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Role of Leptin and metabolism in survival of autoreactive $CD4^+$ T cells

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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 Hypthalamo-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 signaling in immune cells

Leptin, as previously mentioned, is mainly secreted by the adipose tissue, which is also present within both primary and secondary lymphoid organs and has a significant metabolic and immunomodulatory role (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**).

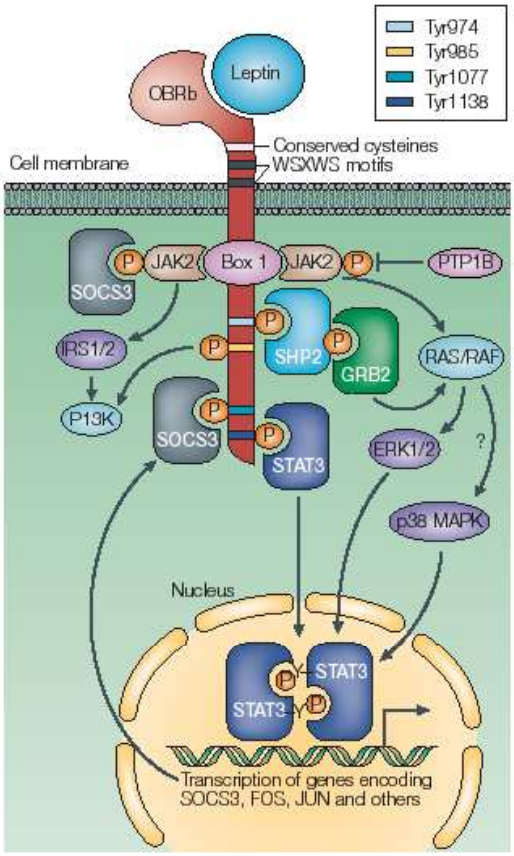

Fig.1 Schematic representation of leptin signaling

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).

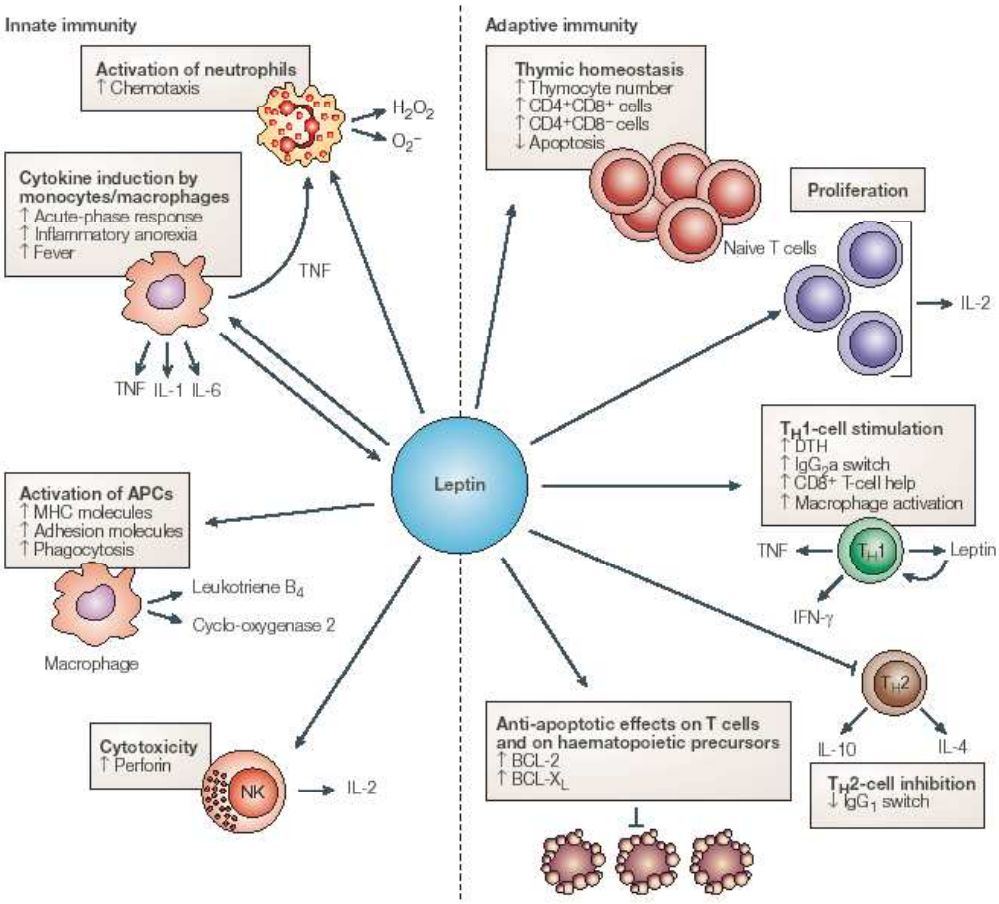

Figure 2. Schematic representation of the effects of leptin on both innate and adaptive immunity.

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).

$CD4^+$ T cells in immunity

The CD4 cell surface marker has come to be associated with a varied group of lymphocytes that orchestrate both innate and adaptive immune responses to pathogens and tumors through a variety of mechanisms. The prototypic member of this group is the $CD4^+$ T-helper (Th) lymphocyte subset, which augments both humoral and cellular immune responses (56, 57). Th cells recognize antigen as peptide epitopes of approximately 12–20 residues long, presented by major histocompatibility complex class II (MHC-II) molecules typically found on specialized antigen-presenting cells (APCs) such as dendritic cells (DCs), macrophages, and B cells (58). In some instances, Th cells can directly recognize antigen on MHC-II-expressing tumor cells, resulting in the production of lymphokines that hinder tumor growth or inducing tumor cell death (59, 61).

Naive $CD4^+$ Th lymphocytes develop in the thymus following a controlled developmental path involving both positive and negative selection to cull potentially autoreactive cells from the repertoire while maintaining the ability to recognize a broad range of pathogen-associated peptides presented by self MHC-II molecules. During an immune response, recognition of the cognate antigen presented on the surface of an APC by the T-cell receptor for antigen (TCR) (Signal 1) along with interaction between appropriate costimulatory molecules such as the CD28 co-receptor with CD80/CD86 (Signal 2) initiates activation of the naive $CD4^+$ T cell. These activated T cells undergo a phase of robust clonal expansion and differentiation into either effector or memory cells. $CD4^+$ memory Th cells can be classified into two main groups based on cell surface markers and functional capacities. Central memory Th cells (Th_{CM}) express high levels of CCR7 and CD62L, lack CD45RA, and traffic through the lymphoid organs (62, 64). Effector memory T cells (Th_{EM}) are CCR7 negative and reside mostly in the blood, spleen, and in non-lymphoid tissues (65). Long-term survival of memory Th cells relies on the participation of costimulatory molecules (OX40/OX40L) and the availability cytokines such as interleukin-7 (IL-7) (66, 68).

The fate and function of the activated Th cells depends in large part upon the microenvironment present at the time of the initial antigen encounter. The composition of the local cytokine milieu will bias development toward one of several alternative differentiation pathways. Likewise, the nature of the antigen acquired by DCs will affect the expression of different sets of costimulatory molecules, which will also dictate the developmental path of the antigen-stimulated Th cells (69). This additional polarizing costimulation has been termed 'Signal 3' and is initiated by various innate pathogen-associated molecular pattern receptors

triggered by the various antigens (70,72). For example, DC exposure to intracellular pathogens programs these APCs to promote Th1-type responses, whereas exposure to helminthes drives DCs to promote Th2 development. A similar situation may exist for the other various regulatory subsets of Th cells (73).

$CD4^+$ T lymphocytes can be grouped into different functional subsets based on function and cytokine secretion patterns (**Figure 3**). Originally, $CD4^+$ T cells were simply classified as Type 1 effector Th cells (Th1) that produce high levels of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) upon antigen stimulation and being responsible for regulating delayed type hypersensitivity (DTH) reactions and cell-mediated immunity to intracellular pathogens and tumor cells. The Th1 developmental pathway is driven by IL-12 activation of signal transducer and activator of transcription 4 (Stat4) and T-bet during immune activation of naive T cells (74). Alternatively, Th2 are characterized by the production IL-4, IL-5, and IL-13 and are responsible for coordinating humoral immunity, eosinophilic inflammation, and controlling helminthic infections. IL-4 is primarily accountable for the differentiation of Th2 cells through Stat6 and GATA (75). The Th1 and Th2 developmental pathways are controlled by a delicate balance of positive feedback loops, as IFN-γ enhances further Th1 development and IL-4 supports continued Th2 differentiation. At the same time, cross-regulation by IFN-γ and IL-4 suppresses Th2 and Th1 differentiation, respectively. In addition to Th1 and Th2 cells, several other subsets of $CD4^+$ T cells participate in the development of immune responses. In many instances, these cells act to control/suppress immune responses and play an important role in the prevention of autoimmune diseases. The best-studied group is the naturally occurring $CD4^+CD25^+$ T-regulatory cells (Tregs) (76, 78). Approximately 5–6% of the $CD4^+$ T cells exiting from the thymus express high levels of CD25, glucocorticoid-induced TNF receptor (GITR), and the transcription factor forkhead box protein 3 (Foxp3) (79, 81). These Tregs mediate immune suppression through a cell-to-cell contact-dependent mechanism that does not require antigenic stimulation (82). While important for the prevention of autoimmunity, in some circumstances Tregs hinder desirable immune responses, for example against tumor-associated antigens. Depletion of this subset in vivo, for example with anti-CD25 monoclonal antibodies, enhances anti-tumor immunity in mice (83, 87), specially when the targeted tumor antigens are expressed to some extent by normal cells (e.g. tissue differentiation antigens or products of overexpressed genes). Antigen-experienced $CD4^+$ T cells can also develop into Tregs that express CD25, Foxp3, and GITR. Although the origins of these adaptively induced Tregs is unclear, they have similar immune suppressive effects as their naturally occurring counterparts. Another subset of regulatory $CD4^+$ T cells

called Th17 has been described recently (88). The evidence suggests that Th17 cells develop independently from either Th1 or Th2 cells and represent a distinct lineage (89).

