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Leptin and Autoimmunity: role of leptin in the modulation of immune response during Experimental Autoimmune Encephalomyelitis

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INTRODUCTION

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 (1). Energy intake and energy expenditure undergo substantial daily and seasonal fluctuations, however.

Immunity requires adequate and balanced energy supply for optimal function (2). Although the risk of infection and death is highest when energy reserves are not sufficient (3), obesity, a state of energy excess, has also been associated with increased susceptibility to infection, bacteremia, and poor wound healing (4).

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 (5). 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 (5). 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 (6). 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 (7), haematopoiesis (8), angiogenesis (9,10), insulin secretion (5), metabolism of bone (11), lipids and glucose (1) and, last but not least, innate and adaptive immunity.

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 (5). 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 (5).

Leptin is classically considered a hormone because it regulates the balance between food intake and energy expenditure, signalling to the brain the changes in stored energy. Synthesized primarily by the white adipose tissue, leptin is secreted at lower levels by the gastric mucosa, placenta, mammary epithelium, and skeletal muscle (5). 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 (5). Moreover, 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 (5).

Many studies have linked the immune and neuroendocrine systems (12, 13). 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 (12, 13). 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 (12, 13). 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 (14). In the 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 (15-21). 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 (22-24). So, although leptin has well documented pro-inflammatory properties, it seems that it might act as an acute-phase reactant in some conditions and not in others.

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.

The fact that leptin has effects on both the neuroendocrine and immune systems should not come as a surprise, given the functional connection and anatomical contiguity between adipocytes and lymphoid cells (6). Morphologically, aggregations of lymphoid tissue, including the lymph nodes, omentum, thymus and bone marrow, are associated with adipose tissue (6). 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 (6). 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). For example, TNF and chemokines promote the differentiation of adipose tissue and leptin secretion, which in turn sustains the differentiation of T helper 1 (T_H1) cells (see later) (25, 26).

Leptin signalling 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 (27, 28). 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) (29). 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 (30, 31). 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 (30, 31). 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 the only form able to transduce the signal and is expressed by the hypothalamus in areas that are responsible for the secretion of neuropeptides and neurotransmitters that regulate appetite, body weight (30, 31) and bone mass (11). Interestingly, OBRb is also expressed by endothelial cells, pancreatic β-cells, the ovary, $CD34^+$ haematopoietic bone-marrow precursors, monocytes/macrophages, and T and B cells (5, 9,10, 30, 31). The expression of OBRb by T and B cells is of interest as it indicates a possible role for leptin in immune-cell activation and signal transduction, and might unveil new effects of leptin on as-yet-unexplored immune-cell functions (32, 33, 34). 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) (30-34) (figure 1).

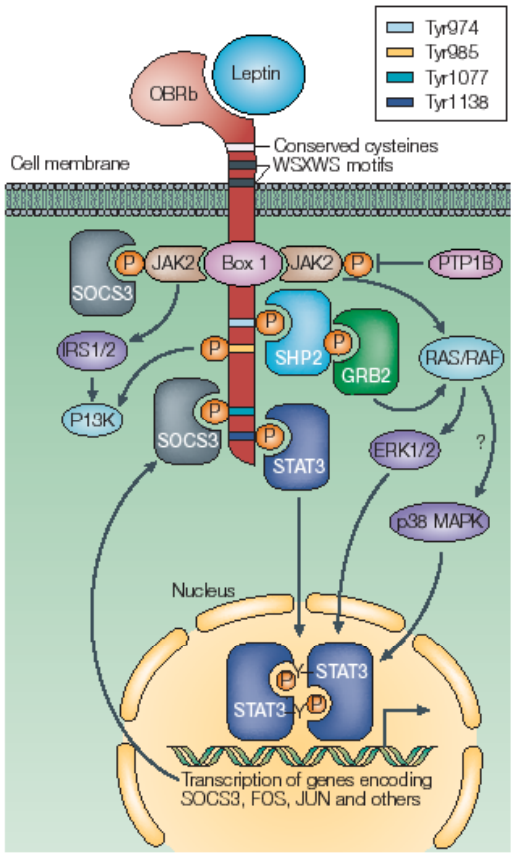


Fig.1 Schematic representation of leptin signalling

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 (30-37). 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) (30-37) (figure 1). Moreover, Src associated in mitosis protein (Sam68), an RNA-binding protein, regulator of RNA metabolism and effector of the PI3'K is currently thought to function as an adaptor protein by binding to activated STAT-3 and to the p85 subunit of PI3'K (35) 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.

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 (38, 39). Similarly, humans with congenital leptin deficiency have a much higher incidence of infection-related death during childhood (40), whereas recombinant human leptin (rmetHuLeptin) administration in two children with congenital leptin deficiency normalized absolute numbers of naive CD4+CD45+RA T cells and nearly restored the proliferation response and the cytokine release profile from their lymphocytes (41). 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 (42, 43) (figure 2). Leptin seems to promote activation of and phagocytosis by monocytes/macrophages and their secretion of leukotriene B4 (LTB4), cyclooxygenas 2 (COX2), nitric oxide and pro-inflammatory cytokines (44-46). 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 (44-46). Moreover, leptin can induce chemotaxis of neutrophils and the release of oxygen radicals (such as superoxide anion and hydrogen peroxide) (47,

48).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 (49). Leptin also affects natural killer (NK)-cell development and activation both *in vitro* and *in vivo* (50-52). 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 (50-52) .

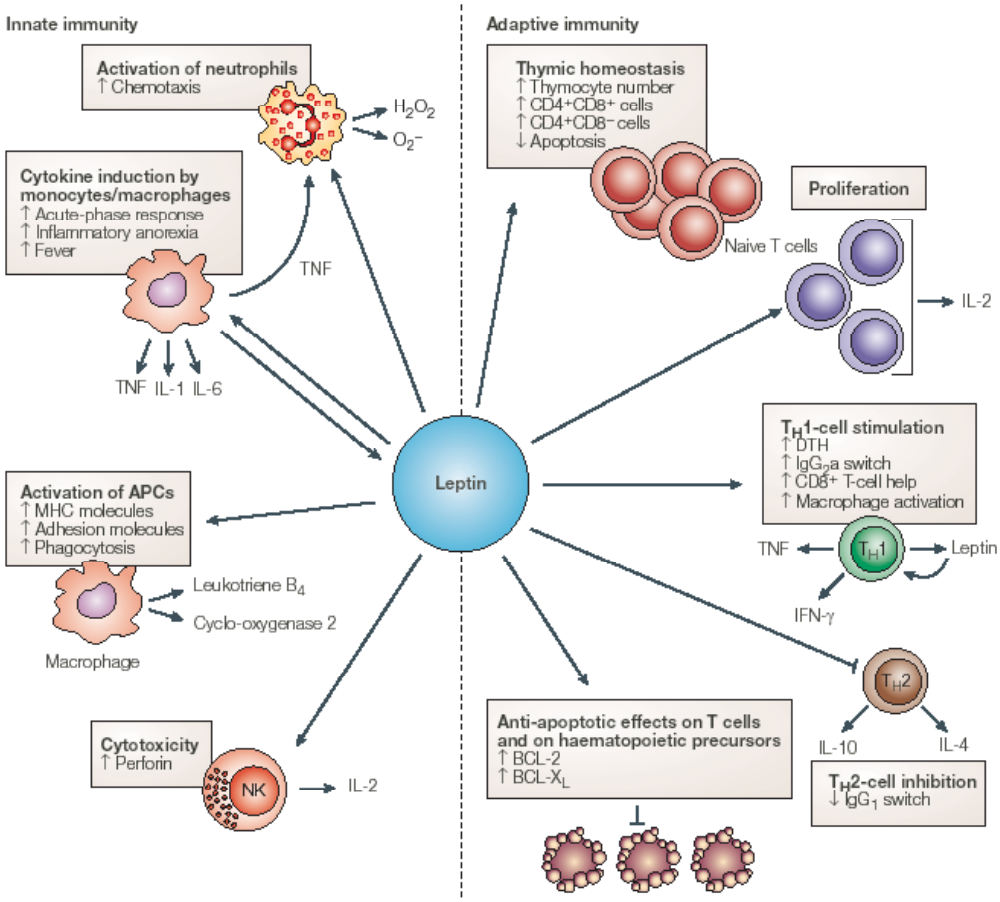


Fig.2 Schematic representation of the effects of leptin on both innate and adaptive immunity.

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 (46).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 immue cells(46).

The effects of leptin on adaptive immune responses have been extensively investigated on human CD4+ T cells (figure 2). Addition of physiological concentrations of leptin to a Mixed Lymphocytes Reaction (MLR) induces a dose-dependent increase in CD4+ T-cell proliferation. 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) (53). 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-γ (IFN-γ). Increased expression of adhesion molecules could then be responsible for the induction of clustering, activation and migration of immune cells to sites of inflammation (53). 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. 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 (54, 55). 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 (54). Despite the evidence of direct effects of leptin on immune responses *in vitro*, a major problem remains in ascertaining 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 (55). 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 abnormalities (55). 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 deficiency (55).

Regulatory T cell

The immune system has devised many checks and balances to circumvent autoimmune disease. Those mechanism have been broadly categorized as influencing central or peripheral tolerance, each having a nonredundant function in maintaining antigen-receptor diversity while providing safeguards to effectively curtail self-reactivity. Peripheral tolerance to self was suggested several years ago to result from the induction of anergy in peripheral self-reactive lymphocytes. More recently, however, it has become clear that avoidance of damage to the host is also achieved by active suppression mediated by regulatory T cell (Tregs) populations.

The $CD4^+CD25^+$ T cell subset is currently the focus of intensive research. T_{Regs} are known to dampen autoreactive responses mediated by $CD4^+CD25^-$ T cells and may influence the onset and progression of autoimmunity (56). 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 (56, 57). These cells represent 5−10% of the $CD4^+$ T lymphocytes in healthy adult mice and humans and are thought to perform a specialized role in

controlling both the innate and the adaptive immune system (58-60) and exhibit a vast spectrum of autoimmunity-preventive activity.

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-β (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 (61, 62). Reverse signalling through cross-linking 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 (63). However, like IL-10 and TGF-β, CTLA-4 does not seem to be a non-redundant mechanism of suppression, as Treg cells isolated from mice with the targeted deletions of genes encoding each of these molecules are suppressive *in vitro* (64, 65). 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 (66). Finally, it has been proposed that Treg cells may suppress immune activation by 'soaking up' T cell growth factors such as IL-2 (67). 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*.

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 (59). 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 (68). 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 (68, 69). Those data and results, suggesting that the maintenance of CD25 expression on Treg cells depends on IL-2 (70) envision the possibility of a relatively simple regulatory network whereby the maintenance and suppressive activity of Treg cells is conditional on IL-2 production by non-regulatory 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 non-immune mice and humans, accurate discrimination between Treg cells and recently activated non-regulatory T cells, which up-regulate 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 non-regulatory 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 (71).

The forkhead/winged helix transcription factor Foxp3 was shown to be specifically expressed by CD25+ Tregs cells, as well as by CD25- T cells with regulatory activity (72-74). This transcription factor is though to program the development and function of this subset and so far is the most unambiguous marker available to identify naturally occurring Tregs cells.

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 (75). 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 (75). 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.

Loss-of –function mutations in this gene, in both humans and mice, caused the absence of Tregs cells (see fatal human autoimmune disorder 'immune dysregulation, polyendocrinopathy, enteropathy, X-linked' (IPEX). Furthermore, 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 (73).

Precise signals that promote the development of Treg cells remain elusive, but considerable evidence suggests that costimulatory molecules and cytokines play important roles (76-79). Depending on these additional signals, thymocytes are then either negatively selected or induce a genetic program for Treg cells, including up-regulation of Foxp3 and CD25. Other mechanisms suggested that something about the nature of the TCR, including its affinity for ligand and/or the level of expression, instructs the cells to become Treg cells (80-83). On the other hand van Santen et al. (84) suggested that perhaps the niche or the context in which the T cell encounters antigen in the thymus is more important than affinity in determining cell fate.

Analysis of TCR from Treg cells has demonstrated that a large proportion of this population has a higher avidity to self-antigen in comparison with TCR from CD4+CD25- cells (82) and that peripheral antigen is required for their development, maintenance, or expansion. Treg cells have been shown to undergo expansion in the periphery, likely regulated by the presence of self-

antigen (85, 86). Neonatally thymectomized mice, which are deficient in Treg cells, develop multiorgan autoimmune disease, which can be overcome by the adoptive transfer of this population of T cells from normal mice. Several clinical observations in humans have supported a link between reduced thymic function, with impaired Treg cell generation, and the induction of autoimmune diseases, suggesting a central role for these cells in self-tolerance (87).

