UNIVERSITÀ DEGLI STUDI DI NAPOLI

"FEDERICO II"

Scuola di Dottorato in Medicina Molecolare

Dottorato di Ricerca in Patologia e Fisiopatologia Molecolare

XXIV ciclo

Regulatory T cell proliferative potential as novel marker to investigate immune tolerance and clinical progression in Multiple Sclerosis

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Abstract

In autoimmune disorders such as Multiple Sclerosis (MS) one of the determining alteration is the breakdown of self-antigen immune-tolerance. Peripheral immune tolerance is maintained, at least in part, by Regulatory T cells (Treg). Several studies have shown that either defects in the frequency or the suppressive capacity of Treg cells can contribute to the development of break of self-tolerance, and that in animal models of autoimmunity, adoptive transfer of Treg cells was able to stop disease process (1). Treg cells are known to be anergic *in vitro* to T cell receptor-induced (TCR) stimulation and this state correlates with their *in vitro* suppressive capacity (2). It has been reported that there are differences in the number of Treg cells in MS patients when compared with healthy controls (3). However there is also extensive evidence indicating a defect in the suppressive function of Treg cells from MS patients (4). In previous studies we showed that Treg cells produce an higher amount of leptin when compared with effector T cells and that leptin acts as a negative signal for the proliferation of Treg cells (5). *In vitro* leptin neutralization results in Treg cells proliferation (5). Although in last few years several studies have been performed to understand the molecular mechanism leading to autoimmune disorders development, there are no surrogate markers to predict the clinical progression of autoimmune diseases and the clinical response to the classical therapeutic regimes.

Introduction

Regulatory T cells

Regulatory T (Treg) cells expressing the transcription factor forkhead box P3 (FOXP3) are key players in the maintenance of peripheral self tolerance and immune homeostasis. Their dysfunction (for example, owing to FOXP3 gene mutation) causes fatal autoimmune disease, immunopathology and allergy (1). $FOXP3^+$ Treg cells, most of which are $CD4^+$ T cells that express CD25 (the interleukin-2 (IL-2) receptor α-chain), can suppress the activation, proliferation and effector functions — such as cytokine production — of a wide range of immune cells, including $CD4^+$ and $CD8^+$ T cells, natural killer (NK) and NKT cells, B cells and antigen-presenting cells (APCs) in vitro and in vivo (1). This unique ability to control immune responses makes $FOXP3^+$ Treg cells central in the prevention of autoimmune disease, immunopathology and allergy, as well as in the maintenance of allograft tolerance and fetal–maternal tolerance during pregnancy (6). As a double-edged sword, $FOXP3^+$ Treg cells can also suppress antitumour immune responses and favour tumour progression. Human Treg cells were first characterized as $CD4^+CD25^+$ T cells in 2001 by several groups (7-9) based on the finding in 1995 that mouse Treg cells constitutively express CD25 (10). Similarly, in 2003, Foxp3 was described as a master control gene for mouse Treg cell development and function (11-13), and subsequent studies have confirmed FOXP3 as a specific marker for human Treg cells (14). It has been show that the normal thymus produces T cells with an autoimmune-preventive function. Direct evidence of the thymic origin of Treg cells and their persistence in the periphery was shown by the adoptive transfer of thymocyte or peripheral T cell suspensions depleted of $CD4^+CD25^+$ T cells, which results in the development of various autoimmune diseases in syngeneic T cell-deficient mice (10,15,16) Ontogenically, $CD4^+CD25^+$ T cells become detectable in the periphery ~3 days after birth, indicating that neonatal thymectomy abrogates thymic production of Treg cells (15,17). Furthermore, depletion of peripheral $FOXP3^+$ T cells for a limited time period results in autoimmunity in mice (17,12). Based on these findings, thymus-derived

$CD4^+FOXP3^+$ T cells are thought to be 'natural' Treg cells. Thymic development of Treg cells requires high-affinity interactions between their T cell receptor (TCR) and self-peptide–MHC complexes presented by thymic stromal cells (18).