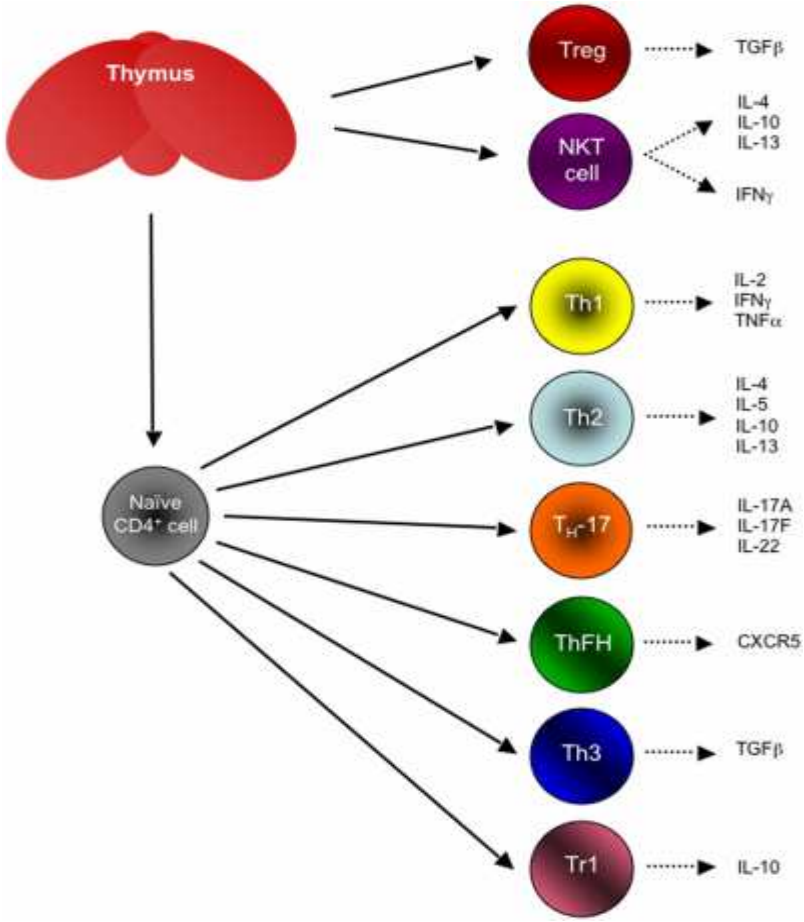

Figure. 3. **Diversity of CD4-expressing cell subsets**. The main $CD4^+$ T-cell subsets develop from naive $CD4^+$ T cells after antigen-dependent T-cell activation. The microenvironment present during priming, including antigen dose, APC type, cytokines, and costimulatory signals, all influence the developmental pathway taken by the responding T cell. The major cytokines and secreted factors contributing to the respective functions of the Th subsets are listed to the right of each cell type. (**Kennedy, R and Celis, E 2008 *Immunol Reviews***)

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 (90). 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 (90). 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 (91). 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 (92).

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 (93). 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 Expeimental Autoimmune Encefalomylaitis (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 Hepatites (EIH) (93, 94). 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. (94) 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) (94). Similar results were obtained in EIH induced by Pseudomonas aeruginosa exotoxin A administration (93). 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 Colites, EIC) (95). 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 (95). 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 (96, 97). 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 (95, 97).

More recently, protection from autoimmunity in *ob/ob* mice has been observed in Experimentally Induced Glomeruloneprhites (98). 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 (99).

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 (100). Leptin accelerates autoimmune diabetes in females NOD/LtJ mice (101, 102). 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 (101). 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 (101). 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 (103, 104). 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 (105). 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 (105).

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 (106).

Multiple Sclerosis (MS) is a chronic, immune-mediated, inflammatory disorder of the central nervous system (CNS) (107). 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 (108, 109). 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 (110, 111). The mechanisms of tissue injury in focal white matter lesions are heterogeneous, resulting in patterns of demyelination that vary between patients or patient subgroups (108). 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 (**Figure 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 (108).

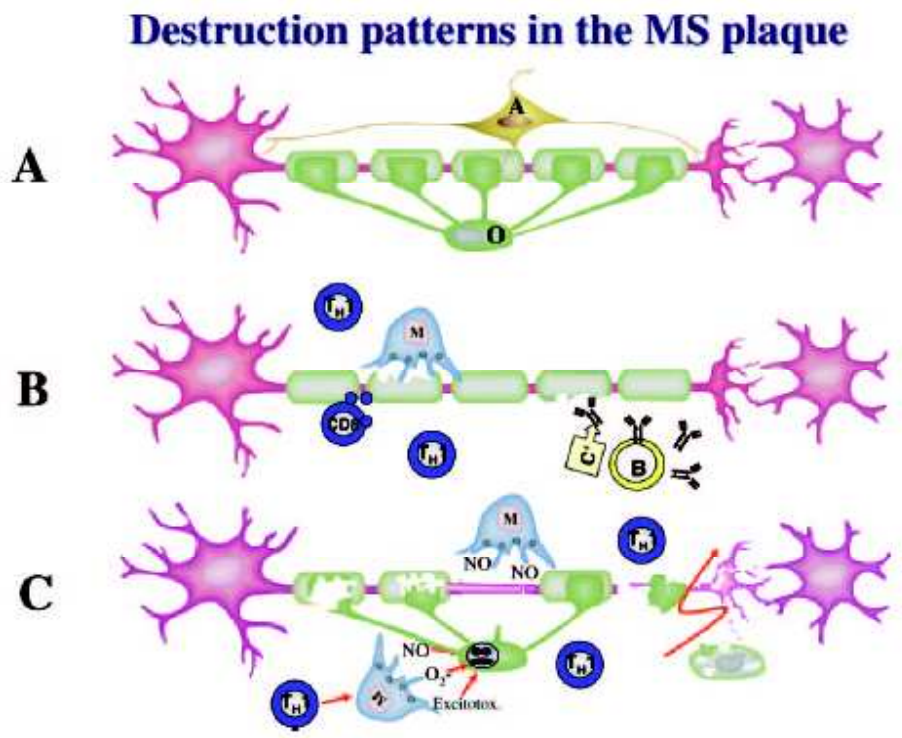

Figure 4. **Destruction patterns in the multiple sclerosis plaque**. A**)** In the healthy CNS oligodendrocytes enheathe the axon and form myelin internodes of regular size. B**)** Cytotoxic attack can destry the myelin sheat via T-cell and macrophage inflammation. Cytotoxic T cells secrete perforin 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 (112). It has been shown that leptin is involved in both the induction and in the progression of EAE (112). 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 (112). Importantly, a surge of serum leptin anticipates the onset of clinical manifestations of EAE (113). The peak of serum leptin correlates with inflammatory anorexia, weight loss, and the development of pathogenic T cell responses against myelin (113). 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 (113) (**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 (114). 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 (114). 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) (114). 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 (115). 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 (113). 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 (114). 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 (114). Finally, recent reports (115) 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 (116). 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.