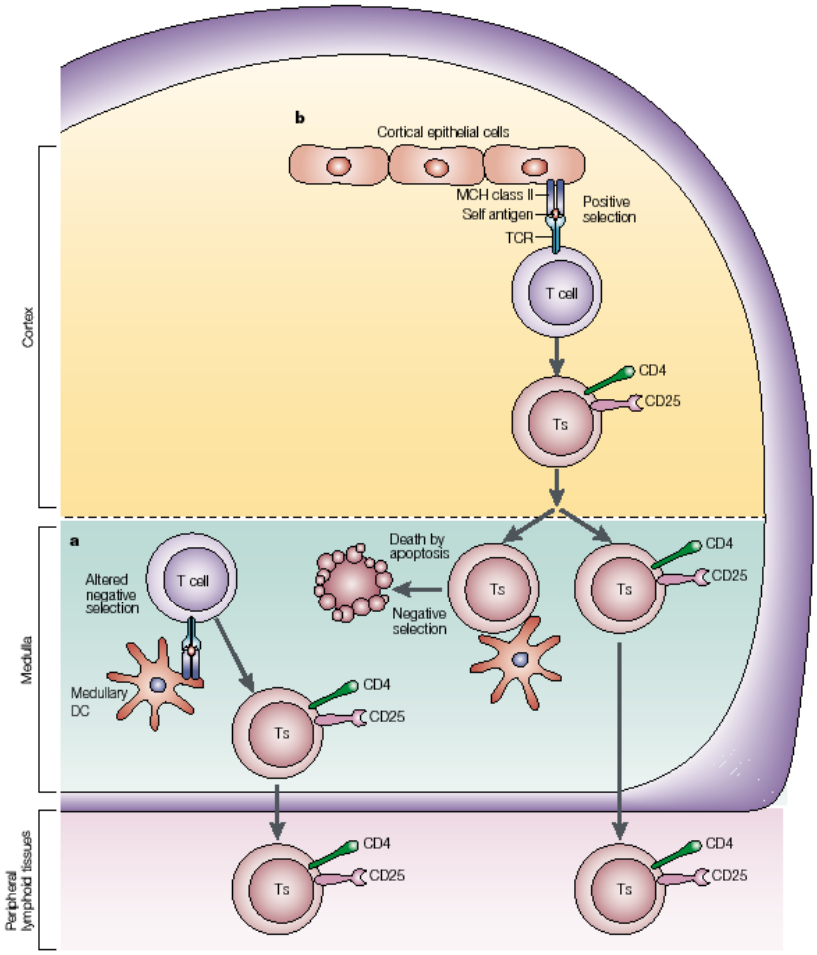


Figure 3. CD4+CD25+ T cells seem to be members of a unique lineage of T cells that are selected during the process of T-cell differentiation in the thymus. It remains unclear where and when this occurs. **A)** One possibility is that CD25+ T cells acquire expression of CD25 and suppressor function in the thymic medulla, where they recognize self-antigens that are presented on MHC class II molecules by medullary dendritic cells (DCs) in a process that is known as 'altered negative selection'. They then migrate directly to peripheral lymphoid tissues. **B)** Studies with the K14 transgenic mouse have indicated that CD25 expression and suppressor function is acquired at a much earlier stage of differentiation in the thymic cortex during the process of positive selection on cortical epithelial cells. Some of these CD25+ T cells then undergo a process of negative selection on bone-marrow-derived cells (such as DCs) in the medulla and die by apoptosis, but others are allowed to migrate to peripheral lymphoid tissues, according to the affinity of their TCR for self-antigens. TCR, T-cell receptor; Ts, suppressor T cell.

Leptin in Autoimmunity

As mentioned earlier, ob/ob mice have several abnormalities that are common to starved animals (5). However, ob/ob (and db/db) mice also have additional endocrine and metabolic disturbances that could affect the immune system indirectly, such as hypercorticosteronaemia and diabetes (5). Similarly, starvation not only associates with hypoleptinaemia, but also with an increased concentration of glucocorticoids and decreased levels of thyroid and growth hormones (which can result in immune suppression) (5). So, the effects of leptin on the immune system should take into account both the direct and indirect effects of this molecule on other hormones. Although the influence of thyroid and growth hormones on the effects of leptin remains elusive, it seems that leptin can affect thymic output and T-cell function independently of glucocorticoids, as congenitally leptin-deficient individuals have glucocorticoid levels within a normal range, but markedly reduced numbers of naive T cells (5).

More importantly, ob/ob mice have reduced secretion of IL-2, IFN-γ, TNF and IL-18, and increased production of T_H2-type cytokines, such as IL-4 and IL-10, after mitogenic stimulation. As a result, ob/ob mice are resistant to the induction of several experimentally induced autoimmune diseases, for example, AIA (ANTIGEN-INDUCED ARTHRITIS), which is a model of immune-mediated joint inflammation induced by administration of methylated bovine serum albumin (mBSA) into the knees of immunized mice (88). The severity of arthritis in leptin and leptin-receptor-deficient mice was reduced. The milder form of AIA seen in ob/ob and db/db mice, as compared with controls, was accompanied by decreased synovial concentrations of IL-1β and TNF-α (Th1-type cytokines), decreased *in vitro* proliferative response to antigen in lymph node cells, and a switch toward the production of Th2 cytokines (88). Serum levels of anti-mBSA antibodies were also significantly decreased in the arthritic ob/ob mice, as compared with controls.

Thus, in AIA, leptin may probably contribute to joint inflammation by regulating both humoral and cell-mediated immune responses. However, joint inflammation in AIA depends on adaptive immune responses, which are impaired in ob/ob and db/db and mice. More recent studies have investigated

the effect of leptin and leptin receptor deficiency on the inflammatory events of zymosan-induced arthritis (ZIA), a model of proliferative arthritis restricted to the joint injected with zymosan A and not dependent on adaptive immune responses (89). ZIA, in contrast to AIA, was not impaired in ob/ob and db/db mice. However, the resolution of acute inflammation was delayed in the absence of leptin or leptin signaling, suggesting that leptin could exert beneficial influences on the evolution of this model of arthritis (90).

In humans, patients with rheumatoid arthritis (RA) with reduced serum leptin levels induced by fasting reportedly had improved clinical and biological measures of disease activity associated with a decrease of $CD4^+$ lymphocyte activation and a shift toward Th2 cytokine production (91). These aspects, resembling somehow those seen in AIA in ob/ob mice, suggested that leptin could also influence inflammatory arthritis in humans through an influence on Th1 responses.

Ob/ob mice are also protected from EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE), whereas administration of leptin to susceptible wild-type mice worsens EAE by increasing the secretion of pro-inflammatory cytokines and directly correlates with pathogenic T-cell autoreactivity (see later for further details). Protection of *ob/ob* mice from autoimmunity is also observed in EXPERIMENTALLY INDUCED HEPATITIS (EIH) (91, 92). Activation of T cells and macrophages is one of the initial events during viral or autoimmune hepatitis. Activated T cells are directly cytotoxic for hepatocytes and release proinflammatory cytokines, which mediate hepatocyte damage. A well-described mouse model of T-cell-dependent liver injury is the one induced by i.v. injection of the T cell mitogen concanavalin A (Con A), which results in fulminant hepatitis. During Con-A-induced hepatitis, TNF-α is a crucial cytokine in the acute disease process because neutralization of this cytokine reduces liver damage. On the other hand, the injection of TNF-α causes acute inflammatory hepatocellular apoptosis followed by organ failure, and TNF-α thus appears to cause hepatoxicity. Siegmund et al. (92) showed that leptin-deficient *ob/ob* mice were protected from Con-A-induced hepatitis. TNF-α and IFN-γ levels, as well as expression of the activation marker CD69, were not elevated in *ob/ob* mice following administration of Con A, suggesting that their resistance was

associated with reduced levels of those proinflammatory cytokines, together with low percentages of intrahepatic NKT cells (which are cells that contribute to progression of this disease) (92). Similar results were obtained in EIH induced by *Pseudomonas aeruginosa* exotoxin A administration (91). Also in this case, leptin administration restored responsiveness of *ob/ob* mice to EIH, and T lymphocytes and TNF-α were required for the induction of liver injury. The authors also showed that leptin played an important role in the production of two proinflammatory cytokines in the liver, namely TNF-α and IL-18 (91). Finally, ob/ob mice are resistant to acute and chronic intestinal inflammation induced by dextran sodium sulphate and to colitis induced by trinitrobenzene sulphonic acid (EXPERIMENTALLY INDUCED COLITIS, EIC) (93).In acute EIC, *ob/ob* mice do not develop intestinal inflammation and show decreased secretion of pro-inflammatory cytokines and chemokines. As expected, leptin replacement increases cytokine production to the levels observed in control mice (93). Of interest, recent reports have shown that leptin secreted by the gastric mucosa is not completely degraded by proteolysis and can therefore reach the intestine in an active form, where it can control the expression of sodium/glucose and peptide transporters on intestinal epithelial cells (94, 95). As a result, leptin might have a dual nature: on one hand, leptin could function as a growth factor for the intestine, because of its involvement in the absorption of carbohydrates and proteins; on the other hand, leptin could function as a mediator of intestinal inflammation (93-95).

More recently, protection from autoimmunity in *ob/ob* mice has been observed in EXPERIMENTALLY INDUCED GLOMERULONEPHRITIS (96). In this immune-complex-mediated inflammatory disease induced by injection of sheep antibodies specific for mouse glomerular basement membrane into mice preimmunized against sheep IgG, the authors observed renal protection of ob/ob mice associated with reduced glomerular crescent formation, reduced macrophage infiltration, and glomerular thrombosis. These protective effects were associated with concomitant defects of both adaptive and innate immune response (testified by reduced *in vitro* proliferation of splenic T cells and reduced humoral responses to sheep IgG, respectively). Finally, evidence that leptin may exert pathogenic effects in immune-mediated disorders of the kidney come from the finding that leptin is a renal growth and profibrogenic

factor that contributes to endocapillary proliferation and subsequent development of glomerulosclerosis during renal damage in conditions possibly including diabetes and obesity, both characterized by high circulating leptin levels (97).

All these studies concern a role for leptin in experimentally "induced" autoimmunity. However, leptin is also important in "spontaneous" autoimmune DIABETES in non-obese diabetic (NOD) mice (98). Leptin accelerates autoimmune diabetes in females NOD/LtJ mice (99, 100). 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 (99). 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 (99). 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 (101, 102). 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. This model nicely complements the previously published data from our group, hypothesizing a key role for leptin in the development of T1D. 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.

Another indication that leptin could be involved in autoimmunity is the sexual dimorphism of serum leptin concentration (higher in females than in males

matched for age and body mass index). In this sense, leptin could be added to the list of hormones, such as oestradiol and prolactin, that have long been known to have a role in favouring the predisposition of females to the development of autoimmunity (103). In particular, only hyperleptinaemic female mice develop autoimmunity, whereas hypoleptinaemic mice are protected, and treatment of EAE-resistant SJL/J males with recombinant leptin renders them susceptible to EAE (103).

Leptin in organ-specific autoimmunity of the central nervous system: the case of Multiple Sclerosis and EAE.

Immunologists look at multiple sclerosis as an autoimmune disease, in which T-lymphocytes specific for myelin antigens start an inflammatory reaction in the central nervous system, which ultimately leads to demyelination and subsequent axonal injury. This view of multiple sclerosis as a T-cell-mediated autoimmune disease is derived primarily from studies on a single animal model, experimental autoimmune encephalomyelitis (EAE). The origins of EAE date back to the 1920s, when Koritschoner and Schweinburg induced spinal cord inflammation in rabbits by inoculation with human spinal cord. Since then EAE was elicited in many different species, including rodents and primates, and from these studies it became clear that EAE can reproduce many of the clinical, neuropathological and immunological aspects of multiple sclerosis (104).

Multiple sclerosis (MS) is a chronic, immune-mediated, inflammatory disorder of the central nervous system (CNS) (105). Clinically the illness may present as a relapsing–remitting disease, or with steady progression of neurological disability. The subsequent course of disease is unpredictable, although most patients with a relapsing–remitting disease will eventually develop secondary progressive disease. Its pathology is, in part, reflected by the formation of focal inflammatory demyelinating lesions in the white matter, which are the characteristic hallmarks in patients with acute and relapsing disease (106, 107). In patients with progressive disease, the brain is affected in a more global sense, with diffuse but widespread (mainly axonal) damage in the normal appearing white matter and massive demyelination also in the grey matter, in

particular in the cortex (108, 109). The mechanisms of tissue injury in focal white matter lesions are heterogeneous, resulting in patterns of demyelination that vary between patients or patient subgroups (106). The destruction patterns in the multiple sclerosis plaque can include a cytotoxic attack via T-cell and macrophages inflammation (with the secretion of perforin and granzyme as effector molecules directed towards the target), as well as a humoral-mediated destruction of the myelin sheat via local deposition of antibodies, which then can activate complement (fugure 4). Furthermore, there is a high inter-individual variability in the extent of axonal damage as well as remyelination and repair. The reason for this complex situation is largely unknown, although it is likely that genetic factors influencing immune-mediated inflammation as well as neuronal and glial survival may play a major role in modulating the phenotype of the disease (106).

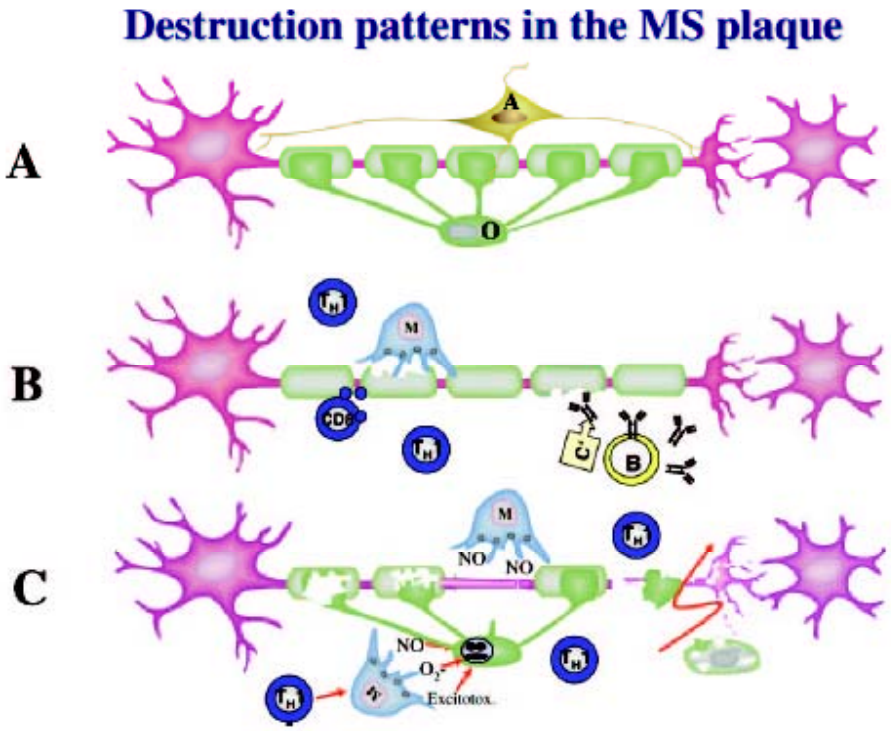


Figure 4. Destruction patterns in the multiple sclerosis plaque. **A)** In the healthy CNS oligodendrocytes enheathe the axon and form myelin internodes of regular size. **B)** Cytotoxic attack can destry the myelin sheat via T-cell and macrophage inflammation. Cytotoxic T cells secrete perforin and granzyme as effector molecules directed towards the target (left). Humoral factors destry the myelin sheat via local deposition of antibodies, which then activate complement (right) or phagocytic effector cells via ADCC (not shown). **C)** Damage towards the oligodendrocyte and the axon is mediated via cytotoxic products of macrophages/microglia (left), with nitric oxide (NO) as one of the major constituents. Note that the oligodendrocyte shows typical morphology of apoptosis. On the right side, the diffuse pattern of axonal and myelin destruction is illustrated, where as yet no unequivocal pathogenetic mechanism has been identified.