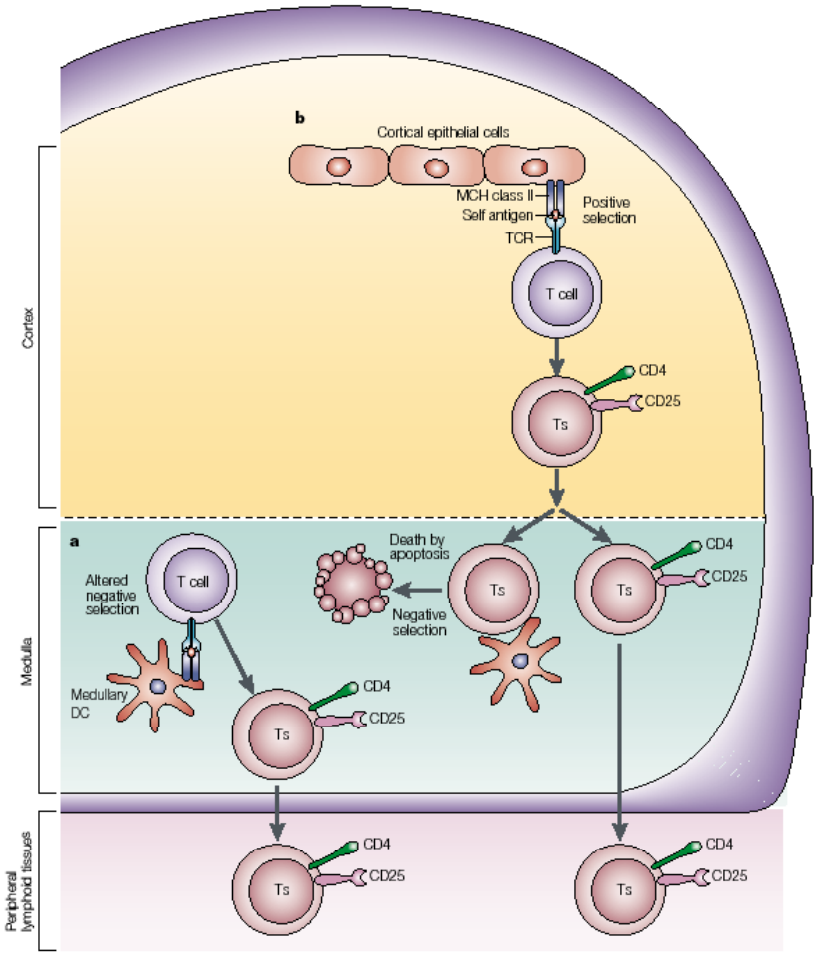


Figure 1. CD4+CD25+ T cells seem to be members of a unique lineage of T cells that are selected during the process of T-cell differentiation in the thymus. It remains unclear where and when this occurs. **A)** One possibility is that CD25+ T cells acquire expression of CD25 and suppressor function in 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.

These cells also provide co-stimulatory signals that are necessary for their development as shown by the decrease in the number of Treg cells generated in the thymus following loss of CD40 or CD28 expression (1). Furthermore, both IL-2 and IL-7 in the thymic microenvironment are required for Treg cell development in mice (19). Given the known similarities between mouse and human thymocyte development (20), it is likely that many of these requirements for Treg cells in mice are similar for human Treg cell development. The key role of FOXP3 gene in the maintenance of self tolerance was first shown in scurfy mice and subsequently in patients with immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome as the causative genetic anomaly that results in severe autoimmune diseases and allergy, which resemble the diseases observed following depletion of $CD4^+CD25^+$ Treg cells in rodents (21-23). Importantly, ectopic expression of FOXP3 in naive mouse $CD4^+$ T cells confers suppressive activity and induces the expression of Treg cell-associated signature molecules such as CD25, cytotoxic T lymphocyte antigen 4 (CTLA4) and glucocorticoid-induced TNF-receptor-related protein (GITR) (11-13). Expression of these receptors also correlates with FOXP3 expression in human $CD4^+$ T cells (24). This induction of suppressive activity in conventional T cells by ectopic FOXP3 expression, together with the development of autoimmune disease in FOXP3 mutant or deficient mice, indicates that FOXP3 is a master regulator for Treg cell differentiation and function (25). Although the transient expression of FOXP3 does not enable suppression, sustained FOXP3 expression by activated T cells can confer regulatory competence (26-28). Furthermore, in human $CD4^+$ Treg cells, stable and high FOXP3 expression is required for suppressive function, and loss of FOXP3 expression over time owing to long-term culture decreases the ability of formerly potent Treg cells to suppress (29,30). Human FOXP3 is expressed by both activated and regulatory T cells in the peripheral blood, as are other known Treg cell biomarkers such as CD25, CTLA4, GITR and CD95 (also known as FAS). This upregulation of FOXP3 in activated T cells could be one component of the homeostatic programme initiated by these cells to exert negative feedback during the course of an immune response. Recently, several groups showed that the lack of cell surface CD127 (also known as IL-7 receptor α-chain) can be a useful alternative to CD25 for the