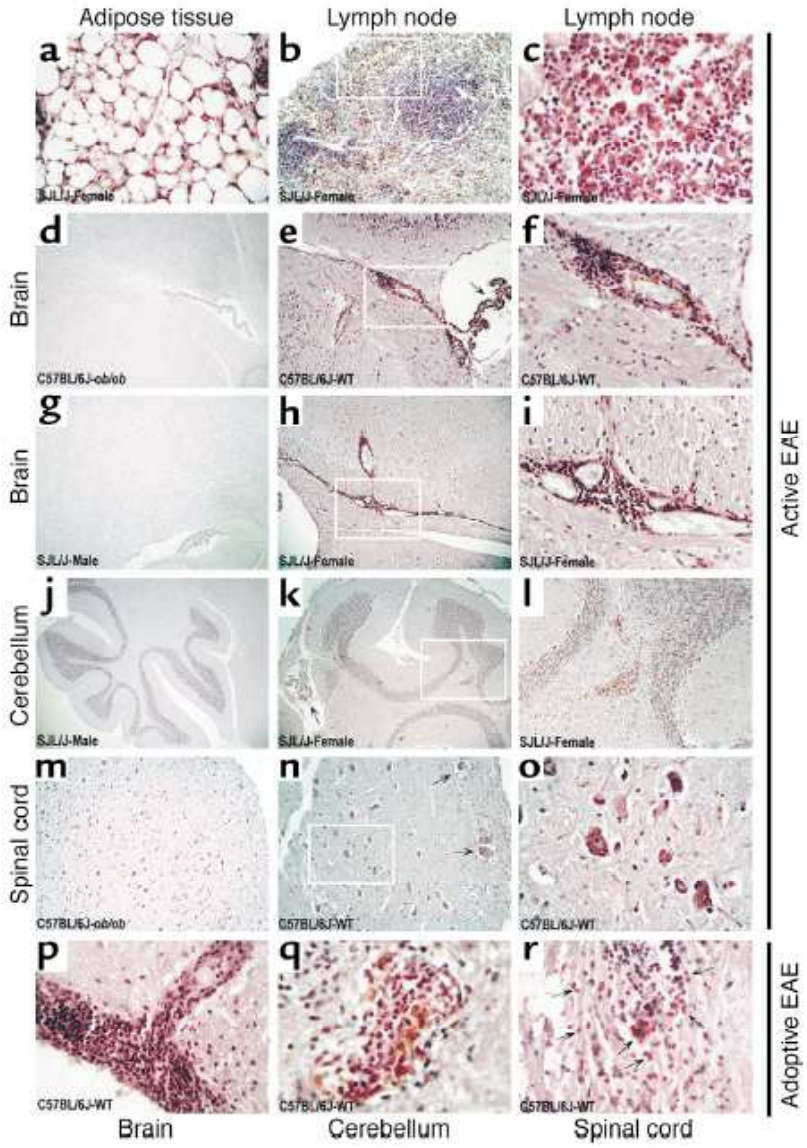

Fig 5. **Lymph node and CNS expression of leptin during acute/active EAE**. A) Leptin expression in SJL/J female mouse adipose tissue used as positive control. (B and C) Expression of leptin in T cells and macrophages in a draining lymph node from SJL/J female mice after immunization with PLP139–151. 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

As discussed before, leptin is a peptide hormone belonging to the helical cytokine family produced primarily from adipocytes, it has been shown to control food intake, basal metabolism and reproductive function (14). Experimental evidence supports a direct role for leptin in the regulation of immunity (117). Obese leptin-deficient (ob/ob) mice and leptin receptor (LepR)-deficient mice (db/db) mice, display numerous immune abnormalities (18, 117), including mild-severe $CD4^+$ T cell lymphopenia, increased absolute number of natural regulatory T (Treg) cells and resistance to a series of inducible or spontaneous autoimmune disorders such as experimental autoimmune encephalomyelitis (EAE) and type 1 diabetes in nonobese diabetic (NOD) mouse, respectively. Leptin enhances also T helper 1 (Th1) proinflammatory cytokine production in vivo and in vitro, conditional on the presence of a functional LepR (119).

Immune homeostasis, the maintenance of lymphocyte numbers, is critical factor for survival. In the thymus, developmental and maturation programs regulate thymocyte numbers and output. In the periphery, regulation of cell survival, proliferation and death ensure the maintenance of T cell numbers. Immune homeostasis is also critical to protect against self-reactivity, which can arise as T cell receptors develop and diversify. Negative selection in the thymus, anergy and a variety of Treg cell populations manage these potentially autoreactive lymphocytes (120).

Loss of immune homeostasis, leading to abnormal lymphocyte numbers, can lead to various disease states. In order to maintain peripheral T cell numbers, lymphocytes undergo homeostatic proliferation (121). Homeostatic proliferation occurs to maintain a full lymphoid compartment in numerous natural settings, such as in newborns, whose immune systems are still developing, and in the elderly, whose thymic output has decreased (122). The regulation of homeostatic proliferation is key for normal immune function. Cytokines, including IL-6, IL-7, IL-15 and IL-21, have all been implicated in homeostatic control (123, 124). Treg cells have also been implicated in the control of homeostatic proliferation, although their exact role remains controversial. Recent reports have shown that increased homeostatic proliferation associated with T cell lymphopenia can expand the pool of autoreactive T cells that promote autoimmunity (125, 128). In striking distinction, the observed $CD4^+$ lymphopenia in ob/ob mice is not associated with an increased homeostatic proliferation of T cells leading to autoimmunity, rather to a resistance to break of self-tolerance leading to autoimmunity.

Our working hypothesis is that reduced susceptibility of ob/ob mice to autoimmunity and EAE could be ascribed to a reduced survival of autoreactive $CD4^+$ T cells in an altered leptin-deficient microenvironment.

Materials and Methods

Mice and *in vivo* experiments

Female C57BL/6J wild-type (WT-B6) and C57/BL/6J-*ob/ob* (*ob/ob*) leptin deficient mice 8-10 week old were purchased from Charles River Italy (Calco, Italy) and from Harlan Italy (Correzana, Italy). The B10.Cg.Tg (TcrAND)53Hed/J (AND-TCR Tg) PCC-specific transgenic mice were purchased from The Jackson Laboratory (Barr Harbor, ME). WT-B6 and *ob/ob* mice were age matched for individual experiments and were group-housed two to six mice per standard cage according to different experimental condition, with a 12-h light-dark cycle. All experiments were performed under approved protocol in accordance with animal use guidelines of the Istituto Superiore di Sanità (Rome, Italy).

WT-B6 mice were injected intraperitoneally (i.p) with either leptin dissolved in (Sigma Aldrich) 200µL of PBS at a dose of 100µg/mouse and rapamacyn (Sigma Aldrich) at a dose 100µg/mouse.

Leptin administration

Mouse recombinant leptin (rleptin) was obtained from R&D Systems Europe (Oxon, U.K.); purity was >97%, assessed by SDS-PAGE and visualized by silver staining analysis. The endotoxin level was <0.1 ng/µg of leptin, as determined by the *Limulus* amebocyte lysate method. Mice comprised two groups (n = 6–11 per group) for *ob/ob* leptin-deficient obese mice (all housed in pairs) and one groups (n = 6–10 per group) for C57BL/6J normal age- and sex-matched control mice (housed two to six mice/cage). For adoptively induced disease, mice were treated starting 3 days before the transfer of MOG_{35-55} T cells and continuing over a period of 30 days. Of the groups of leptin-deficient mice, one was injected with 200 µl of PBS twice daily (at 10:00 a.m. and 6:00 p.m.); the second group was injected with murine rleptin (0.5 µg/g initial body weight twice daily in 200 µl volume i.p., for a total of 1 µg/g/day of rleptin); according to the same schedule (112, 129). For the group of WT-B6 mice, was injected with PBS twice daily according to the same schedule of obese mice. All mice were weighed and their food intake was recorded daily.

Antigens

The peptide used in this study were the immunodominant MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK) and the PCC_{88-104} peptide (KAERADLIAYLKQATAK) (130). It was obtained from Inbios srl (Napoli, Italy) and purity was verified by HPLC (97% pure); the amino acid composition was assessed by mass spectrometry. In all experiment, we used MOG_{35-55} peptide and PCC_{88-104} peptide from the same preparations, initially solubilized in LPS-free saline solution at 4 mg/ml concentration, and stored at -80°C.

Induction of adoptive EAE

For induction of adoptive EAE (101), 10 female donor C57Bl/6J mice (6-8 week old) were primed s.c with 300 µg of MOG_{35-55} peptide in CFA distributed over four sites. After 9-10 days, draining lymph nodes and spleen were harvested, homogenized into a single cell suspension, and cultured separately *in vitro* in 24-well plates (Falcon, Becton Dickinson, Franklin Lakes, NY) with RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (Life Technologies), 2 mM L-glutammine (Life Technologies), 0.1 mM nonessential amino acids (Life Technologies), mM sodium pyruvate (Life Technologies), 50 µM 2-ME (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies), and 25 µg/ml of $MOG_{35–55}$ peptide. After 3 days in culture and addition to medium of 2 U/ml of rIL-2 (Roche Biochemicals, Monza, Italy), the cells were harvested and centrifuged over a Ficoll gradient (Pharmacia Biotech, Uppsala, Sweden) to remove debris Recipient syngeneic naive female leptin-deficient, PBS or rleptin treated, and WT-B6 control mice were i.v. injected with 2.5 x 10^6 T cells in a final volume of 500 µl of PBS. Mice also received 200 ng of pertussis toxin immediately after cell transfer and 1 day later.

Clinical assessment

Individual mice were observed daily for clinical signs of disease for up to 30 days after adoptive transfer. Mice were weighed and scored daily according to the clinical severity of symptoms. We used on a scale of 0 to 6 (112) by a "blinded" to mice identity experimenter, 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 of these signs; 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 (score = 0) were included in the calculation of the daily mean clinical score for each group.