As previously said, 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 (110). It has been shown that leptin is involved in both the induction and in the progression of EAE (110). Genetically, leptin-deficient ob/ob 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 (110). Importantly, a surge of serum leptin anticipates the onset of clinical manifestations of EAE (111). The peak of serum leptin correlates with inflammatory anorexia, weight loss, and the development of pathogenic T cell responses against myelin (111). 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 (111) (figure 5). 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 T_{Regs} – a key subset of lymphocytes involved in the suppression of immune and autoimmune responses that is reduced in patients with MS as compared with healthy matched controls (112). Of note, the number of peripheral T_{Regs} in patients with MS inversely correlates with the serum levels of leptin, suggesting a link between the number of T_{Regs} and leptin secretion (112). Considering that T_{Regs}

are generated in the thymus, it is not known whether peripheral leptin or that produced in the perithymic adipose tissue could affect T_{Regs} 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) (112). 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 (113). 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 (110, 111). 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 (112). 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 (112). Finally, recent reports (114) 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 (114). 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.

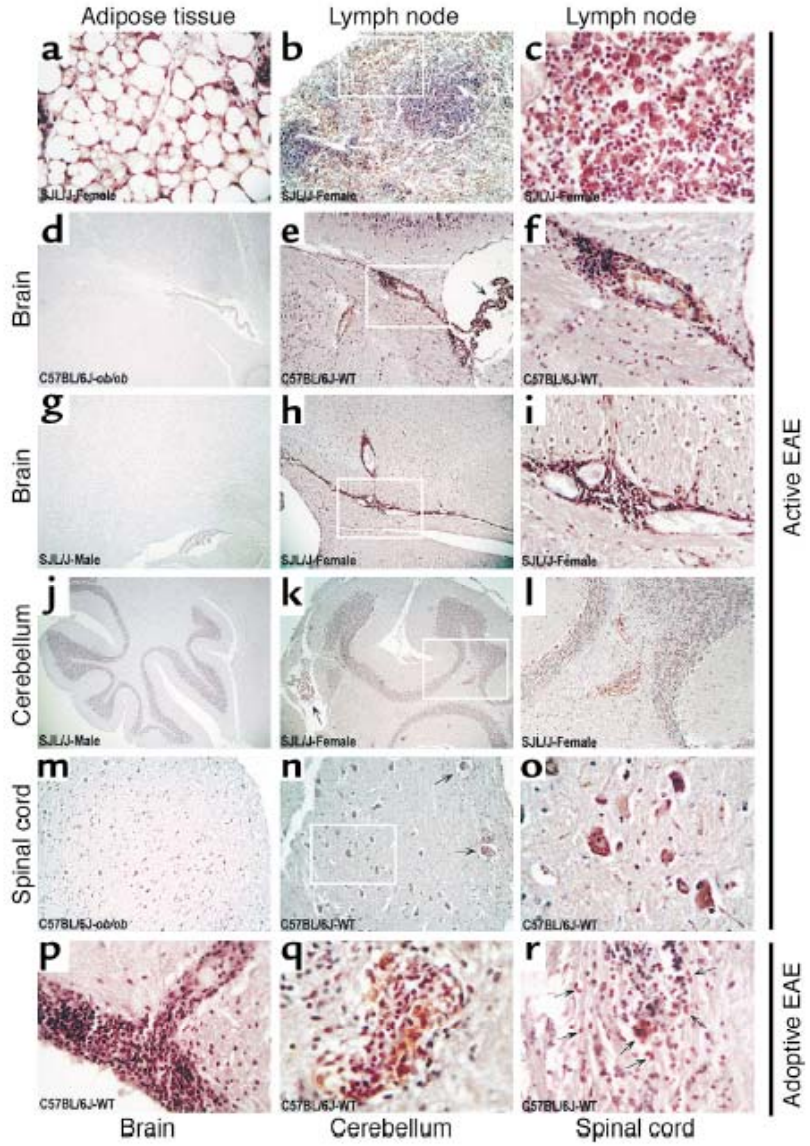


Figure 5. Lymph node and CNS expression of leptin during acute/active EAE. (**A**) Leptin expression in SJL/J female mouse adipose tissue used as positive control. (**B** and **C**) Expression of leptin in T cells and macrophages in a draining lymph node from SJL/J female mice after immunization with PLP139–151. (**D**) Leptin was not expressed in the brain of C57BL/6J ob/ob mice after immunization with MOG35–55 peptide (n = 4). (**E** and **F**) Expression of leptin in inflammatory infiltrates (white square) and in choroid plexus (arrow) during the acute phase of EAE in C57BL/J6 WT mice (n = 4). (g) Leptin was not expressed in the brain of SJL/J male mice after immunization with PLP139–151 peptide (n = 6). (**H** and **I**). Leptin expression in inflammatory lesions in the acute phase of EAE in SJL/J female mice (n = 6). (**J**) Cerebellum of SJL/J male mice did not express leptin after immunization with PLP139–151 peptide, whereas in k and l leptin was expressed in inflammatory infiltrates (white square) and choroid plexus (arrow) of SJL/J females. (**M**) Spinal cord C57BL/J6 ob/ob mice immunized with MOG35–55 peptide did not express leptin. (**N** and **O**) Expression of leptin in neurons (white square in n) and two inflammatory infiltrates around blood vessels (arrows in n) detectable during the acute phase of EAE in C57BL/6J WT mice spinal cord. (**P-R**) Leptin expression was revealed in T cells present in inflammatory infiltrates of the brain, cerebellum, and spinal cord (arrows) of C57BL/J6 WT mice after adoptive transfer, but it was not detectable in the CNS of C57BL/6J ob/ob mice after adoptive transfer (not shown). The white squares in b, e, h, k, and n represent the zone of higher magnification shown in c, f, i, l, and o, respectively.

AIM OF THE STUDY

Leptin is a cytokine-like hormone ,mainly produced by adipose tissue, that links nutritional status with the immune system. Data from literature suggest that, acting as a cytokine, leptin is able to promote 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. Moreover the pivotal role of leptin in autoimmune disease has been highlighted by the observation that leptin-deficient *ob/ob* mice are resistant to induction of EAE, an animal model of MS.

We have recently reported that leptin administration to susceptible mice worsens EAE, by increasing secretion of pro-inflammatory cytokines. In a similar fashion, leptin replacement in leptin-deficient *ob/ob* mice restores susceptibility to both active and passive EAE. Since we and others have also described that leptin is expressed in active inflammatory lesions of the CNS during acute EAE and MS, the first aim of this study was to investigate the molecular and cellular effects induced by leptin neutralization either with anti-mouse leptin Abs or with soluble mouse leptin receptor chimera-Fc (ObR:Fc), on induction and progression of EAE (phenotypically evaluated in terms of changing in clinical score). More in detail, we analyzed whether leptin blockade could interfere with autoreactive T cells proliferation, cytokine profile secretion, expression of regulatory (Foxp3) and activation markers (ICAM-1, VLA-4, OX-40). We also evaluated the biochemical changes induced by leptin neutralization in the molecular machinery of autoreactive CD4+ T cells, analyzing a series of biochemical markers specific for T cell activation, anergy, and Th2/regulatory-type cytokine secretion.

In the second part of this study we evaluated the possible interaction between leptin and regulatory T cell (CD4+CD25+Foxp3+ cells), to determine whether the protection from EAE onset and progression, mediated by leptin neutralization, could be ascribed to a direct effect of this treatment on regulatory T cell subset, which are known to regulate immune tolerance, inhibiting the autoreactive responses occuring during autoimmune diseases. For this purpose we analyzed leptin receptor expression on Tregs,

and how leptin neutralization could interfere with their state of hyporesponsiveness and suppressive function. Finally to better clarify and further characterize the phenomenon of Tregs expansion, we performed in vivo experiments in animal models of acute (anti-leptin treated mice) and chronic leptin deficiency (ob/ob mice). The full understanding of Tregs biology will allow us to imagine a scenario of a huge number of autoimmune settings where T_{regs} are isolated from patients either during remission or soon after disease onset and after their expansion, mediated by leptin neutralization, they could be reintroduced at the time of maximal disease activity to moderate the inflammatory response.

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 (which is a chimeric antagonist of leptin, constituted by the extracellular domain of mouse leptin receptor fused to the Fc region of human IgG_1) to affect induction and progression of EAE, after subcutaneous immunization with proteolipid protein 139–151 ($PLP_{139–151}$) myelin peptide (day 0). Treatment with anti-leptin Abs, ObR:Fc or with affinity-purified IgG_1 (used as a control) 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 5, and Table 1; see Methods for details).

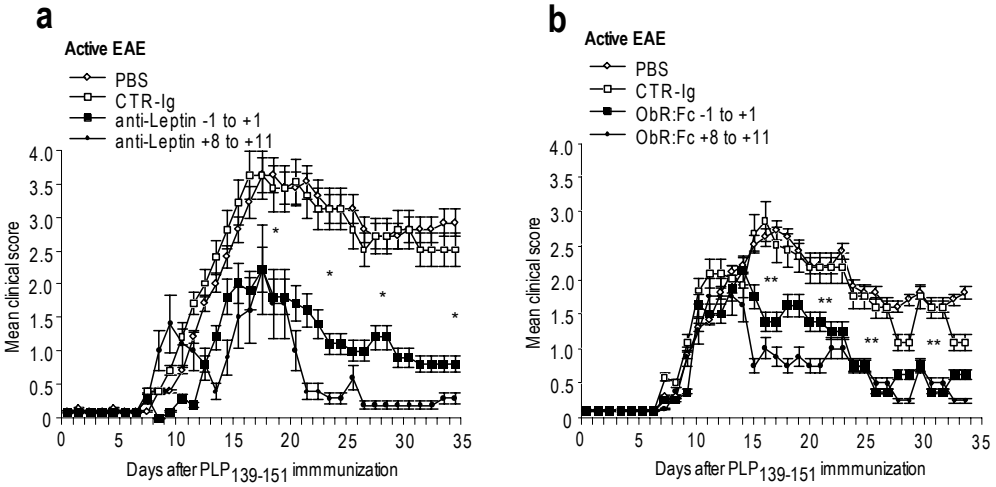


Figure 5. **Leptin blockade during actively induced EAE with either anti-leptin Abs or ObR:Fc reduces the clinical severity of EAE**. (**A**) Mean clinical score of SJL/J female mice treated with anti-mouse leptin Abs injected either from day –1 to day 1 or on days 8–11 (100 μg/d i.p.). Data are from 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.

Individual mice were observed daily for clinical signs of disease for up to 40 days. CTR-Ig treated mice developed the pathology and exhibited complete hind limb paralysis or fore limb weakness (score=4/4,5) after 17-20 days from the immunization. While in both cases, leptin neutralization led to a

statistically significant reduction in EAE clinical score (about 60%), to a re-establishment of motor functionality, as well as a decrease in the percentage of initial body weight loss and a reduced number of inflammatory infiltrates (Figure 5, 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.

Group of mice	Incidence No/tot (%)	Day of onset (range)	Peak clinical score	No of Inflammatory foci	Post treatment Relapse rate*
PBS (**Active EAE**)	18/18 (100.0)	9.0 ± 0.5 (8-9)	3.6 ± 0.9	45.5 ± 10.0	2.0
CTR-IgM	18/18 (100.0)	8.1 ± 0.5 (8-9)	3.5 ± 1.0	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^{A}	15.5 ± 10^{A}	0.55
anti-Leptin +8 to +11	18/18 (100.0)	12. 1 ± 3.0 (8-16)	1.6 ± 0.8^{B}	9.0 ± 4.0^{B}	0.33
PBS (**Active EAE**)	18/18 (100.0)	8.0 ± 0.5 (8-9)	2.7 ± 0.8	31.0 ± 10.0	1.55
CTR-IgG$_1$	18/18 (100.0)	8.1 ± 0.4 (8-9)	2.6 ± 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^{A}	15.0 ± 7.0^{C}	0.55
ObR:Fc +8 to +11	17/18 (94.4)	9.0 ± 0.5 (8-10)	0.9 ± 0.5^{B}	11.0 ± 5.0^{D}	0.55
PBS (**Passive EAE**)	18/18 (100.0)	7.1 ± 1.0 (6-8)	2.8 ± 1.0	27.0 ± 5.0	1.38
CTR-IgM	18/18 (100.0)	7.0 ± 0.7 (6-8)	2.6 ± 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^{A}	10.5 ± 9.0^{C}	0.61
anti-Leptin +8 to +11	14/18 (77.7)	17.8 ± 1.1 (17-20)	0.3 ± 0.4^{B}	0.2 ± 0.5^{D}	0.05
PBS (**Passive EAE**)	18/18 (100.0)	7.2 ± 1.2 (7-8)	3.1 ± 1.0	35.5 ± 6.0	1.27
CTR-IgG$_1$	18/18 (100.0)	7.5 ± 0.7 (7-8)	3.0 ± 0.9	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^{B}	0.5 ± 0.1^{D}	0.0
ObR:Fc +8 to +11	13/18 (72.2)	10.0 ± 0.7 (8-10)	0.5 ± 0.0^{B}	0.5 ± 0.4^{D}	0.0

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**.
The data shown are cumulated and averaged from three independent experiments each performed with 6 mice/group and they are presented as mean ± SD. CTR-Ig, control immunoglobulins (see Methods for details of isotype controls utilized). *Post-treatment relapse rate, total number of relapses in a group divided by the total number of mice in that group (group of mice were observed up to 90-120 days after disease induction to asses the relapse rate). $^{A}P = 0.001$ and $^{B}P = 0.0001$ as compared with the respective PBS and CTR-Ig group.

