extracellular matrix can significantly impact the function of the Tregs.
155 In particular, both HMW and LMW HA bind to Tregs, but the size of HA differentially impacts the functional
156 activity of these cells, and HMW-HA enhances the suppressive capacity of activated Tregs - causing an
157 upregulation of their intracellular Foxp3 expression (52). These findings suggest that the state of HA in the
158 local environment is one mechanism by which Tregs can receive cues about an inflammatory milieu (52)
159 and respond accordingly. In this context, it could be suggested that although no specific homing receptor
160 has been identified as specific for Tregs, the integrin $\alpha E\beta 7$ (CD103) and the chemokine receptor CCR6 –
161 which are selectively expressed by these cells at high levels - might help the direction and retention of
162 Tregs in inflamed sites to locally balance the activity of effector T cells (53).
163 Additional work pointing at the ability of Tregs to respond to the local environment comes from recent
164 findings indicating that Tregs in mice can depend on folic acid for their maintenance in the periphery, and
165 expression of a subtype of the receptor for folic acid, the folate receptor 4 (FR4), may enable them to bind
166 and incorporate folic acid efficiently (Table 1 and Figure 1). Folic acid is a vitamin that downmodulates
167 plasma homocysteine levels - which are frequently elevated in patients with autoimmune disease such as
168 lupus and rheumatoid arthritis (54). The high constitutive surface expression on Tregs of FR4 – which is
169 controlled by Foxp3 - makes the administration of FR4 monoclonal antibody responsible for a reduction of
170 the number of Tregs and subsequent autoimmune disease in normal mice (55).

Another example is the expression on Tregs of membrane-bound CD39 and CD73, which are ectonucleotidases involved in catabolism that causes the generation of extracellular adenosine from extracellular nucleotides such as ATP and UTP (56, 57). For the immune system, extracellular ATP represents an indicator of tissue damage. Adenosine has important suppressive functions on activated T cells through the activation of adenosine receptors that lead to increased intracellular levels of cyclic adenosine monophosphate (cAMP), a second messenger present in cells with a high metabolic rate and well known for its ability to inhibit T cell proliferation (58). Tregs have a high intracellular content of cAMP (59). Of note, CD39 is expressed by Tregs, where it can convert extracellular ATP into the catabolite exerting suppressive properties on T cell proliferation and IL-2 secretion (56, 57). In normal conditions, after T cell activation through the TCR-MHC-CD28 complex, a peak of intracellular concentration of cAMP is detected rapidly, and is followed by rapid degradation by cAMP-phosphodiesterases (PDE) recruited into the cell membrane lipid rafts (58). Tregs could rapidly generate and directly provide cAMP to effector T cells in a cell contact-dependent fashion, through the formation of gap-junctions (59).

Also considering that the local environment reflects the metabolic state of the host, Tregs may need to be able to rapidly adjust their activity depending on what would be most expedient for the overall economy of the host. The presence of the proinflammatory cytokine-like hormone leptin in the *milieu* – which reflects the caloric balance of the host - can provide an excellent example of how the presence of a molecule reflecting metabolic excess can promote negative signalling for the proliferation of Tregs through inhibitory signalling (*via* the leptin receptor - which is highly expressed on the cell surface of Tregs (60) (Figure 1). When Tregs are stimulated *in vitro* with anti-CD3 and CD28 and leptin is blocked with anti-leptin neutralizing antibodies, hyporesponsiveness of these cells is abrogated, and Tregs can proliferate vigorously, maintaining their suppressive capacity (60).

194 Another aspect worth consideration is how intracellular metabolism can affect the suppressive capacity and
195 ability of Tregs to expand *in vitro.* A possible answer to this question may come from the analysis of Tregs
196 responses to rapamycin, an immunomodulator that can both inhibit the proliferation of effector T cells and
197 facilitate the expansion of natural Tregs *in vitro* expansion (61, 62). Rapamycin is an inhibitor of the
198 nutrient-sensitive kinase mammalian target of rapamycin (mTOR) (63), a macromolecular protein complex
199 with serine-threonine kinase activity that regulates cell-cycle progression and growth by sensing changes in
200 the energy status of the extracellular *milieu* (Figure 1). mTOR represents a cellular "fuel sensor" activated
201 by amino acids, glucose availability, growth factors, and certain hormones (64). The fact that rapamycin
202 exerts opposite effects on Tregs and effector T cells (65) reiterates the concept that in basal conditions the
203 two cell types may be under different metabolic conditions. Effector T cells may have mTOR inactive, and
204 the inhibition would prevent cell growth and expansion. Conversely, Tregs may have mTOR active, and can
205 thus proliferate *in vivo*. The fact that inhibition of mTOR with rapamycin results in the capacity of Tregs to
206 grow and expand *in vitro* (61, 66) is in line with the observation that Tregs produce high levels of cAMP -
207 which can activate mTOR (67) - suggesting an involvement of the intracellular metabolism of Tregs in the
208 mechanisms of proliferation of these cells following influences by the local environment.
209
210 The "on/off hypothesis"
211 In view of the above considerations, we propose the "on/off hypothesis" to explain why Tregs, which have a
212 very high metabolic rate, can be influenced in their activity and/or proliferative potential by the local
213 environment more significantly than effector T cells (Table 1 and Figure 1). Tregs and effector T cells both
214 appear somehow as lymphocytes in a resting phase, yet they differ significantly for their metabolic activity -
215 high for the Tregs, slow for the T cell effectors. This condition could be compared to two cars that are
216 aligned at a specific position and that are not moving. Looking at the two cars, an external observer would
217 not know whether the engine of each car is "on" (high metabolic rate) or "off" (low metabolic rate). The car

consuming fuel and energy with the engine on would be the Tregs, the car not consuming fuel and energy, with the engine off, would be the T effector. Despite that the status of the two cars would not be readily apparent from afar, visibly different outcomes would appear when depressing the accelerator pedal in the two cars: Tregs would rapidly go, T effector would stay still unless “pre-activation” (ignition) had been provided. Ultimately, the environment (ignition) would maximally affect the subsequent ability to operate in the two conditions - the high metabolic rate making the Tregs more sensitive to immediate stimulation and response. While this would contrast with the T effectors, the latter cells would have more energy available for longer lasting responses (because they did not consume much energy). In general, this phenomenon would contribute to a capacity of Tregs to operate quickly and in accordance with environmental signals (i.e. catabolizing extracellular ATP and amino acids and/or using cAMP to inhibit effector T cell proliferation), yet this activity might be confined in time because of the elevated metabolic rate of these cells (Table 1 and Figure 1).

Concluding remarks

It has been suggested that tissues can send signals that initiate immunity and guide the type of effector responses (46). Since the delicate function of peripheral immune tolerance can be easily compromised by powerful, disruptive effector mechanisms, immune regulation needs to be preserved in the face of opposing immune effector mechanisms. We think that one such mechanism could be the ability of Tregs to probe the environment for rapid adjustment. As immune cells, Tregs are in constant communication with their environment through soluble mediators or *via* cell contact, to respond to cues that can positively or negatively tune their function. The recent discovery of biochemical events linked to the responsiveness of Tregs to environmental influences could be possibly exploited to harness the beneficial potential of these cells in the control of autoimmune responses.

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246

Disclosure

The authors declare no conflicts of interest.

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384 Table I. Schematic aspects of the “on/off hypothesis”

	Treg	Teff	References
Metabolic rate	++	-	55-67
Sensitivity to environmental stimuli	+++	+	39, 55-60
Rapidity of response	+++	+	20, 40, 41, 44,
Length of response	+	+++	30-32

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