Induction of delayed-type hypersensitivity (DTH) (footpad-swelling assay)

DTH responses to adoptively transferred $MOG_{35–55}$ specific T cells were quantitated using a time-dependent footpad-swelling assay. Briefly, mice previously adoptively transferred with $5x10^6$ $MOG_{35–55}$ specific $CD4^+$ T cells were challenged by s.c. injection of into the right hind footpad 50μg MOG_{35-55} peptide . PBS alone was injected into the left footpad to serve as control for measurements. As negative control, we used immunized mice (sensitized with CFA alone). Footpad thickness was measured either after 7 days after transfer at 12, 24, 48, and 72 h after challenge, and for long-time DTH assessment footpad swelling was measured at respectively at 1, 7, 14 days by a "blinded" to sample identity experimenter using a caliper-type engineer's micrometer. The footpad-swelling response was calculated as the thickness of the right footpad (receiving Ag) minus the baseline thickness of the left footpad (receiving PBS).

Cytokine measurement

Leptin, IL-7, IL-15 and IL-21 were measured using ELISA detection kits purchased from R&D Systems (Minneapolis, MN, USA), Bioo Scientific (Austin, TX USA) and Biolegend (San Diego CA USA), respectively. Measurements were performed according to the manufacturer's instructions. Soluble IL-1α, IL-2, IL-4, IL-5, IL-10, IL-17A, IFN-γ GM-CSF,

TNF-a, mouse cytokine were measured using beads based Analyte Detection Assay (Th1/Th2 FlowCytomix Kit, Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions.

Proliferation assays

Spleen cells were obtained from mice at different time points after adoptive transfer, dissociated into single cell suspension, and cultured in flat-bottom 96-well microtiter plates (Falcon) at a density of $5x10^5$ viable cells/well in a total volume of 200µl of RPMI 1640 medium (Life Technologies), supplemented with 1% autologous mouse serum from each different groups of mice, 2 mM L-glutamine (Life Technologies), 0.1mM nonessential amino acids (Life Technologies), 1mM sodium pyruvate (Life Technologies), 50µM 2-ME (Sigma), 100U/ml penicillin, and 100µg/ml streptomycin (Life Technologies). Cells were cultured at 37°C in 100% humidity and 5% CO_2 in the presence or absence of varying concentrations of $MOG_{35–55}$ peptide (from 0 to 100µg/ml peptide). For experiments with AND-TCR-Tg mice DCEK transfectants (murine fibroblasts cells transfected with the E^k mouse class II molecule, (131) were used as antigen presenting cells to activate *in vitro* $CD4^+$ AND-TCR-Tg mice T cells. T cells were incubated for 72h and an additional 16h, pulsed with 0.5µCi/well of [^{3}H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ), harvested on glass-fiber filters using a Tomtec (Orange, CT) 96-well cell harvester, and counted in a 1205 Betaplate liquid scintillation counter (Wallac, Gaithersburg, MD). Results are expressed as mean cpm ± SD from triplicate cultures.

Flow cytometry, cell sorting and biochemical analyses

$CD4^+$ T cells from donor mice were stained with the fluorescent dye CFSE (5-, 6-carboxyfluorescein diacetate succinimidyl ester) from Molecular Probes (Eugene, OR) used at 1µg/ml $5x10^6$ $CD4^+$ $CFSE^+$-labelled T cells were injected in tail vein of WT-B6, *ob/ob* and leptin-treated *ob/ob* mice. For flow cytometric analyses of $CFSE^+$ $CD4^+$ T cells 7 and 14 days after transfer, spleen of three mice groups were harvested and $1x10^6$ cells were analyzed to Facscalibur (Becton Dickinson, San Diego, USA) using CellQuest software (Becton Dickinson, San Diego, USA). For biochemical analyses ($0.5\text{-}1x10^6$) $CD4^+CFSE^+$ cells were obtained from the spleen of each group of WT, *ob/ob* and *ob/ob*-leptin-replaced mice, after High-Speed Cells Sorting (MoFlo, Dako, Denmark); cells were 99% pure. For western blotting, sorted cells were lysed in 50 mM HEPES (pH 7.5), 250 mM NaCl, 1 mM EDTA,

1.0% Triton X-100, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin. 50 μg of total proteins were loaded on SDS-PAGE gel under reducing conditions. After electrophoresis, proteins were transferred onto a nitrocellulose filter membrane (Protan, Schleicher & Schuell) with a Trans-Blot Cell (Bio-Rad) and transfer buffer containing 25mM Tris, 192mM glycine, 20% methanol. Membranes were placed in 5% nonfat milk in PBS, 0.5% Tween 20 (PBST) at 4°C for 2 hr to block the nonspecific binding sites. Filters were incubated with specific antibodies before being washed three times in PBST and then incubated with a peroxidase-conjugated secondary antibody (Amersham Biosciences). After washing with PBST, peroxidase activity was detected with the ECL system (Amersham, Biosciences) or Femto (Pierce). The antibodies used were the following: anti-$p27^{Kip-1}$, anti-pAKT, anti-AKT and anti-Bcl-2 and anti-PS6 (all from Cell Signaling Technology, Beverly, MA); all filters were quantified by densitometric analysis of the bands utilizing the program ScionImage 1.63 for Mac (Scion Corporation, Frederick, MD). Finally, FACS analyses for intracellular signalling were also performed by intracellular staining of P-S6, using Pe-conjugated P-S6 antibody (Cell Signaling Technology, Beverly, MA), was performed *ex-vivo* on $CFSE^{+}$ cells after fixation and permeabilization procedures.

Results

Chronic leptin deficiency associates with hypoplasia of lymphoid organs

We examined lymphoid organs in leptin-deficient (*ob/ob*), leptin-replaced *ob/ob* and wild-type (WT-B6) age-matched control mice. In *ob/ob* mice, thymus, spleen showed marked macroscopic hypoplasia as compared with normal WT counterparts and recombinant leptin-replaced *ob/ob* mice (**Figure 6A**). Microscopic analyses in *ob/ob* mice (hematoxylin&eosin) confirmed the atrophy of thymus in both cortical and medullar areas (**Figure 6B**). In addition, the spleen reduced in size and characterized by almost the absence of white pulp and primary follicles. These latter were confined only in the polar zones of the spleen (**Figure 6B arrows**). Finally, lymph nodes were very difficult to be found in *ob/ob* mice within the massive adipose tissue and the few that were isolated showed a marked "adipose-metaplasia", in which the adipocytes represented the majority of cells (data not shown). In all the different lymphoid organs leptin-replacement restored both normal macroscopic and microscopic architecture (**Figure 6A-B**).

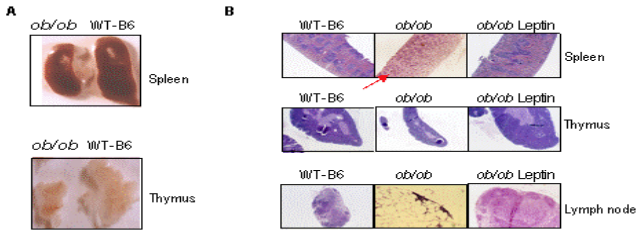

Figure 6. Leptin deficiency is associates with hypoplasia of lymphoid organs.
A) Macroscopic hypoplasia of spleen and thymun in *ob/ob* as compared with normal WT-B6 counterpats. B) Hemotoxylin&eosin staining of spleen, thymus and lymph node from WT-B6, *ob/ob* and *ob/ob* rleptin-treated mice.

Resistance to adoptively-transferred EAE in *ob/ob* mice associates with a progressive decline in the *in vivo* myelin-antigen-specific $CD4^+$ T cell responses and reduced Th1/Th17 cytokine secretion