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 6, 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), following the same scheme of the experimental design descrived above . Mice treated with control Igs all developed severe EAE (Figure 6A and Table 1). In contrast, mice treated with anti-leptin Abs from day –1 to day 1 displayed a milder disease (Figure 6A 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 B, and Table 1). A significant reduction in the relapse rate was also found in animals observed for 90–120 days (Table 1).

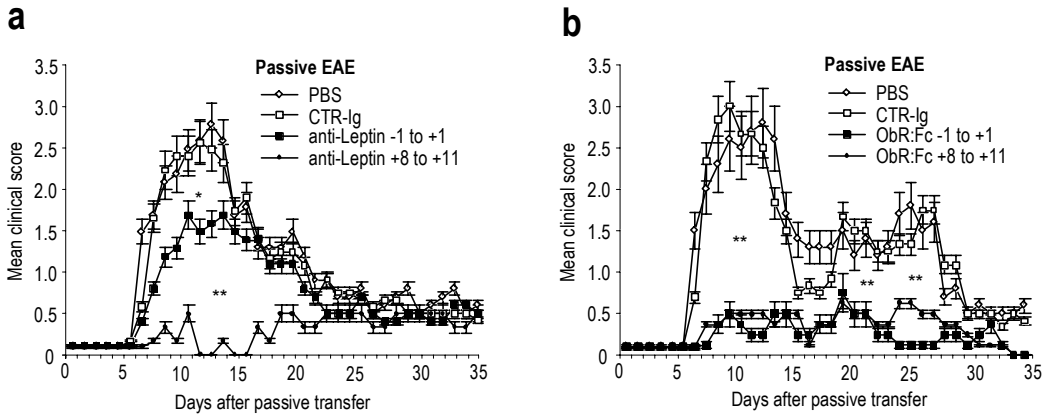


Figure 6. **Leptin blockade during adoptively transferred EAE with either anti-leptin Abs or ObR:Fc chimera reduces the clinical severity of EAE**. (**A**) Mean clinical score of SJL/J female mice treated with anti-mouse leptin Abs injected either from day –1 to day 1 or on days 8–11 (100 µg/d i.p.). 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; ‡$P = 0.02$, 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.

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 treated 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 significantly reduced in mice treated with anti-leptin Abs and ObR:Fc, whereas the controls exhibited a marked DTH reaction (Figure 7A and Figure 8A). We also examined whether leptin blockade could affect proliferation of $PLP_{139–151}$-specific T cells in vitro, measured as thymidine incorporation. 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 7B and Figure 8B, 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 7C and Figure 8C, respectively; see Methods), indicating that the effect of leptin neutralization was restricted only to the autoreactive CD4+ T cells. Of note, inhibition of in vitro anti-$PLP_{139–151}$ proliferation was more efficient in mice treated with the ObR:Fc chimera (Figure 7B and Figure 8B).

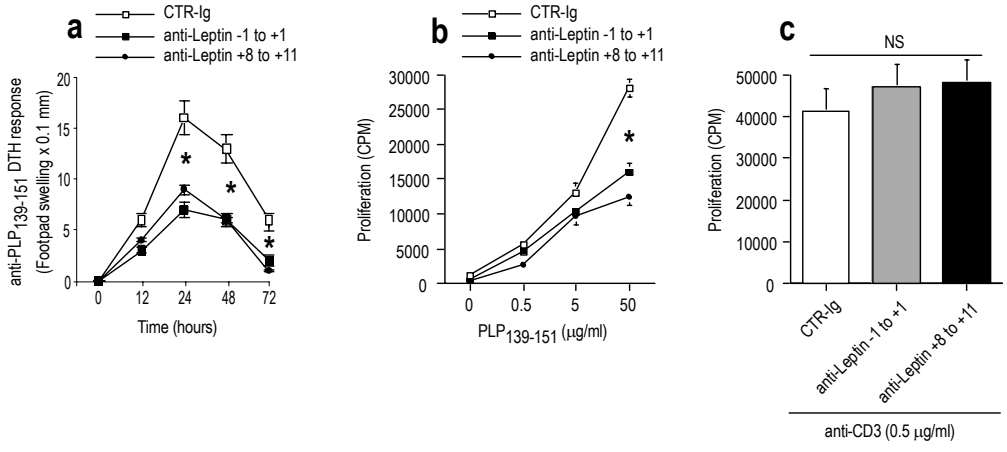


Figure 7. In vivo leptin neutralization with anti-mouse leptin Abs in SJL/J mice inhibits DTH response and induces T cell hyporesponsiveness to PLP139–151 myelin peptide. (**A**) DTH reaction in leptin-neutralized and control Ig–treated mice measured as footpad swelling. Data are from 1 representative experiment of 2. (**B**) Proliferative response of lymph node–derived T cells against PLP139–151. Data are from 1 representative experiment of 3. (**C**) Polyclonal T cell proliferation induced with anti-CD3ε stimulation. #*P* = 0.02, day –1 to day 1 and days 8–11 versus control Ig.

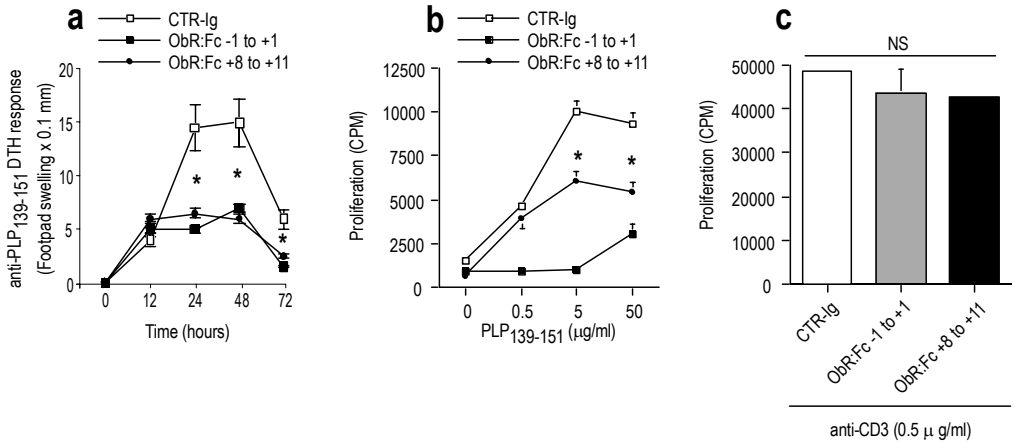


Figure 8. **In vivo leptin neutralization with mouse ObR:Fc chimera in SJL/J mice inhibits DTH response and induces T cell hyporesponsiveness to PLP139–151 myelin peptide**. (**A**) DTH reaction in ObR:Fc leptin-neutralized and control Ig–treated mice measured as footpad swelling. Data are from 1 representative 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 PLP139–151. 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**) Polyclonal T cell proliferation induced with anti-CD3ε stimulation.

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.

It's well known from the literature that Th1 CD4+ Ag-specific cells are able to induce encephalomyelitis when adoptively transferred in normal mice and that cytokines secreted by Th1 cells (IFN-γ) are present in the inflammatory lesions of EAE in the CNS. So we next asked whether EAE protection, derived by leptin neutralization, was associated with an immune response characterized by considerable switching in the cytokine profile secretion towards a Th2 phenotype. We checked for the presence of both pro- and anti-inflammatory cytokines by ELISA tests in all the different treated group of mice. 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 9, A and B, and Figure 10, A and B). Conversely, a significant increase in the Th2/regulatory-type cytokines such as IL-4 (Figure 9, C and D, and Figure 10, C and D) and IL-10 (Figure 9, E and F, and Figure 10, E and F) was observed in the cells isolated from leptin-neutralized mice during both anti-$PLP_{139–151}$–specific proliferation and polyclonal stimulation with anti-CD3ε, suggesting a key role for leptin

blockade in the improvement of EAE, through the modulation of the cytokine secretion .

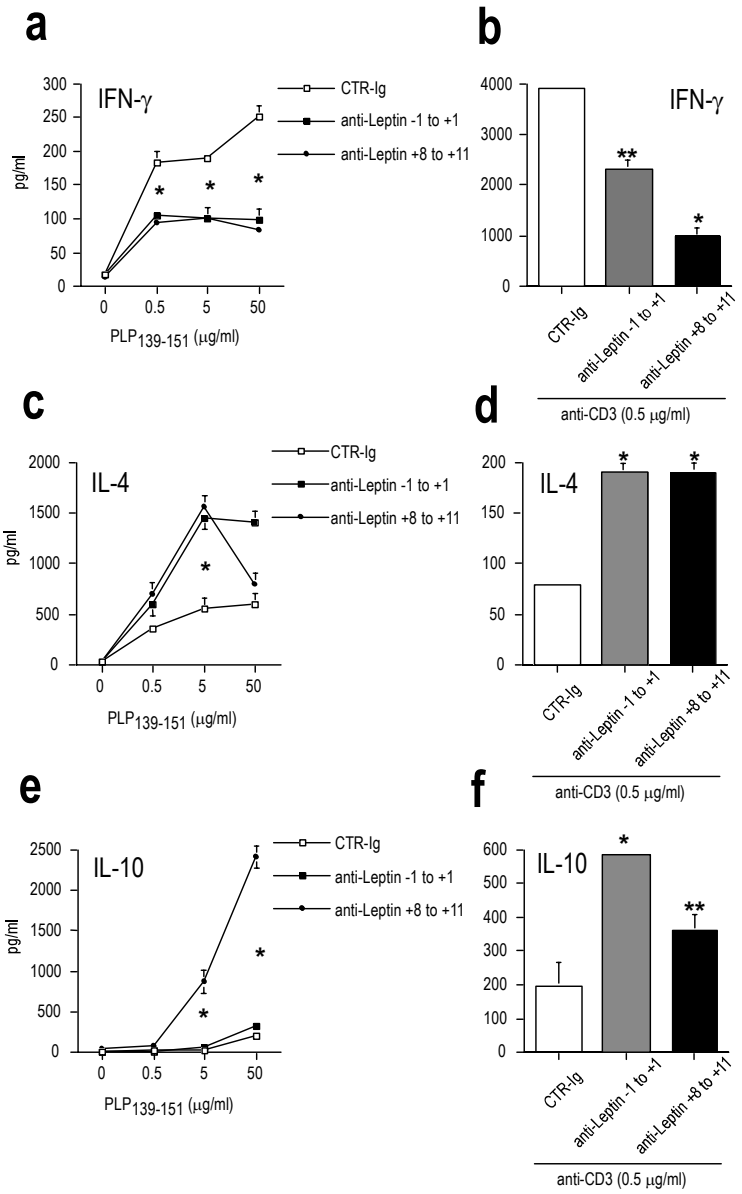


Figure 9. In vivo leptin neutralization with anti-mouse leptin Abs inhibits IFN-γ production and induces the secretion of IL-4 and IL-10 regulatory cytokines. IFN-γ secretion of lymph node–derived T cells stimulated with the myelin antigen PLP139–151 (**A**) and by anti-CD3ε (**B**). IL-4 secretion of lymph node–derived T cells stimulated with the myelin antigen PLP139–151 (**C**) and by anti-CD3ε (**D**). IL-10 secretion of lymph node–derived T cells stimulated with the myelin antigen PLP139–151 (**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**) #$P = 0.02$, **$P = 0.001$, *$P = 0.002$, † $P = 0.04$ versus control Ig.