We have previously suggested that leptin is required for induction and progression of EAE (112). Our hypothesis is that leptin controls survival and proliferation of myelin-antigen-specific $CD4^+$ T cells. We adoptively transferred $5x10^6$ encephalitogenic MOG_{35-55}-specific $CD4^+$ T lymphocytes into the tail veins of WT-B6, leptin-deficient *ob/ob* mice treated or not with mouse recombinant leptin (rleptin), respectively. As shown **Figure 7A**, MOG_{35-55}-specific $CD4^+$ T cells were unable to transfer EAE when injected into *ob/ob* recipients. In contrast, these T cells induced EAE when transferred into WT-B6 and *ob/ob* treated with rLeptin, respectively, as suggested by a similar frequency of disease and clinical score in these groups of mice. We dissected *in vivo* and *in vitro* the magnitude, the efficiency and the progression overtime of the Th1-metiated immune response transferred by the MOG_{35-55}-specific T cells. More specifically, 7-days after adoptive transfer of MOG_{35-55}-specific $CD4^+$ T cells mice were challenged with 50μg of MOG_{35-55} peptide into the footpad to measure the delayed-type hypersensitivity (DTH) response. *Ob/ob* mice showed a reduced kinetics in the DTH reaction (12-72h) as compared with WT-B6 and rleptin treated *ob/ob* mice (**Figure 7B**). In addition, to evaluate whether leptin deficiency could influence DTH response over a long time frame, we analyzed at different days from the adoptive transfer (1, 7, and 14 days) the maintenance of footpad swelling in all the above mentioned groups. MOG_{35-55}-specific $CD4^+$ T cells were unable to maintain DTH responses over time, when adoptively transferred into *ob/ob* mice (**Figure 7C**). Finally, to define whether leptin deficiency could affect *ex-vivo* proliferation of previously-transferred MOG_{35-55} specific T cells, we performed dose-depended MOG_{35-55} specific *in vitro* stimulation of splenocytes pulsed with MOG_{35-55} from the three groups of mice. all in autologous mouse serum to preserve also *in vitro* the *in vivo* condition of leptin deficiency and/or treatment. Cell proliferation of WT-B6 cells obtained from *ob/ob* mice was strongly reduced at all the different concentrations of MOG_{35-55} peptide, whereas rleptin treatment restored the proliferative capacity (**Figure 7D**). Next, secretion of cytokines from WT-B6 MOG_{35-55}-specific T cells obtained from *ob/ob* mice was reduced in terms of production of pro-inflammatory cytokines such as IL-1α, IL-2, IL-6, IFN-γ, TNF-α, GM-CSF, IL-17A , restored by rleptin administration (**Figure 7E**). Leptin deficiency did not alter the production of IL-4 and IL-10 classical Th2/regulatory type cytokines by WT-B6 cells in response to MOG_{35-55} antigen, whereas IL-5 was reduced similarly to Th1 cytokines.

Finally, WT-B6 MOG_{35-55}-specific $CD4^+$ T cells from *ob/ob* mice showed impaired release of survival cytokines, IL-15 and IL-21, when stimulated *in vitro* with MOG_{35-55}-specific peptide. The levels of IL-7 *in vitro* secretion were undetectable after MOG_{35-55}-peptide stimulation. In addition, the level of surface expression of the IL-7 receptor was not different in all three groups of mice (data not shown).

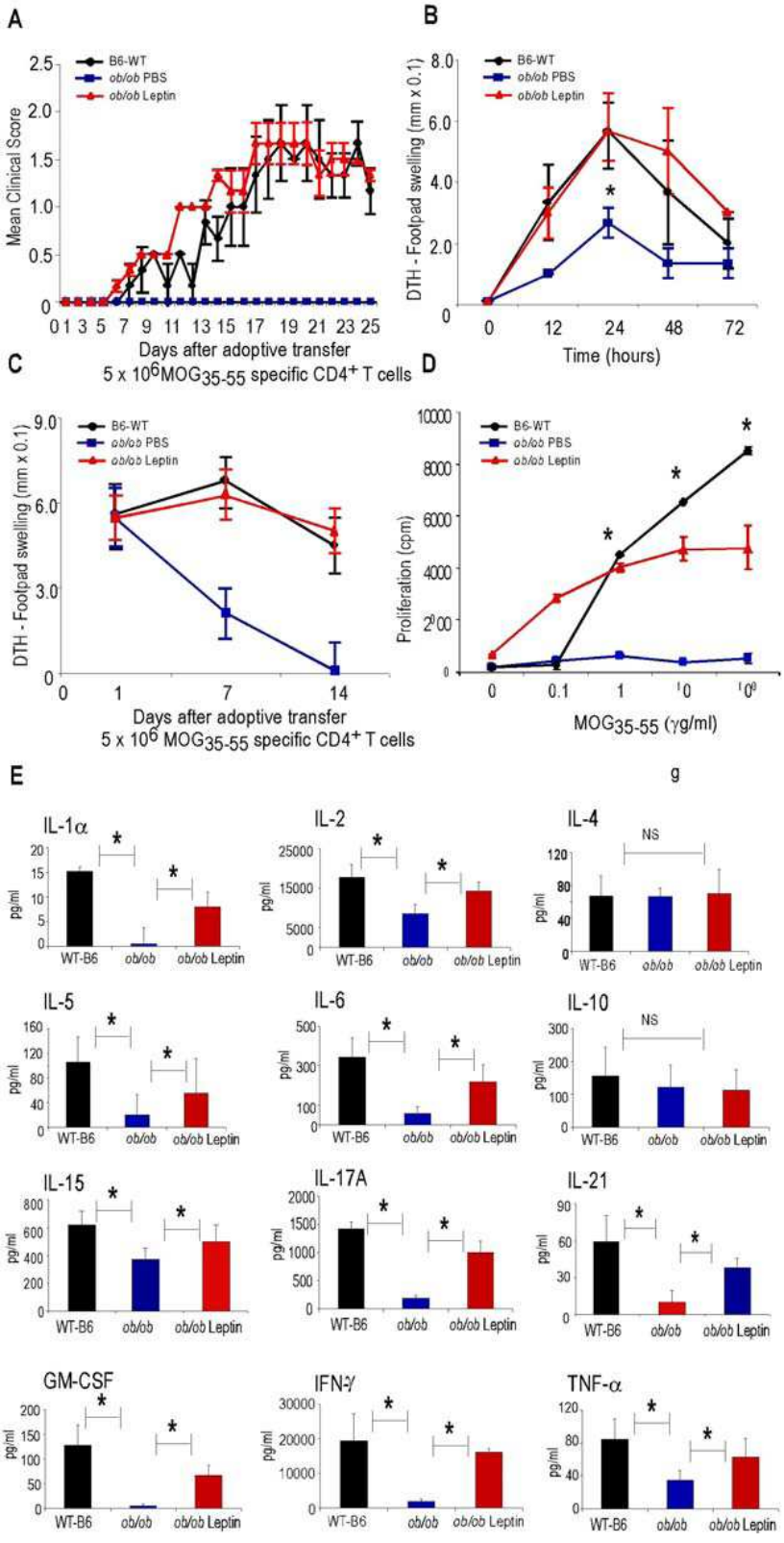
A
B6-WT
ob/ob PBS
ob/ob Leptin
Mean Clinical Score
Days after adoptive transfer
5 x 10^6 MOG35-55 specific CD4+ T cells
B
DTH - Footpad swelling (mm x 0.1)
Time (hours)
C
DTH - Footpad swelling (mm x 0.1)
Days after adoptive transfer
5 x 10^6 MOG35-55 specific CD4+ T cells
D
Proliferation (cpm)
MOG35-55 (γg/ml)
E
g
IL-1α
IL-2
IL-4
IL-5
IL-6
IL-10
IL-15
IL-17A
IL-21
GM-CSF
IFN-γ
TNF-α
pg/ml
WT-B6
ob/ob
ob/ob Leptin
NS

Fig. 7 Leptin deficiency associates with resistance to passively-induced EAE, reduced DTH responses and decreased proliferation of $CD4^+$ MOG_{35-55} specific T cells. A) Mean clinical score of passively induced EAE disease in WT-B6, *ob/ob*-PBS treated and *ob/ob*-leptin treated mice. Only WT-B6 and *ob/ob*-leptin treated mice groups develop clinical signs of disease, and showed a similar disease score. *ob/ob*-PBS treated mice were resistant to EAE induction when adoptively transferred with $5x10^6$ encephalitogenic MOG_{35-55}-specific $CD4^+$ T lymphocytes. *ob/ob*-leptin treated group were injected with rleptin starting 3 days before the transfer until day 25. Data are representative of three independent experiments with similar results (n = 5 mice per group). B) DTH reaction in WT-B6, *ob/ob*-PBS treated and *ob/ob*-leptin treated mice. 7-days after adoptive transfer of MOG_{35-55}-specitif $CD4^+$ T cells, mice were challenged 50mg of MOG_{35-55} peptide into the footpad to measure the delayed-type hypersensitivity (DTH) response. C) DTH response over time, 7-14 days after adoptive transfer MOG_{35-55}-specitif $CD4^+$ T. Data are representative of two independent experiments with similar results, showing the means ± SD of footpad-swelling responses. $*p < 0.001$ compared with WT-B6. D) Dose-depended MOG_{35-55} specific *in vitro* stimulation of splenocytes pulsed with MOG35-55 from the three groups of mice previously adoptively transferred with pathogenic MOG_{35-55}-specific $CD4^+$. Data are representative of two independent experiments showing the means ± SD of footpad-swelling responses. $*p < 0.05$. E) *In vitro* cytokines release on cell culture supernatent upon MOG_{35-55} specific *in vitro* stimulation of splenocytes from the three groups of mice previously adoptively transferred with WT-B6 pathogenic MOG_{35-55}-specific $CD4^+$.