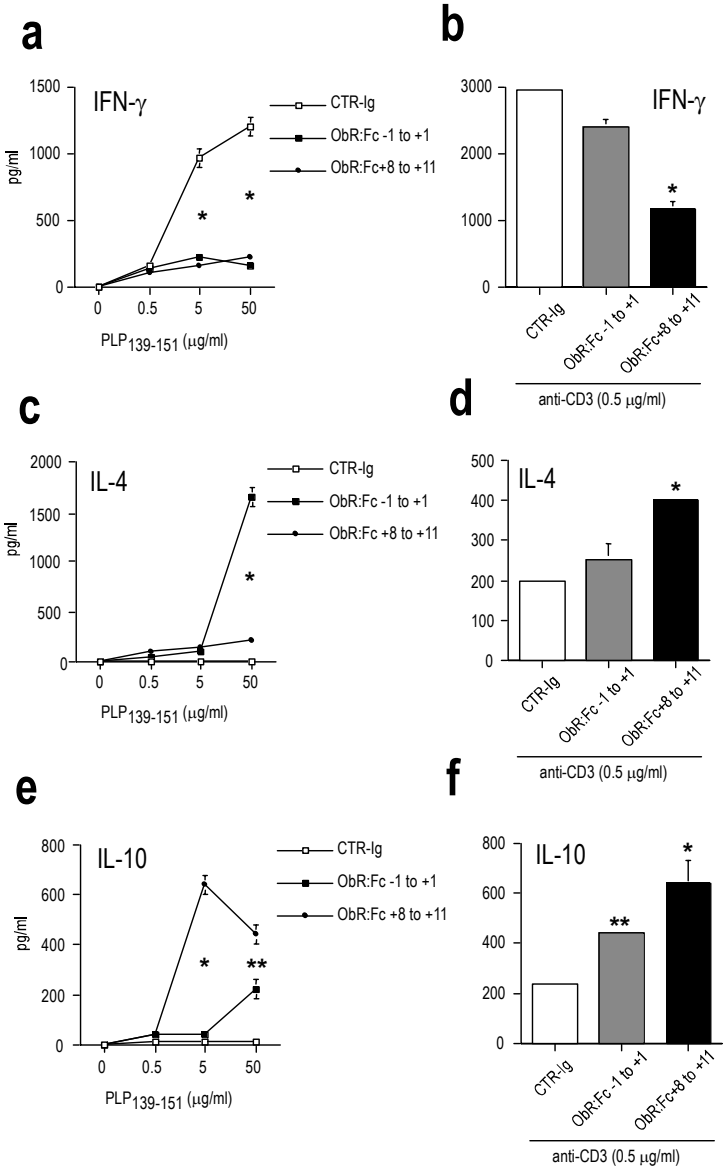


Figure 10. In vivo leptin neutralization with ObR:Fc inhibits IFN-γ production and induces the secretion of IL-4 and IL-10 regulatory cytokines. IFN-γ secretion of lymph node–derived T cells stimulated with the myelin antigen PLP139–151 (**A**) and by anti-CD3ε (**B**). IL-4 secretion of lymph node–derived T cells stimulated with the myelin antigen PLP139–151 (**C**) and by anti-CD3ε (**D**). IL-10 secretion of lymph node–derived T cells stimulated with the myelin antigen PLP139–151 (**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 † $P = 0.04$, 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**) $\#P = 0.02$, $^{*}P = 0.002$, † $P = 0.04$ versus control Ig.

In addition, to determine whether treatment with ObR:Fc induced forkhead box p3 (Foxp3) expression, (a marker of cellular subsets with regulatory phenotype able to dampen the autoimmune response), 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 11,

A and B), suggesting induction of regulatory T cell markers in leptin-neutralized mice.

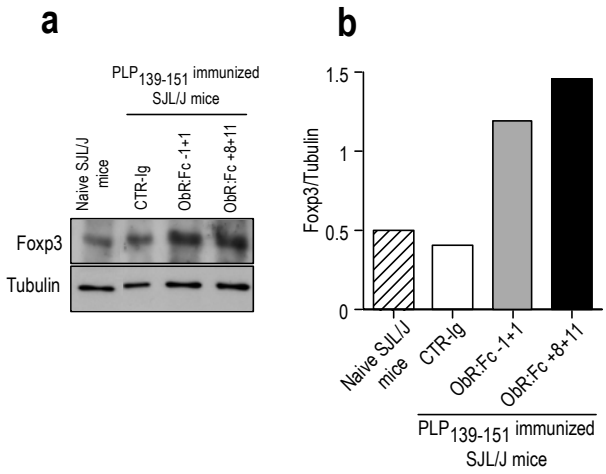


Figure 11. Increased expression of Foxp3 in CD4+ T cells induced by leptin neutralization in mice with EAE. (**A**) Western blot analysis for Foxp3 on purified CD4+ T cells obtained from SJL/J mice immunized with PLP139–151 treated or not with ObR:Fc. (**B**) Results are presented as Foxp3 protein level normalized to tubulin expression. Data are from 1 representative experiment of 3.

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 OX-40 (Figure 12, A and B, respectively), both classically involved in the pathogenesis of EAE (115-117), suggesting that leptin may affect expression of key molecules on T lymphocytes, involved in the mechanisms of immune tolerance. Conversely, the overall expression of the α4β1 integrin, very late antigen-4 (VLA-4) (118), was increased, with particular upregulation of the population at high fluorescence intensity (Figure 12C). 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).

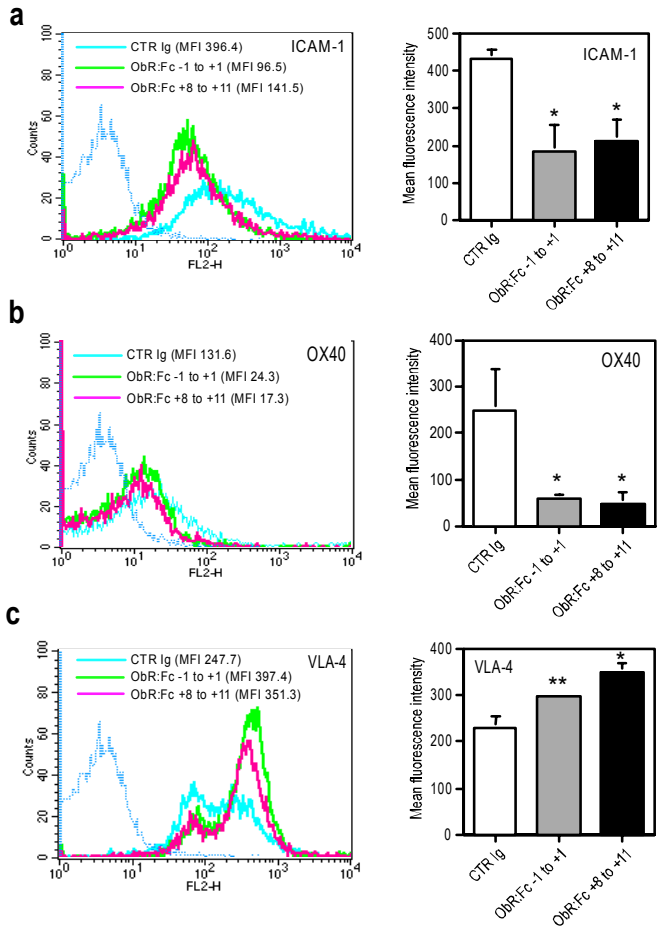


Figure 12. Leptin neutralization suppresses ICAM-1 and OX-40 expression on CD4+ cells but upregulates VLA-4 in mice with EAE. (**A**) Flow cytometric analysis of cell-surface ICAM-1 molecules (left) and mean fluorescence intensity (MFI) from 3 independent experiments (right). (**B**) OX-40 surface expression. (**C**) VLA-4 expression on CD4+ T cells from ObR:Fc-treated mice and controls. #P = 0.02, *P = 0.002, † P = 0.04 versus control Ig.

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 (119-123). More specifically, we studied ex vivo purified CD4$^+$ autoreactive T cells from spleens and lymph nodes of ObR:Fc-treated mice (both -1+1 and +8+11 treatement) 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 13A) associated with increased tyrosine phosphorylation of ERK1/2 (Figure 13C). Moreover, we observed an upregulation of STAT6 tyrosine phosphorylation levels (Y641, known to be associated with Th2/regulatory-type cytokine secretion; (123) in ObR:Fc-treated mice compared with control mice (Figure 13E). Taken together, these data underline the capability of leptin neutralization in modulating functionality of pro-pathogenic T cells, by altering their proliferative/anergy rate and inducing the expression of molecules with regulatory functions.

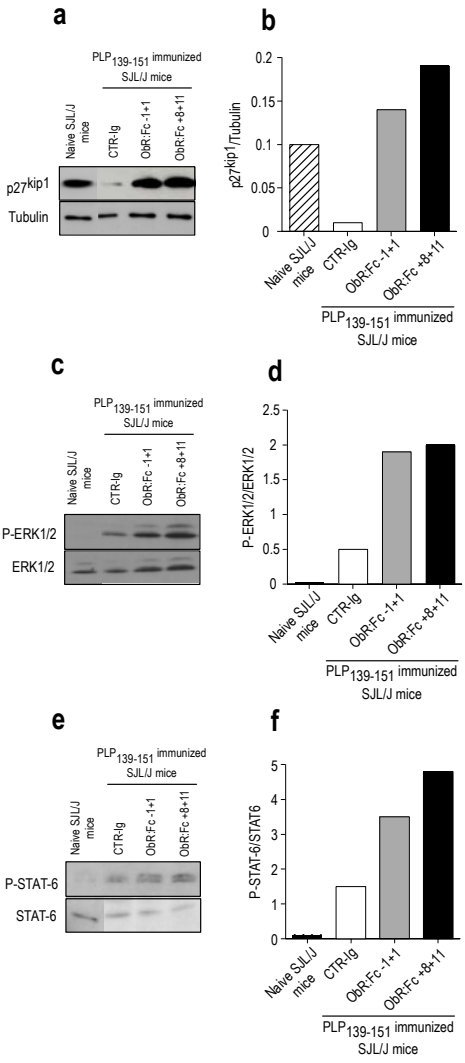


Figure 13. Leptin neutralization determines the failure to downmodulate the anergy factor p27Kip-1 and is associated with sustained phosphorylation of ERK1/2 and STAT6. (**A** and **B**) Western blot analysis for p27Kip-1 and tubulin on purified CD4+ T cells obtained from naive (**C** and **D**) Western blot analysis for phosphorylation of ERK1/2 in resting CD4+ T cells from naive and SJL/J mice immunized with PLP139–151, treated or not with ObR:Fc. (**E** and **F**) Western blot analysis for phosphorylation of STAT6 in resting CD4+ T cells from naive and SJL/J mice immunized with PLP139–151, treated or not with ObR:Fc. For each panel, 1 representative experiment of 5 is shown.

Human T_{reg} cells express higher level of Leptin receptor (ObR) than do $CD4^+CD25^-$ effector T cells.

To evaluate whether clinical improvement of EAE, induced by leptin neutralization, could be ascribed to the effect of this kind of treatment on the homeostasis and function of regulatory T cells (a specific cellular subset, known to control the mechanisms of immune tolerance), we performed a series of experiments to check and confirm a possible link between leptin and/or its neutralization and Tregs. 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 (124). We sought to analyze the expression of ObR also on T_{reg} cells and to correlate its expression with Foxp3, to check whether leptin could act directly on Tregs subpopulation. First of all $CD4^+CD25^+$ T_{reg} cells and $CD4^+CD25^-$ effector T cells purified from human healthy donors (Figure 14A, left) were analyzed for Foxp3 expression by FACS analysis and immunoblot (Figure 14A, right, and Figure 14B, respectively), to confirm the purity of our preparation. 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 14A and 14B). 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 14C).

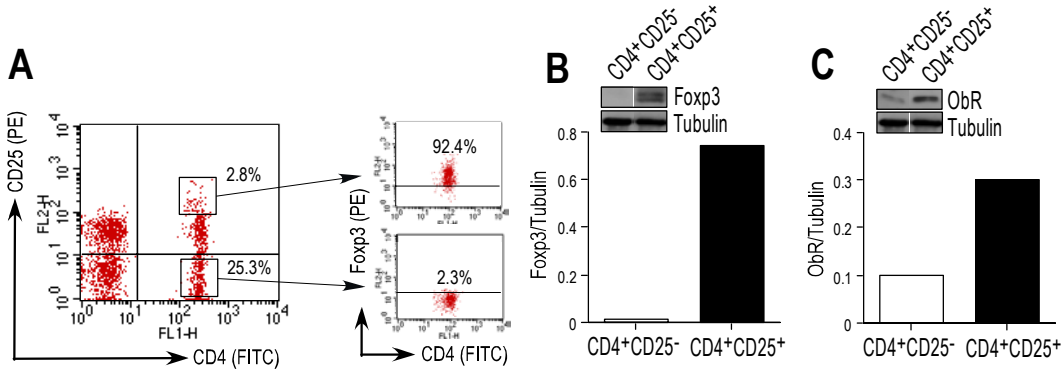


Figure 14. Human Treg Cells Express High ObR, and (**A**) Representative flow cytometry plot of human T cells stained for CD4, CD25, and Foxp3. (**B and C**) Immunoblot analysis of sorted CD4+ T

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.

Leptin neutralization induces proliferation of human T_{reg} cells upon anti-CD3 and anti-CD28 stimulation.

Human T_{reg} cells were hyporesponsive to anti-CD3 and anti-CD28 stimulation (Figure 15A), in agreement with previous findings from the literature (125). Addition of exogenous recombinant leptin to the cultures did not alter T_{reg} cells hyporesponsiveness (Figure 15A). However, addition of neutralizing leptin monoclonal antibody (mAb) reversed their state of hyporesponsiveness and promoted T_{reg} cell proliferation (Figure 15A), in a dose-dependent fashion (Figure 15B). 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 15A). Moreover, dose-dependent increase of the proliferation of stimulated T_{reg} cells in the presence of leptin mAb (Figure 15B) was reversed by addition of increasing doses of recombinant leptin (Figure 15C). Conversely, leptin mAb inhibited the proliferation of purified effector $CD4^+CD25^-$ T cells, a phenomenon that was reversed by addition of exogenous leptin (Figure 15D). 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 15D), whereas it promoted expansion of the latter subpopulation (Figure 15A). 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 15E).

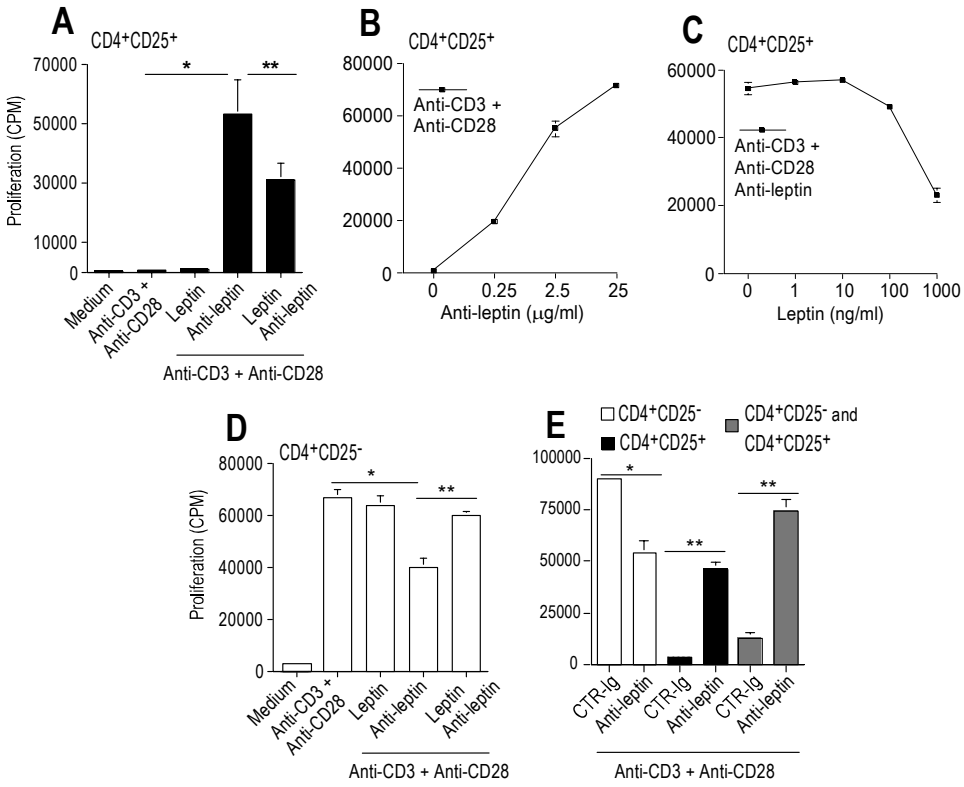


Figure 15. Leptin neutralization reverses Tregs hyporesponsiveness. (**A**) Proliferation of CD4+CD25+ Treg cells treated with recombinant human leptin (250 ng/ml) in the presence or absence of leptin mAb (10 μg/ml). The data are shown as mean ± SD (n = 5, *p < 0.0001; **p < 0.01). (**B**) Dose dependency of Treg 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). (**C**) Proliferation of Treg 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). (**D**) 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). (**E**) Proliferation of CD4+CD25- effector T cells, Treg 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).

Anti-leptin-expanded 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 16A). In contrast, suppression of proliferation was apparently abrogated in the presence of leptin mAb (Figure 16A, 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 16A, gray columns). To understand whether T_{reg} cells during anti-leptin-induced proliferation could exert suppressive capacity, since it seemed from the amount of incorporated thymidine in the coculture experiments, that they apparently lost this function, we performed coculture experiments with CFSE-labeled $CD4^+CD25^-$ T cells in the presence of unlabeled T_{reg} cells (Figures 16B–16E). As expected, T_{reg} cells suppressed the expansion of $CD4^+CD25^-$ effectors (Figure 16D), and suppression was partially maintained in the presence of leptin mAb (Figure 16E)—albeit at a lower degree when compared with untreated T_{reg} cells (Figure 16D). 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 16A, gray columns) was apparent only because $CD4^+CD25^-$ effectors were inhibited in part in their proliferation, as indicated by the CFSE dilution (Figure 16E). 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 15E and 16A) has to be ascribed mainly to T_{reg} cell proliferation and partly to $CD4^+CD25^-$ T cells. In parallel 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 16F) and was significantly inhibited in the presence of T_{reg} cells (Figure 16H). 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 16I). 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 16E).

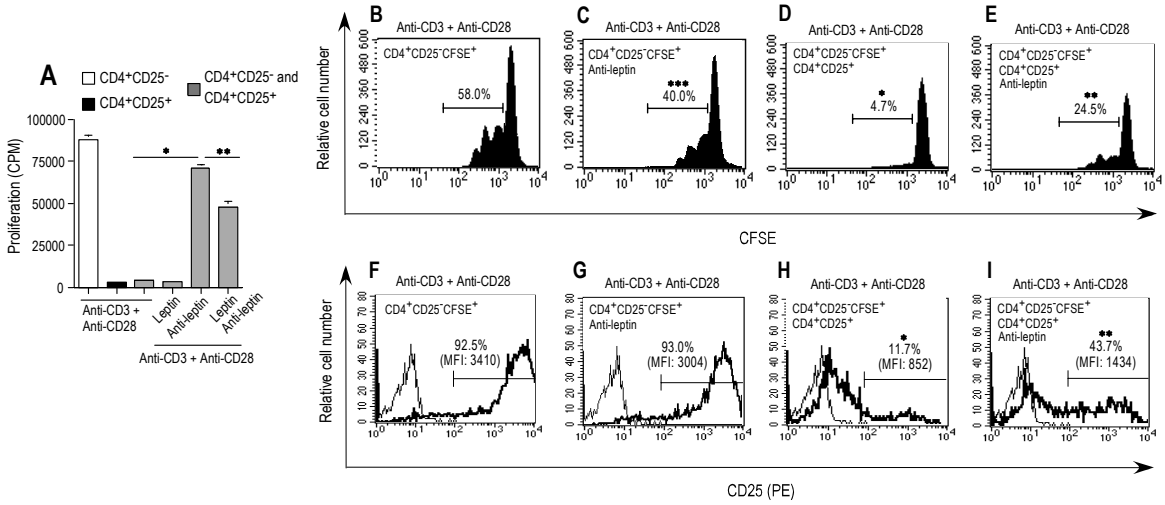
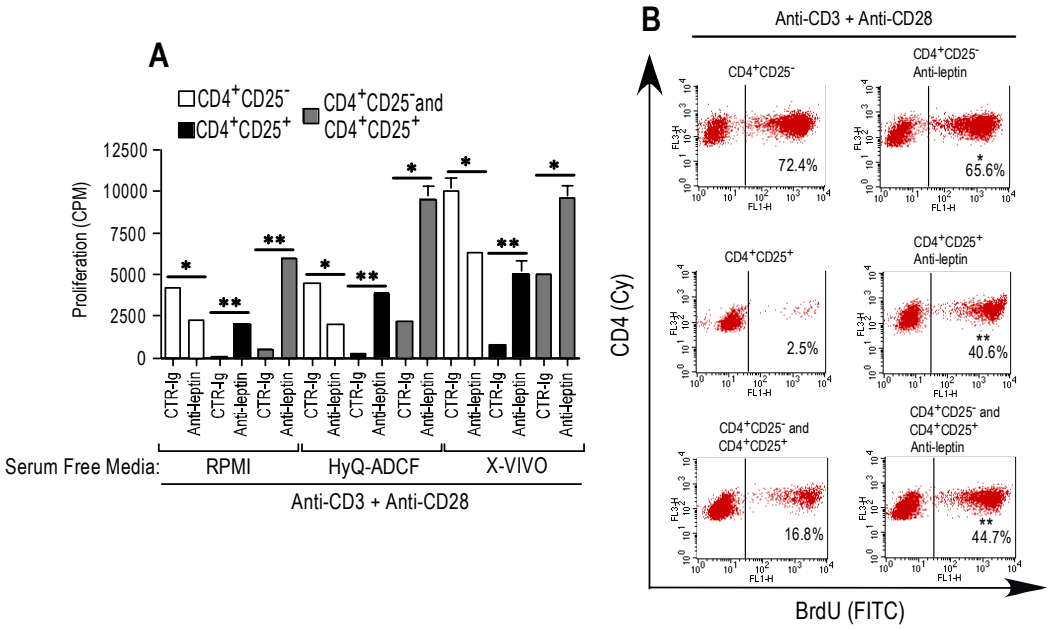


Figure 16. Human Treg cells exhibit partial suppressive capacity upon leptin-mAb-induced Proliferation. (**A**) Proliferation of CD4+CD25- effector T cells, Treg cells, and of both cell types in coculture treated with recombinant human leptin (250 ng/ml) in the presence or absence of leptin mAb (10 μg/ml). 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 Treg 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+).

Leptin production from human T_{reg} cells

Leptin is present in media supplemented with human serum, such as the medium used in our 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 17A), RPMI, HyQ-ADCF and X-VIVO. 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 expansion (Figure 17A). This finding was also confirmed by BrdU incorporation in serum-free medium cultures (Figure 17B), where it's shown that leptin neutralization was able to increase the percentage of BrdU positive cells from 2.5 to 40 in Tregs subset. This finding suggests the possibility that leptin may be produced directly by T_{reg} cells in a fashion really similar to the production of leptin by $CD4^+$ T cells and monocytes in multiple sclerosis (111, 112). To test this possibility, we examined leptin and ObR expression on T_{reg} cells and $CD4^+CD25^-$ T cells, by confocal microscopy. Both freshly isolated T_{reg} cells and $CD4^+CD25^-$ T cells stained positive for leptin and ObR, with

different intensity patterns (Figure 17C). 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 17C). Parallel quantitation by immunoblotting analysis for leptin on cell lysates confirmed the difference (Figure 17D). Indeed, the presence of a basal production of leptin increased significantly after anti-CD3 and anti-CD28 stimulation (Figure 17D), and T_{reg} cells always produced more leptin than did $CD4^{+}CD25^{-}$ T cells (Figure 17D). 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 17C and 17D). These results were confirmed at 12 hr by confocal microscopy and immunoblotting studies (data not shown).



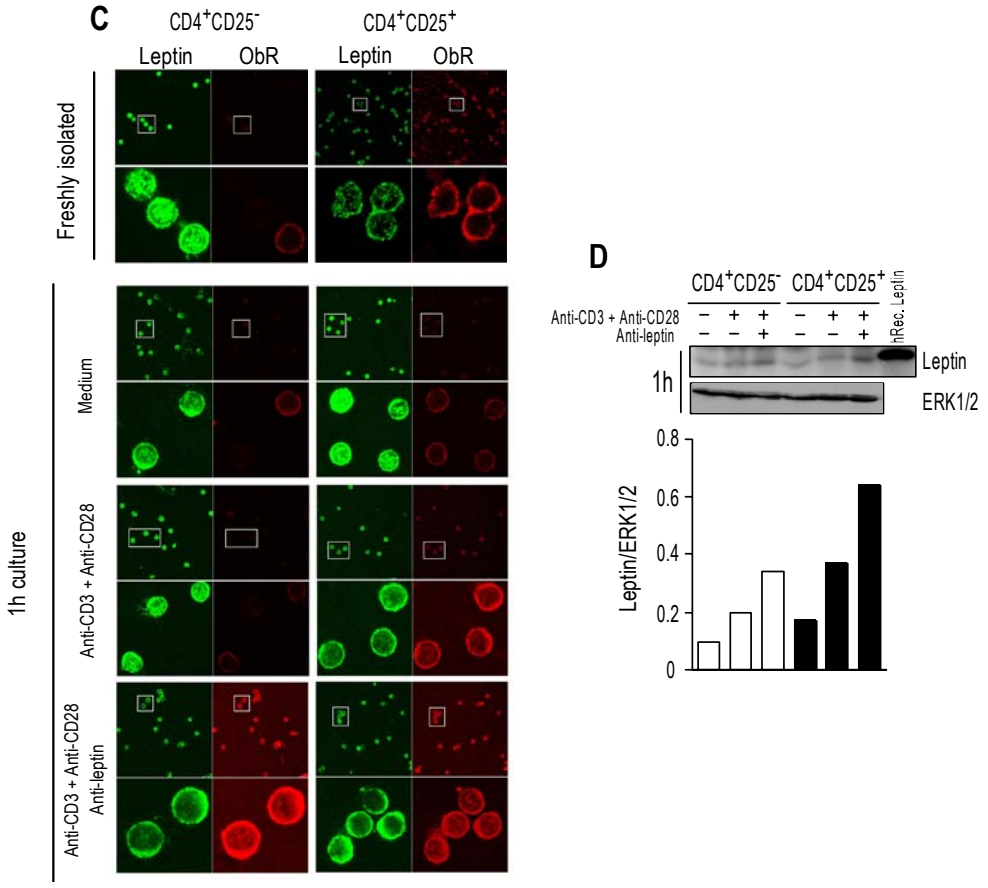


Figure 17. Treg cells produce leptin and express high amounts of ObR. (**A**) Proliferation of CD4+CD25- effector T cells, Treg cells, and of both cell types in coculture in three different serum- and leptin-free media in the presence or absence of leptin mAb. The data are shown as mean ± SD (n = 6, *p < 0.01; **p < 0.001). (**B**) Flow cytometry plot of BrdU incorporation of CD4+CD25- effector T cells, Treg 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 Treg 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 Treg 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.

Additionally, to address the capacity of T_{reg} cells and $CD4^{+}CD25^{-}$ T cells to secrete leptin, we performed a human leptin-specific ELISA at different time points (12 hr and 36 hr) on culture supernatants (Figure 18). 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 18). 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 and acting as a negative signal, constraining Tregs expansion.

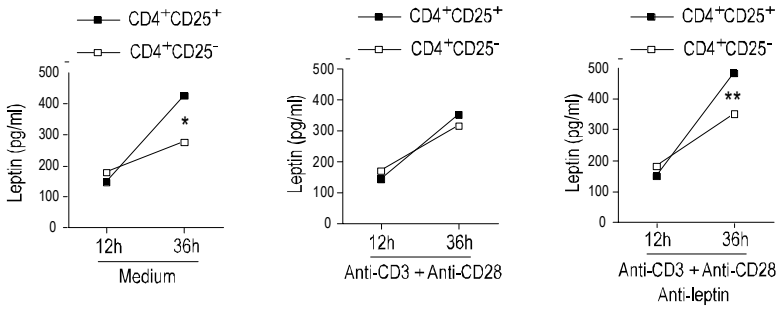


Figure 18. Tregs cells secrete leptin in the culture supernatant. Human leptin specific ELISA performed on cell culture media at 12h and 36h after stimulation.