Leptin controls homeostasis and survival of autoreactive and antigen-specific $CD4^+$ T cells

To define whether leptin deficiency influences survival and proliferation *in vivo* of MOG_{35-55}-specific $CD4^+$ T cells, $CD4^+$ T purified from immunized MOG_{35-55} peptide WT-B6 mice 7 days after immunization were activated *in vitro* for three days with MOG_{35-55}-specific peptide, CFSE-labeled and adoptively transferred into WT-B6, *ob/ob* and rleptin-treated *ob/ob* recipient mice, respectively (**Figure 8**). At different time points the cells were monitored longitudinally in the spleens; we analyzed the recovery of $CFSE^+$ MOG_{35-55}-specific $CD4^+$ T cells in all groups of mice. Seven days post-transfer the percentage of $CFSE^+$ $CD4^+$ T cells in *ob/ob* mice was dramatically reduced when compared with the other groups (**Figure. 8A-B**). At later time points (day 14) post transfer $CD4^+$ T cells further collapsed as suggested by a reduced percentage and number (**Figure. 8B)**. As the *in vivo* expansion rate measured as CFSE dilution was similar among the groups we tested whether altered recovery of $CFSE^+$ $CD4^+$ T in *ob/ob* mice could be ascribed to their increased apoptotic rate, we evaluated the expression of Annexin-V apoptotic marker on surface of $CFSE^+$ MOG_{35-55}-specific $CD4^+$ T cells recovered from the spleens of WT-B6, *ob/ob* treated or not with rleptin. We found that Annexin-V levels were significantly increased in WT-B6 $CD4^+$ T cells derived from *ob/ob* mice (**Figure 8C**).

To expand and confirm our observations to an homogeneous and clonal T cell population, we utilized also $CD4^+$ cells from TCR transegnic mice (132) (AND-TCR-Tg) against pigeon cytochrome c peptide (PCC_{88-104}) and analyzed their CFSE dilution and survival overtime. We adoptively transferred $CFSE^+$ PCC_{88-104}-specific $CD4^+$ T cells after three days *in vitro* activation with the PCC_{88-104} peptide loaded on DCEK transfectants (104) into WT-B6, *ob/ob* treated or not with rleptin. Once again leptin deficiency was responsible of the reduced recovery of $CFSE^+$ PCC_{88-104}-specific $CD4^+$ transgenic T cells from *ob/ob* mice (**Figure 8D**) at 7 and 14 days after transfer (**Figure 8D-E**). Levels of apoptosis measured as Annexin-V were increased in *ob/ob* mice similarly to autoantigen specifc MOG_{35-55} cells (data not shown).

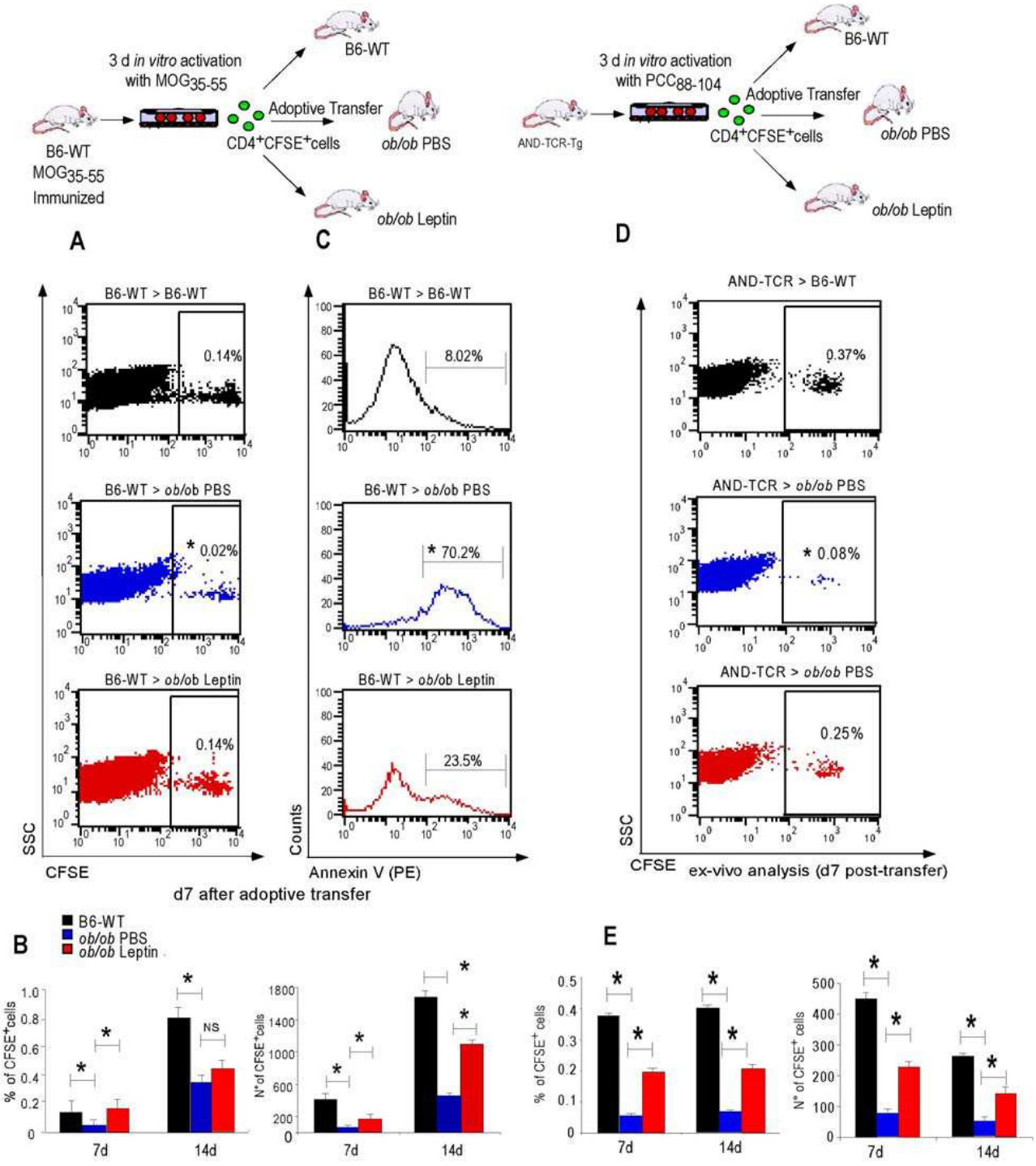

Figure 8. Leptin affects homeostasis of antigen-specific CD4$^+$ T cell

Left Schematic model of the experimental procedure for MOG_{35-55} specific CD4$^+$ T cells: WT-B6 mice were immunized with MOG_{35-55}-petide. After 9-10 days, draining spleen CD4$^+$ T cells were cultured *in vitro*, for 3 days in presence of MOG_{35-55}-petide. Next, 10 x 10^6 CD4$^+$ T cells were stained with CFSE and injected i.v into WT-B6, ob/ob PBS-treated and ob/ob rLeptin-treated recipient mice. After 7 and 14 days mice were harvested and citofluorimetric analysis was performed. **Right** Schematic model of the experimental procedure for PCC_{88-104} specific CD4$^+$ T cells: WT-B6 mice were immunized with PCC_{88-104}-petide. After 9-10 days, draining spleen CD4$^+$ T cells were cultured *in vitro*, for 3 days in presence of PCC_{88-104}-petide.. Next, 10 x 10^6 CD4$^+$ T cells were stained with CFSE and injected i.v into WT-B6, ob/ob PBS-treated and ob/ob rLeptin-treated recipient mice. After 7 and 14 days mice were harvested and citofluorimetric analysis was performed.

A) Representative flow cytometry plots of CFSE$^+$ CD4$^+$ T cells recovered in the spleen from WT-B6 (upper panel), *ob/ob*-PBS treated (middle panel) and *ob/ob*-leptin (lower panel) treated mice 7 days after adoptive

transfer of *in vitro*-activated MOG_{35-55}-specific CFSE-labelled $CD4^+$ T cells from WT-B6. B) Histograms represent the percentage (left) and absolute number (right) of $CFSE^+$ T cells detected at 7 and 14 days post adoptive transfer in spleen and B6-WT (dark bars), *ob/ob* PBS-treated (blu bars) and *ob/ob* rleptin-treated mice (red bars). Data are representative of three independent experiments. Data are shown as mean +/- SD. *$p < 0.05$. C) Representative flow cytometry plot of Annexin V staining $CFSE^+$ $CD4^+$ T cells recovered in the spleen from WT-B6 (upper panel), *ob/ob*-PBS treated (middle panel) and *ob/ob*-leptin (lower panel) treated mice 7 days after adoptive transfer of *in vitro*-activated MOG_{35-55}-specific CFSE-labelled $CD4^+$ T cells from WT-B6. Data are representative of three independent experiments and indicate the percentage of Annexin V^+ cells. D) Representative flow cytometry plots of $CFSE^+$ $CD4^+$ T cells recovered in the spleen from WT-B6 (upper panel), *ob/ob*-PBS treated (middle panel) and *ob/ob*-leptin (lower panel) treated mice 7 days after adoptive transfer of *in vitro*-activated PCC_{88-104}-specific CFSE-labelled $CD4^+$ T cells from WT-B6. E) Histograms represent the percentage (left) and number (right) of $CFSE^+$ T cells detected at 7 and 14 days post adoptive transfer in spleen B6-WT (dark bars), *ob/ob* PBS-treated (blu bars) and *ob/ob* rLeptin-treated mice (red bars). Data are representative of three independent experiments. Data are shown as mean +/- SD. *$p < 0.05$.