Leptin neutralization increases the expression of Foxp3 in T_{reg} cells

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 (early time point) and 12 hr (late time point) during anti-CD3 and anti-CD28 stimulation. As shown in Figure 19A, 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} , whose expansion is mediated by leptin blockade, showed increased Foxp3 amounts when compared to those simply activated with anti-CD3 and anti-CD28 alone (Figure 19B). As expected, Foxp3 was undetectable in $CD4^{+}CD25^{-}$ T effectors after 1 hr of stimulation in all the experimental conditions (Figure 19A), 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 19B). All these data were confirmed by FACS analysis and real-time PCR.

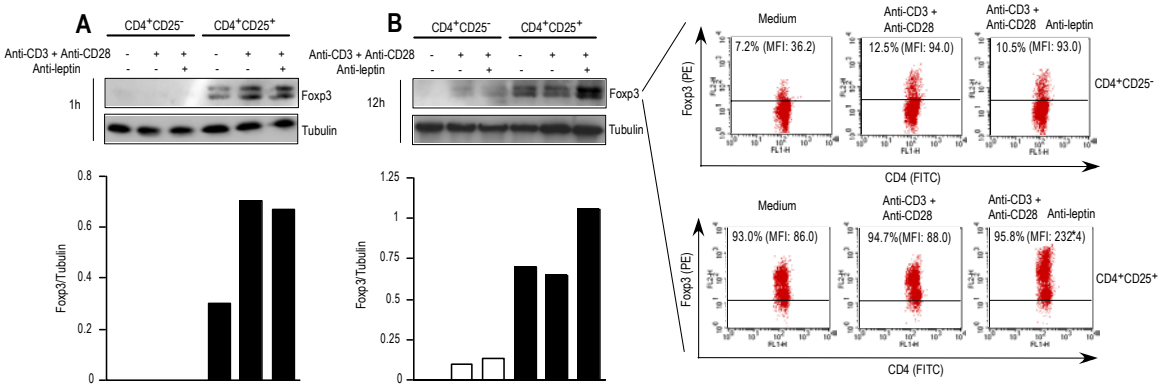


Figure 19. **Leptin neutralization increases the expression of Foxp3 in T_{reg}** (**A**) Immunoblot analysis of CD4+CD25- effector T cells and Treg cells in the presence or absence of leptin mAb, at 1 hr stimulation with anti-CD3 and anti-CD28. The graph shows quantitation of Foxp3 with respect to tubulin. One representative out of five independent experiments is shown. (**B**) Immunoblot analysis and flow cytometry plot of CD4+CD25- effector T cells and Treg 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).

Leptin deficiency promotes T_{reg} cells proliferation in mice

T_{reg} cells, despite their in vitro hyporesponsiveness, can expand in vivo in normal, nonlymphopenic hosts (126, 127). We used nonirradiated, nonlymphopenic recipient mice to avoid a homeostatic expansion of the T_{reg} cells that would occur in lymphopenic hosts. 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 20A). 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 20A). 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, because of the absence of leptin, expanded more robustly and earlier (day 4 and day 7 after transfer) when compared with cells transferred into normal WT mice (Figure 20B). 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). We confirmed these results by looking at the modulation of Foxp3

expression, by western blot, in all the differently treated mice, and we showed that T_{reg} cells from the leptin-neutralized WT mice exhibited higher amount of this key molecule (Figure 20C).

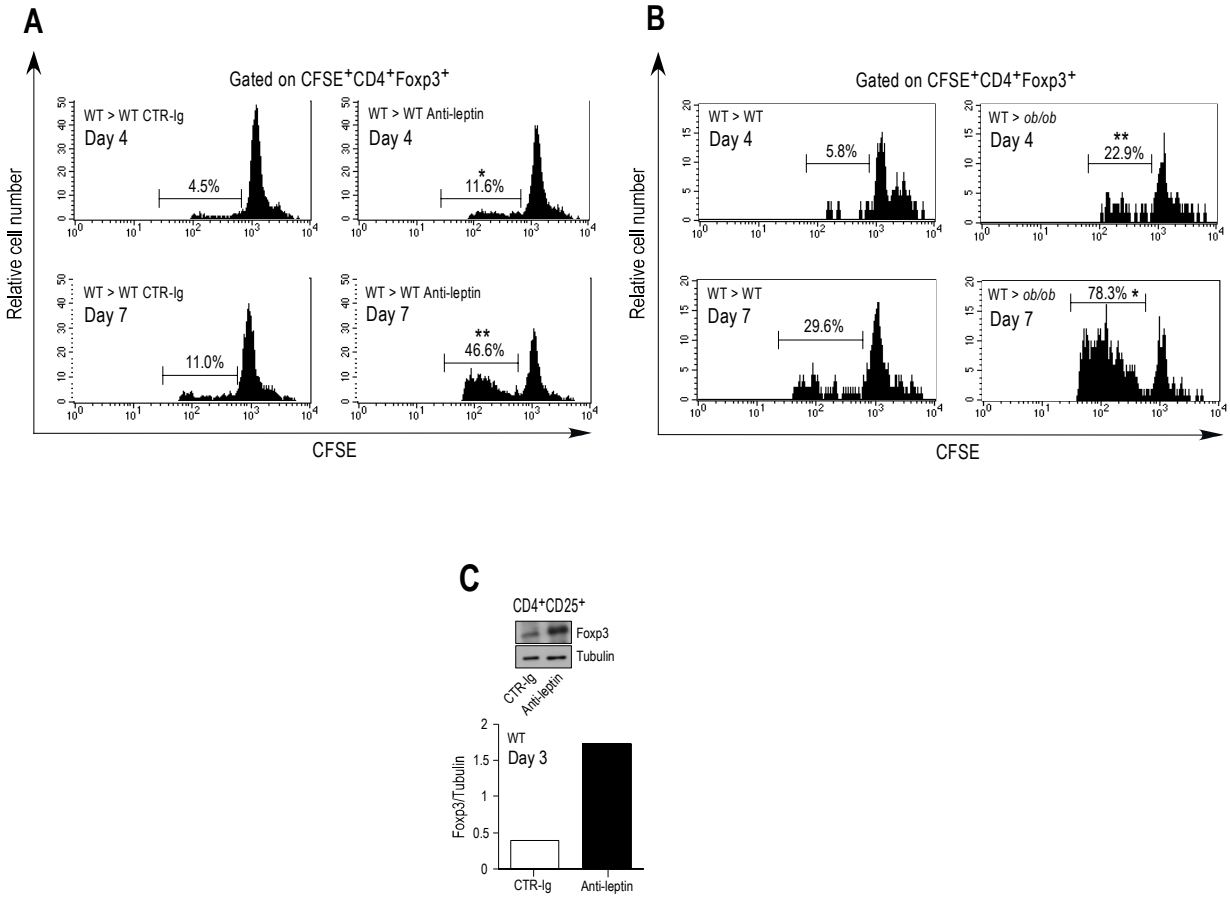


Figure 20. In vivo leptin neutralization or congenital leptin deficiency associate with proliferation of Treg cells. (**A**) Proliferation measured as CFSE dilution of CFSE-labeled Treg cells obtained from WT mice and transferred into control (CTR)-Ig or mouse leptin neutralizing Ab-treated WT mice. The histogram shows the percent of CFSE+ Treg 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 Treg 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**) Ex vivo immunoblot and protein quantification of Foxp3 protein in Tregs cell from CTR-Ig and Leptin-Ab-trated WT mice. One representative out of three indipendent experiments with 3 mice per group is shown.

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 (91, 110, 111). T_{reg} cells play a central role in regulating autoimmune responses (128, 129). So we tested whether

protection from autoimmune attacks observed in genetic deficiency of leptin, could be associated with altered T_{reg} cells expansion. A significant increase of the percentage of peripheral T_{reg} (expressed as CD4+CD25+ cells(A) or CD4+Foxp3+ cells(B)) cells was observed in *ob/ob* as compared to WT (Figures 21A and 21B, 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 21A and 21B), confirming the negative role played by leptin in the control of T_{reg} cells homeostasis and function.

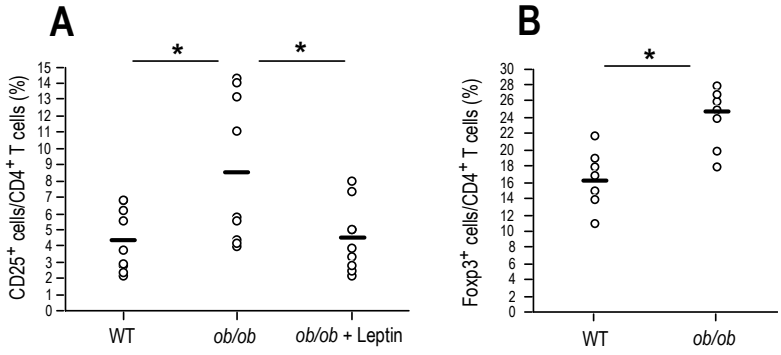


Figure 21. Effect of congenital leptin deficiency on the number of Treg ells in vivo (**A**) Percentage numbers of peripheral CD4+CD25+ Treg cells in WT, leptin deficient *ob/ob* mice, and recombinant leptin-treated *ob/ob* mice. One representative out of three independent experiments with 8 mice per group is shown (**P* < 0.02). (**B**) 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).

Proliferative potential and functional capacity of Treg 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 (Figures 22A–22C). 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 22A). 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 22B). 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, because 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 22C), confirming the finding that addition of exogenous leptin did not alter the suppressive capacity of Treg cells in vitro.

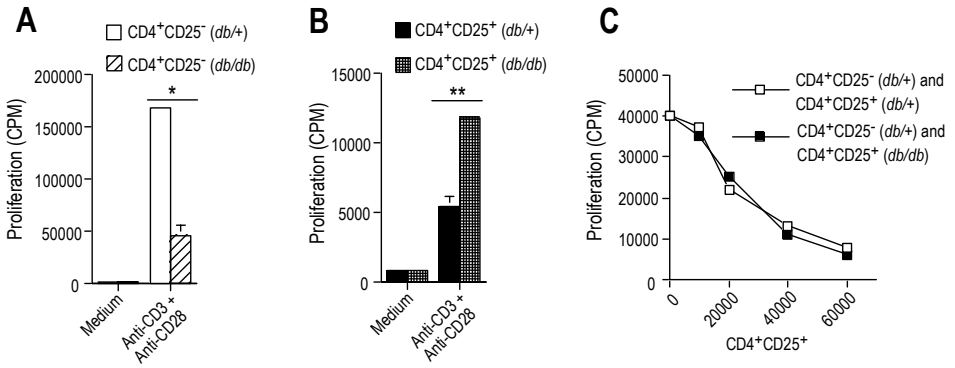


Figure 22. ObR deficiency increases Treg cells proliferative potential, does not alter their suppressive capacity, and impairs CD4+CD25- proliferation. (**A**) Proliferation of CD4+CD25- effector T cells from db/+ and leptin receptor-deficient db/db mice stimulated with anti-CD3 and anti-CD28. The data are shown as mean ± SD (n = 5, *p < 0.001). (**B**) Proliferation of Treg 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 Treg cells, stimulated with anti-CD3 and anti-CD28. The data are shown as mean ± SD (n = 5).

MATERIAL AND METHODS

Mice. Female SJL/J mice (H-2^s), 6–8 weeks old, were obtained from Harlan-Italy. 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); 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). 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. 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)..

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 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.

In vivo Ab treatment. For EAE experiments, 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). *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. In adoptive transfer experiments, 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.

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).

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). Flow cytometric analysis of CFSE dilution was performed by gating on $CFSE^+CD4^+Foxp3^+$ cells with a FACScalibur (Becton-Dickinson, San Diego, CA) and analyzed by Cell Quest software (Becton-Dickinson.)

Proliferation assays and cytokine analysis (ELISA). 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 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. Human leptin-specific ELISA was purchased from R&D Systems and measurements were performed according to the manufacturer's instructions. Serum-free media were RPMI (Life Technologies), HyQ-ADCF (Animal Derived Component Free, from Hyclone-Pierce), and X-VIVO (BioWittaker).

Purification and culture of Tregs.. 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. Briefly, isolated tregs and effector t cells were labelled in vitro with BrdU (10μM final concentration), then, after 48 hours we performed an immunofluorescent staining of cell surface antigens (CD4, CD25). After fixation and permeabilization process (with BD Cytofix/Cytoperm buffer) the cells were treated with DNase (30μg of DNase for 10^6 cells) to expose BrdU epitopes and incubate fotr 1 hour at 37°. Then an immunofluorescent staining with fluorochrome conjugated (FITC) anti-BrdU was performed on cultured cell, which were analyzed by flow cytometry.

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). 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)..

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 binding 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.)

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.

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.

DISCUSSION

Over the last few years the intricate interaction between immune system and adipose tissue or neuroendocrine system has been recognized. These systems influence reciprocally through the production of mediators such as leptin, the product of the obese gene (ob). So it has been suggested that the adipose tissue is not only a mere site of lipid and energy storage, but can be considered as an "immune-related" organ producing a series of molecules named adipokines. Among these, leptin seems to play a pivotal role in the regulation of several neuroendocrine and immune functions.

Previous studies by our group and others have shown the relevance of leptin in the pathogenesis of EAE (110-112). In particular, it has been previously reported that *ob/ob* mice are resistant to induction of the disease and leptin replacement was able to revert this state of resistance into a susceptible one. (110). Furthermore in wild-type, EAE-susceptible controls, a surge of serum leptin precedes acute EAE clinical onset, which correlates with inflammatory anorexia and disease susceptibility (111). In this study, we show that in vivo neutralization of leptin is effective at blocking initiation, progression, and clinical relapses of EAE, an animal model of MS.

We and others have previously reported that in the CNS of EAE mice, both infiltrating T cells and neurons express high levels of 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 (111). Because of the possibility of an autocrine loop, sustaining autoreactive Th1 lymphocytes proliferation in EAE ,we analyzed the reactivity and the modulation of immune response in leptin-neutralized mice. More in detail, 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, characterized by the secretion of IL-4 and IL-10. 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.

It is interesting to note that in vivo leptin neutralization differentially affected proliferative responses and regulatory cytokine switch during polyclonal anti-CD3 stimulation. 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 (130). 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. Intercellular adhesion molecule (ICAM)-1, or CD54, is a member of the immunoglobulin superfamily that binds to lymphocyte function-associated antigen-1 and macrophage-1 antigen. ICAM-1/LFA-1/Mac-1 interaction may be involved in both activation and extravasation of leukocytes. To determine the roles of ICAM-1 in the development of autoimmune disease, Samoivola et al studied experimental autoimmune encephalomyelitis (EAE) in mice deficient in ICAM-1 (131). They found that T cell proliferation and TH1-type cytokine production in response to myelin antigen were significantly reduced in ICAM-1-deficient mice, whereas TH2-type cytokine IL-10 was increased, resulting in a significantly attenuated EAE, characterized by markedly reduced spinal cord T cell infiltration. These results suggest that ICAM-1 is involved in the activation of autoreactive Th1 cells and plays an important role in down-regulating autoimmune inflammation in the central nervous system..

In particular, in our results, reduced expression of ICAM-1 observed in leptin-neutralized mice, was consistent with our previous findings, showing that leptin treatment increases surface expression of this adhesion molecule on T cells (110). This finding suggested the possibility that neutralization of leptin directly affects the cognate interaction leading to reactive and/or autoreactive T cell activation. Moreover, marked reduction of OX-40 was also observed after leptin blockade. OX-40 is an important costimulatory molecule with pro-

survival activity for $CD4^+$ T cells, and signaling through this molecule breaks peripheral T cell tolerance. The OX-40 protein was selectively up-regulated on encephalitogenic myelin basic protein (MBP)-specific T cells at the site of inflammation, during the onset of experimental autoimmune encephalomyelitis (EAE). An OX-40 immunotoxin was used by Weinberg et al. to target and eliminate MBP-specific T cells within the central nervous system without affecting peripheral T cells (132). When injected in vivo, the OX-40 immunotoxin bound exclusively to myelin-reactive T cells isolated from the CNS, which resulted in amelioration of EAE. The same authors also showed that in vivo administration of soluble OX-40R at the onset of actively induced or adoptively transferred EAE reduced ongoing signs of disease, and the mice recovered more quickly from acute disease (116). The data imply that OX-40L, expressed by CNS-derived APC, acts to provide an important costimulatory signal to EAE effector T cells found within the inflammatory lesions. In this context our data suggest that leptin may affect expression of key molecules on T lymphocytes involved in the mechanisms of immune tolerance, modulating the expression of OX-40 as well as ICAM-1. 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. 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 (118). 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 (133, 134).

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 $p27^{Kip-1}$. Boussiotis et al., using anergic human T-cell clones and tolerant alloreactive mouse T cells, that do not induce graft-versus-host disease, showed that p27kip1 cyclin-dependent kinase inhibitor is an essential regulator responsible for the blockade of clonal expansion of anergic T cells in vitro and in vivo. Moreover, in anergic cells, p27kip1 associates with the c-Jun co-activator JAB1, resulting in defective transactivation of AP-1 and interleukin 2 transcription. Therefore, pharmacological agents that up-regulate the expression or prevent the degradation of p27kip1, during antigen recognition, should be part of new therapeutic strategies to induce antigen-specific T-cell unresponsiveness. So the effect mediated by leptin neutralization on p27kip1 expression can lead us to think to leptin as a potential target to induce T cell anergy. (119, 122, 135). Activation of T cells via the TCR and other co-stimulatory receptors triggers a number of signalling cascades. Among them, the Ras-activated Raf-mitogen-activated protein/extracellular signal-related kinase (ERK) kinase (MEK)-ERK signaling cascade has been demonstrated to be crucial for both T cell development and activation. It has previously been demonstrated that high doses of Ag or anti-CD3 mAb are able to induce in T cells a non-responsive state to subsequent treatment with cytokines, such as IL-2. The precise biochemical mechanisms underlying this effect are not fully characterized. In 1999 Chen et al. demonstrated that cytokine non-responsiveness is accompanied by the induction of the cyclin-dependent kinase inhibitor $p21^{Cip1}$ that is mediated, at least in part, by the activation of the Raf-MEK-ERK pathway (136). Furthermore, they demonstrated that selective activation of the Raf-MEK-ERK signalling pathway in T cells is sufficient to induce cytokine non-responsiveness in both a T cell clone and naive primary T cells. In this case, non-responsiveness is accompanied by the induction of $p21^{Cip1}$ and the prevention of $p27^{Kip1}$ down-regulation, leading to inhibition of cyclin E/cyclin-dependent kinase 2 activity (136). These data suggest that anti-CD3 mAb-induced cytokine non-responsiveness may be a consequence of hyperactivation of the Raf-MEK-ERK pathway, leading to alterations in the expression of key cell cycle regulators. 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 (136). 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 (137), promote Th2 responses, and improve EAE (138) by disabling downregulation of $p27^{Kip-1}$ and upregulating phosphorylation of ERK1/2 (139). Experimental autoimmune encephalomyelitis (EAE) is mediated by myelin-specific CD4(+) T cells secreting Th1 cytokines, while recovery from disease is associated with expression of Th2 cytokines. Investigations into the role of individual cytokines in disease induction have yielded contradictory results. Animals with targeted deletion of the STAT4 or STAT6 genes were generated to determine the role of these signalling molecules in EAE. The STAT4 pathway controls the differentiation of cells into a Th1 phenotype, while the STAT6 pathway controls the differentiation of cells into a Th2 phenotype. Indeed Chitnis et al demonstrated that mice deficient in STAT4 are resistant to the induction of EAE, with minimal inflammatory infiltrates in the central nervous system. In contrast, STAT6-deficient mice, which have a predominantly Th1 phenotype, experience a more severe clinical course of EAE as compared with wild-type or STAT4 knockout mice. Finally, we also observed at the biochemical level the induction of phosphorylation of the STAT6 transcription factor, after leptin neutralization. As said before, this molecule is a 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 (123). So, our results confirm the induction of a regulatory phenotype, induced by leptin neutralization, in the autoreactive T cells infiltrating CNS during EAE.

In conclusion, in the first part of this study, 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.

In the second part of this study, to evaluate whether clinical improvement of EAE, induced by leptin neutralization, could be ascribed to the effect of this kind of treatment on the homeostasis and function of regulatory T cells (a specific cellular subset, known to control the mechanisms of immune tolerance), we performed a series of experiments to check and confirm a possible link between leptin and/or its neutralization and Tregs. The supporting evidence for the hypothesis of a direct interaction between leptin and Tregs base on some results we recently obtained in our laboratory, in which we showed that leptin deficiency associates with resistance to autoimmunity, higher frequency of Tregs and increased susceptibility to infections; we also demonstrated the presence of an inverse correlation between circulating Tregs and serum leptin levels in naive-to-treatment multiple sclerosis patients.

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 (data confirmed in serum and leptin-free conditions). 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 (140) 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.

Naturally occurring CD25+CD4+ regulatory T cells are engaged in the maintenance of immunological self-tolerance and down-regulation of various immune responses (129). Recent studies with mice showed that Foxp3, which encodes the transcription factor Scurfin, is a master regulatory gene for the development and function of CD25+CD4+ regulatory T cells. The FOXP3 gene and its protein product were preferentially expressed in peripheral CD25+CD4+ T cells, in particular CD25+CD45RO+CD4+ T cells in normal individuals and, interestingly, in some human T cell leukemia virus type 1-

infected T cell lines, which constitutively express CD25. Ex vivo retroviral gene transfer of FOXP3 converted peripheral CD25-CD45RO-CD4+ naive T cells into a regulatory T cell phenotype similar to CD25+CD4+ regulatory T cells. For example, FOXP3-transduced T cells exhibited impaired proliferation and production of cytokines including IL-2 and IL-10 upon TCR stimulation, up-regulated the expression of regulatory T cell-associated molecules such as CD25 and CTL-associated antigen-4 and suppressed in vitro proliferation of other T cells in a cell-cell contact-dependent manner. Thus, human FOXP3 is a crucial regulatory gene for the development and function of CD25+CD4+ regulatory T cells, and can be used as their reliable marker. In our experimental conditions Foxp3 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 suppressive 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 (141).

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. Tang et al. already described a robust method to expand antigen-specific Tregs from autoimmune-prone nonobese diabetic mice: briefly purified CD4+ CD25+ Tregs were expanded up to 200-fold in less than 2 weeks in vitro, using a combination of anti-CD3, anti-CD28, and interleukin 2 (142). The expanded Tregs express a classical cell surface phenotype and function both in vitro and in vivo to suppress effector T cell functions. So current strategies to expand T_{reg} cells generally employ ex vivo addition of cytokines to cultured cells during TCR stimulation (142, 143). 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 (126). 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. The finding 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 (144, 145), 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.

There are still many questions concerning the role of several molecules, including leptin and other adipokines, at the interface between metabolism and immunity in the regulation of these two systems. Taken together these results allow us to imagine a scenario of a huge number of autoimmune settings where T_{regs} are isolated from patients either during remission (as would be the case for systemic lupus erythematosus or multiple sclerosis) or soon after disease onset (as would be the case for T1D). The cells would be expanded and reintroduced at the time of maximal disease activity to moderate the inflammatory response. In some cases this could be combined with rapamycin, anti-CD3, or other drugs that cause deletion of the pathogenic cells without affecting the T_{regs} . Together, these therapies could both reduce the short-term pathogenic responses while reinstating a homeostatic balance for long-term tolerance induction and recent studies aim at developing a procedure to selectively and reproducibly expand antigen-specific T_{regs} from polyclonal populations for therapeutic use. Significant leaps of knowledge have been done in recent years in expanding the

field of adipobiology and adipopharmacology of leptin. While new information is unveiling the complexity connecting metabolism and immunity, further research is still needed. This should consider the adipose tissue an active participant in the regulation of essential body process, with prominent roles, particularly in the balance of inflammation and immune homeostasis.

CONCLUSIONS

In the first part of this study we analyzed the possible effects of leptin blockade on the pathogenesis of Experimental Autoimmune Encephalomyelitis (EAE), the animal model of human Multiple Sclerosis. More in detail, we reported 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 (a selective marker for regulatory T cells, a cellular subpopulation known to be involved in the control of immune tolerance) 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, induced by leptin neutralization, 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 (a 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). We also evaluated whether clinical improvement of EAE, induced by leptin neutralization, could be ascribed to a direct effect of this kind of treatment on the homeostasis and function of regulatory T cells**.**

We report in this study that leptin can act as a negative signal for the proliferation of human naturally occurring Foxp3+CD4+CD25+ regulatory T (Treg) cells. Freshly isolated Treg cells expressed high amounts of leptin receptor (ObR) and produced substantial amounts of leptin, that are responsible for an autocrine inhibitory loop that constrains the expansion of T_{reg} cells (data confirmed in serum and leptin-free conditions). Although leptin neutralization inhibits the proliferation of effector $CD4^+CD25^-$ T cells, in vitro neutralization with leptin monoclonal antibody (mAb), during anti-CD3 and anti-CD28 stimulation, resulted in Treg cell proliferation and these cells, that proliferated in the presence of leptin mAb, had increased expression of Foxp3 and retained partial suppressive capacity, which attains full activity when they enter the resting phase. The opposite effects of leptin blockade on $CD4^+CD25^-$ T cells and T_{reg} cells associated with a differential expression of intracellular leptin

and cell-surface ObR in the two cell subsets. We also showed that mouse Treg cells expanded in vivo more robustly and earlier (day 4 and day 7 after transfer) when adoptively transferred into leptin-neutralized WT mice, or leptin deficient mice. Moreover leptin-deficient mice have increased numbers of Treg cells that can be reduced by administration of leptin, confirming the role of leptin as a negative regulator of Tregs homeostasis in constraining their proliferative response. Taken together these results suggest a potential for innovative therapeutic leptin-based interventions in immune and autoimmune diseases, since they describe a novel strategy to expand human peripheral T_{reg} cells, on one hand, and inhibit autoreactive T cells proliferation, on the other, via leptin neutralization.

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PUBLICATIONS

1. 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***. *2007 Feb 23;26 (2):241-255*.

2. 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***. *2006 Feb 1;116(2):447-455*.(* equally contributed).

3. **Procaccini C**., De Rosa V., Galgani M., Abanni L., Matarese G. Leptin: role of metabolism in the regulation of inflammation. ***Biomed Rev***. 2006; 17:1-10.

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CHAPTERS OF SCIENTIFIC BOOKS

1. Matarese G, De Rosa V, Aufiero D, **Procaccini C**, Alviggi C, De Placido G, Fontana S, Zappacosta S. Immune response and metabolism: novel targets for future therapeutic strategies in inflammation and autoimmunity. ***Trends in Immunology Research***. 2005 Nova Science Publishers, Inc. Hauppauge, NY, USA.

2. Matarese G., **Procaccini C**., De Rosa V. Leptin and immune function, inflammation and angiogenesis. ***Leptin: Endocrinology update series*.** 2005 Humana Press, USA.

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