Leptin deficiency associates with reduced expression of the survival gene Bcl-2, impaired activation of P-ERK1-2 pathway and increased expression of the cell cycle inhibitor $p27^{kip1}$

To understand the intracellular molecules and biochemical pathway leading to impaired survival of MOG_{35-55}-specific $CD4^+$ T cells in leptin-deficient mice, we dissected the molecular pathways involved in cell survival and proliferation. Western blot analysis showed that FACS-sorted WT-B6 MOG_{35-55}-specific $CD4^+$ T cells, derived from *ob/ob* mice, displayed lower levels of the anti-apoptotic protein Bcl-2 and higher amount of cell cycle inhibitor $p27^{Kip1}$ as compared to WT-B6 $CD4^+$ T cells from WT-B6 and leptin-treated mice (**Figure 9**). Finally, leptin deficiency affected P-ERK1/2 pathway of WT-B6 MOG_{35-55}-specific $CD4^+$ T cells transferred in *ob/ob* mice. These findings are in agreement with the increased apoptosis and cell cycle arrest observed in the condition of leptin deficiency.

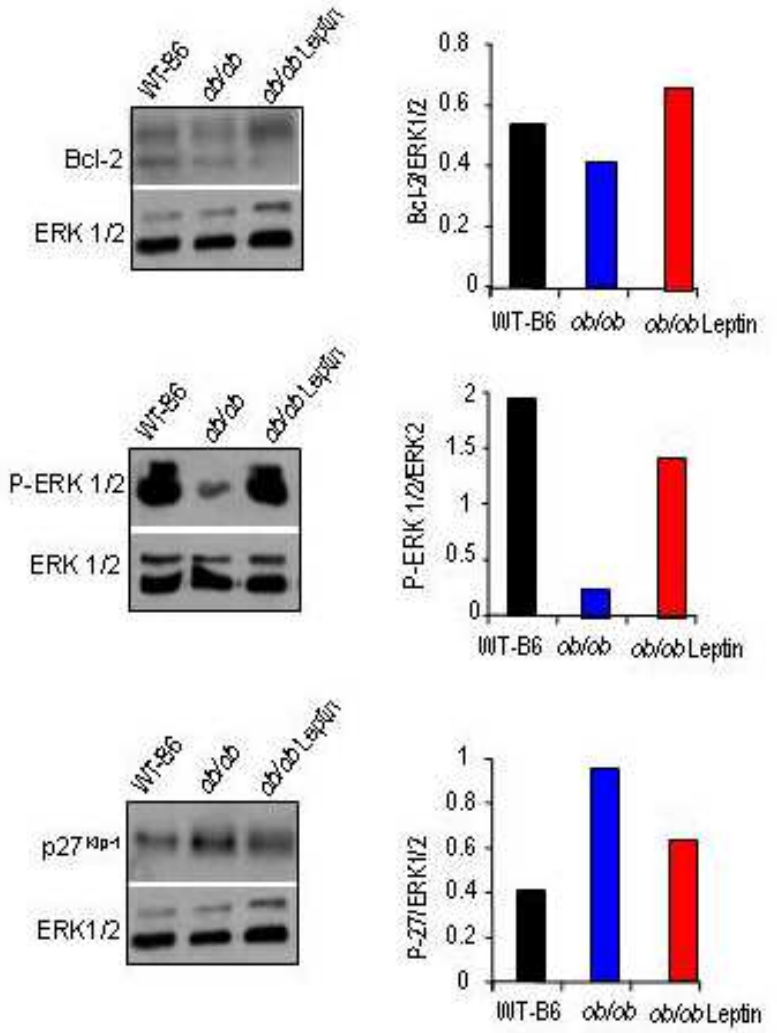

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Fig. 9 Leptin deficiency is associates with impaired ERK1/2 (P-ERK1/2) phosphorylation, reduced expression of survival gene Bcl-2 and increased expression of the cell cycle inhibitor $p27^{kip1}$ in MOG_{35-55} specific T cells

Immunoblot for, phospho-ERK1/2 (P-ERK1/2)**,** $p27^{kip1}$, Bcl-2, on FACS-Sorted MOG_{35-55} $CD4^+$ T cells from WT, *ob/ob* and *ob/ob*-treated mice 7 day post transfer, respectively. Graphs show quantitation of each specific protein. One representative out of three independent experiments is shown.

Leptin controls survival of antigen-specific autoreactive $CD4^+$ T cells through the nutrient/energy-sensing AKT-mTOR pathway

Next, we tested whether leptin controls the expression of the protein kinase B (AKT) and its downstream enegy-sensing mTOR pathway (133, 135). We found that FACS-sorted MOG_{35-55}-specific $CD4^+$ T cells from *ob/ob* mice, displayed lower level of phosphorilation of both AKT and S6 ribosomal protein (S6) (**Figure 10A**), two important upstream and dowstream molecules involved in mTOR signalling cascade, respectively. These results on S6 phosphorilation were also confirmed by flow cytometric detection of P-S6 on $CFSE^+$ MOG_{35-55}-specific $CD4^+$ T cells (**Figure 10B**).

As the P-S6 appeared to be impaired by leptin deficiency we performed a series of experiments utilizing rapamycin, an mTOR specific inhibitor. Interestingly, acute/short term rapamycin (RAPA) treatment *in vivo* resembled the effects of leptin deficiency in terms of recovery of MOG_{35-55}-specific $CD4^+$ T in WT-B6 mice. Indeed, seven days after adoptive transfer of WT-B6 $CFSE^+$ MOG_{35-55} pathogenic T cells into WT-B6 mice alternatively treated with PBS, RAPA, rleptin and rleptin+RAPA (**Figure 11**). RAPA treatment significantly reduced the number of WT-B6 $CFSE^+$ MOG_{35-55} T cells and leptin treatment alone or with RAPA prevented reduction of these T cells. These effects were secondary to inhibition of the mTOR pathway and reversed by exogenous rleptin administration during RAPA treatment (**Figure 11**).

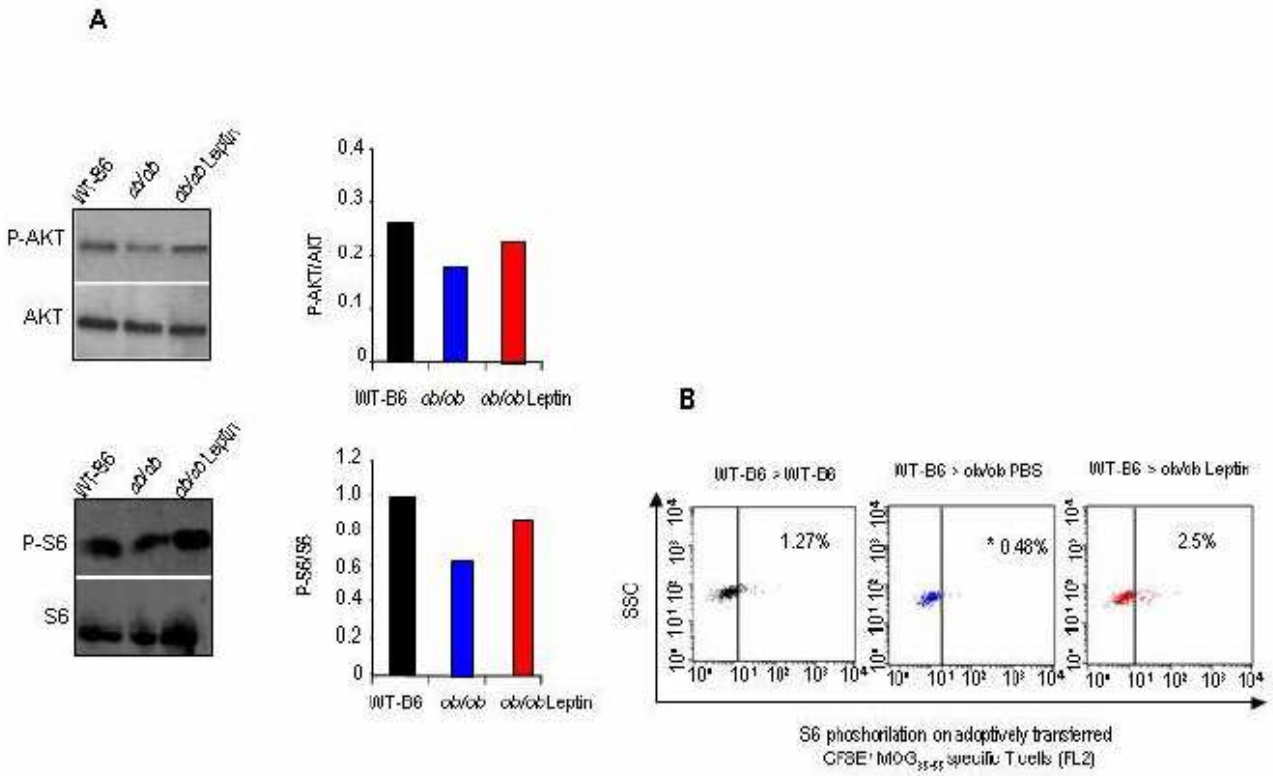

Fig. 10 Leptin affects survival antigen-specific autoreactive CD4$^+$ T cells through the nutrient/energy-sensing AKT-mTOR pathway

A) Immunoblot for pAKT, pS6 on FACS-Sorted MOG_{35-55} CD4$^+$ T cells from WT, *ob/ob* and *ob/ob*-treated mice 7 day post transfer, respectively. Graphs show quantitation of each specific protein. One representative out of five independent experiments is shown.

B)Flow cytometric analysis of S6 phosphorilation on FACS-Sorted CFSE$^+$ MOG_{35-55} CD4$^+$ T cells from WT-B6 transferred into WT-B6 (left), *ob/ob* (middle)and *ob/ob*-treated (right) mice. MOG_{35-55} CD4$^+$ T cells were sorted from the spleen of WT, *ob/ob* and *ob/ob*-treated mice respectively. CFSE$^+$ MOG_{35-55} CD4$^+$ T cells were than fixed and stained using P-S6-Phycoerytrine (PE) specific antibody. One representative out of three independent experiments is shown. (* $p < 0.001$)

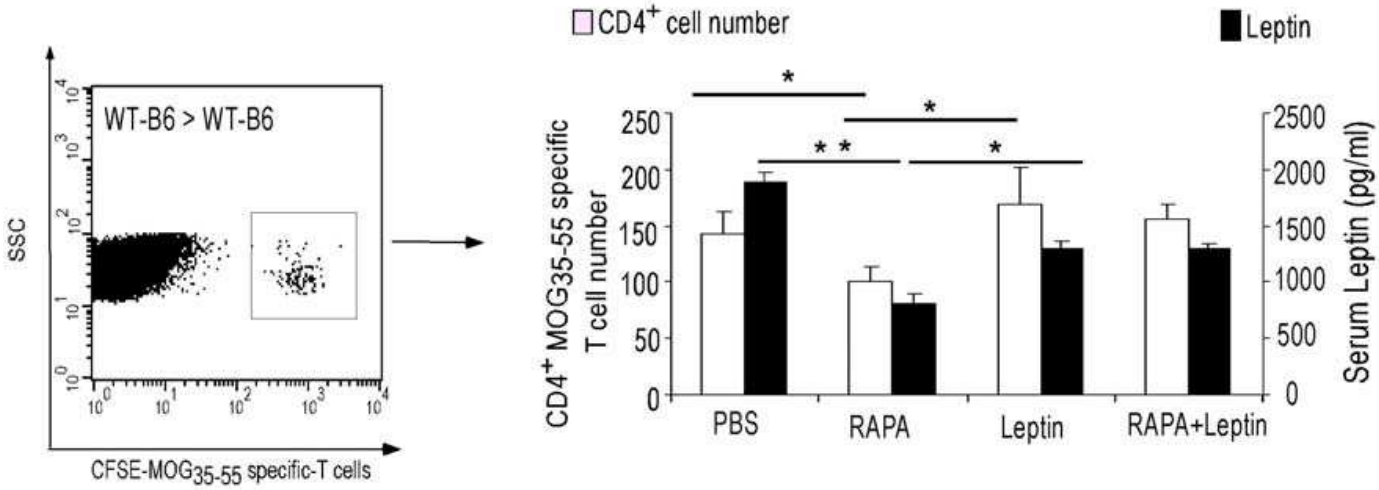

Figure 11. Rapamacyn treatment reduced the pool of MOG_{35-55}-specific $CD4^+$ T in WT-B6 mice upon adoptive transfer

Number of MOG_{35-55} $CD4^+$ T recovered in WT-B6 alternatively treated with PBS, RAPA, rleptin and rleptin+RAPA seven days post adoptive transfer (With Bars). Circulating Leptin levels in WT-B6 mice alternatively treated with PBS, RAPA, rleptin and rleptin+RAPA seven days post transfer (Black Bars). Bars represent mean + 3 SD of three replicates experiments. * $p<0.05$; ** $p<0.001$

Discussion and Conclusion

In this study, we provide data suggesting that leptin is essential for survival of autoreactive, antigen-specific $CD4^+$ T cells. Leptin deficient *ob/ob* mice were almost protected from adoptively-transferred EAE. Protection of *ob/ob* mice mirrored a progressive decline in survival of autoreactive myelin-peptide specific T cells and was associated with a reduced secretion of Th1/Th17 pro-inflammatory cytokines by $CD4^+$ T cells from WT-B6 mice after they "experienced" an environment devoid of leptin in *ob/ob* mice. These effects were secondary to a significant downregulation of the survival protein Bcl-2, and associated with cell cycle arrest as testified by a reduction in the P-ERK1/2 and the upregulation of the cell cycle inhibitor $p27^{kip1}$. In addition, as leptin activates the nutrient energy-sensing mTOR pathway (136), we investigated also the AKT-S6 levels in $CD4^+$ T cells from WT-B6 adoptively transferred into *ob/ob* mice. Interestingly, we observed a reduction in the P-AKT and P-S6 levels in these cells that was reversed by recombinant leptin treatment. As rapamycin-induced downregulation of the S6 levels and consequent reduction of circulating leptin in serum of normal WT-B6 mice, we enumerated the circulating $CD4^+$ antigen-specific T cells following transfer before and after rapamycin treatment and observed a significant reduction of autoreactive myelin-specific T cells. These data suggest that either chronic leptin deficiency in *ob/ob* mice or induced leptin deficiency by rapamycin treatment are able to reduce survival of autoreactive T cells in mice through the impairment of the leptin-mTOR signalling. Our results indicate that nutritional status can affect and influence survival of potentially autoreactive T cells. This is in line with the epidemiological evidence that autoimmunity is more common in more effluent countries and that susceptibility to autoimmune diseases in some circumstances can correlate with body fat mass and body weight at birth (137). Moreover, other studies published by Fontana et al. (138) and our group have shown that nutritional deprivation or caloric restriction are able to profoundly modulate

and reduce magnitude and disease score during EAE (139, 140). The so called “frugal phenotype” in which survival of chronically food restricted mice is higher than freely feeding mice fits into this view, in which the environment can influence the break of immune tolerance through nutritional status and that interventions aimed at modulating cytokines and mediators of metabolism can have profound immunemodulating activities on autoimmune disease curse and progression. Similar results have also been obtained with chronic treatment with rapamycin of mice in which survival was increased significantly. The precise mechanisms for these results are still not fully clarified but it is clear that rapamycin treatment is able to induce pharmacologically a “frugal phenotype” similar to that observed in caloric restricted animals. Indeed, rapamycin, thorugh mTOR inhibition, is able to reduce the absorption of amino acids, glucose and also, to dampen the level of a series of adipocytokines produced by adipocytes including leptin (141). Also in our experimental settings we observed that rapamycin administration was able to reduce circulating leptin levels and survival of autoreactive T cells. Exogenous administration of leptin was able to partially revert the rapamycin-induced reduction of $CD4^+$ T cells.

Leptin represents a nutritional signal in which the environment communicates to the center (the brain and hypothalamus) that sufficient amount of nutrients are present in the environment, and these are stored in form of fat depots. Factors involved in survival of autoreactive T cells are still not well characterized and specific microenvironmental requirements are not completely identified. In this context, one of the most recent advances are represented by the study of IL-21, a long helical cytokine, involved in survival and homeostatic proliferation of T cells and their possible conversion into autoreactive T cells (142). This view in *ob/ob* mice despite it is present a mild-to-severe lymphopenia homeostatic proliferation of autoreactive T cells is not present as IL-21.

In summary, our study demonstrates previously unrecognized role of leptin in survival of autoreactive antigen-specific T cells that mediate autoimmunity. Leptin regulates autoantigen-

specific $CD4^+$ T cell survival directly, through activation of the nutrient energy-sensing mTOR pathway and the survival gene Bcl-2, and indirectly, through a reduced secretion of cytokines important in T cell survival such as IL-6, IL-15, IL-21, GM-CSF. IL-7, another key cytokine in homeaostatic proliferation and survival, was not produced and detectable upon antigen stimulation by T cells that experienced a leptin-free environment, and also its receptor was not found different in terms of surface expression in all different group of mice treated or not with leptin (data not shown). Interestingly, IL-7 circulating serum levels were significantly higher in *ob/ob* mice (data not shown) suggesting that its secretion by other sites was increased to compensate the reduced number of $CD4^+$ T cells observed in these mice. These data are in agreement with studies in which it has been suggested that in conditions of $CD4^+$ T cell lymphopenia, IL-7 circulating concentration increase.

In conclusion, manipulation of the leptin axis can represent a novel tool for control of T cell tolerance and inflammation, and our study sets the molecular basis for a novel immune intervention in autoimmunity.

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