delineation and purification of human Treg cells: FOXP3 expression and suppressive ability are enriched in $CD4^+$ T cells that express low levels of CD127 (31,32). Treg cells have potent in vitro suppressive activity and show hyporesponsiveness following activation in vitro (7,8) analysis of effector Treg cell turnover in vivo indicates that these cells undergo rapid turnover, which is not indicative of a long-lived memory T cell population (33). The precise molecular mechanisms of suppression by human Treg cells remains to be determined, although in vitro and in vivo mouse studies have implicated several mechanisms. These include modulation of the cytokine microenvironment, metabolic disruption of the target cell, alteration of DC activating capacity and cytolysis (1,34,35). For example, human Treg cells can be suppressive in vitro even in the absence of APCs (7), indicating that target cell suppression can occur through direct contact between Treg and effector T cells. A recent study in mice has shown that CTLA4 is crucial for the suppressive function of $FOXP3^+$ Treg cells both in vitro and in vivo (36). CTLA4 expressed by Treg cells can modulate CD80 and CD86 expression by DCs and thereby inhibit the activation of effector T cells. Dysfunctional Treg cells can be a cause of autoimmune disease, allergy and immunopathology. But it remains to be determined how these cells are involved in the pathophysiology of common immunological diseases such as autoimmune disease (6,37).

Leptin as an immunoendocrine mediator

Leptin is a 16-kDa nonglycosylated protein encoded by the *ob* gene, which is located on human chromosome 7 and on mouse chromosome 6. In humans and mice, mutations of the *ob* gene are associated with hyperphagia and obesity, reduced energy expenditure, and other reproductive, neuroendocrine, and metabolic dysfunctions. Serum leptin is usually higher in obese individuals and has a strong sexual dimorphism—higher in females than males matched by age and body weight (38). Leptin is classically considered a hormone, as it regulates the balance between food intake and energy expenditure, signaling to the brain the changes in stored energy. Serum leptin is correlated directly with

the body-fat stores, increasing with fat accumulation and decreasing during fasting. Leptin gene expression is regulated by several factors, including other hormones such as insulin, glucocorticoids, and sex hormones. Insulin stimulates leptin secretion during feeding, and a decrease in insulin levels anticipates a fall in leptin during starvation. Glucocorticoids also operate synergistically with insulin in the secretion of leptin from cultured adipocytes, although an inverse relationship between leptin and glucocorticoids is generally observed (38). Finally, leptin expression is inhibited by testosterone, increased by ovarian sex steroids, and directly influences the hypothalamic-pituitary-adrenal axis, the reproductive system, hematopoiesis, and angiogenesis. A series of studies has linked the immune and neuroendocrine systems. Leptin is one of the mediators that is common to the neuroendocrine and immune systems (39). 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 (39-41). 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, and HIV infection and during anti-inflammatory therapy (42-44). 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 estrogens 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 (38). The fact that leptin has effects on neuroendocrine and immune systems should not come as a surprise, given the functional connection and anatomical contiguity between adipocytes and lymphoid cells (45). Morphologically, aggregations of lymphoid tissue, including the lymph nodes, thymus, and bone marrow, are associated with adipose tissue (45). Fat deposits do not simply have a structural, metabolic, and heat-insulating function but also provide a microenvironment that helps the immune system to sustain immune responses. In particular, lymphoid and

adipose tissues 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 proinflammatory cytokines).

Leptin in innate and adaptive immunity

Humans with congenital leptin deficiency have a much higher incidence of infection-related death during childhood (46), whereas recombinant human leptin administration in children with congenital leptin deficiency normalized absolute numbers of naive CD4CD45RA T cells and nearly restored the proliferation response and the cytokine release profile from their lymphocytes (47). Studies in mice have shown that the effect of leptin on the immune system is direct and indirect, i.e., via modulation of central or peripheral pathways. The effects of leptin on adaptive immune responses have been investigated extensively on human $CD4^+$ T cells. Addition of physiological concentrations of leptin to a MLR induces a dose-dependent increase in $CD4^+$ T cell proliferation (48). 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 toward Th1 cell responses). 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 (48). 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 (48-50). 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 (49). Despite the evidence of direct effects of leptin on immune responses in vitro, a major problem remains whether leptin can

influence immune responses in vivo. This task is particularly difficult, because of the complexity of the network of interactions that link leptin to several endocrine pathways. It is notable that T cells are sensitive to the supply of cellular nutrients, such as glucose (51), as they do not have glycogen stores and therefore, depend on the import of extracellular glucose to meet their metabolic needs (52). By stimulating glucose uptake through ERK1/ERK2- and PI-3K-dependent pathways, leptin might help to restore the impaired T cell function caused by starvation (49).

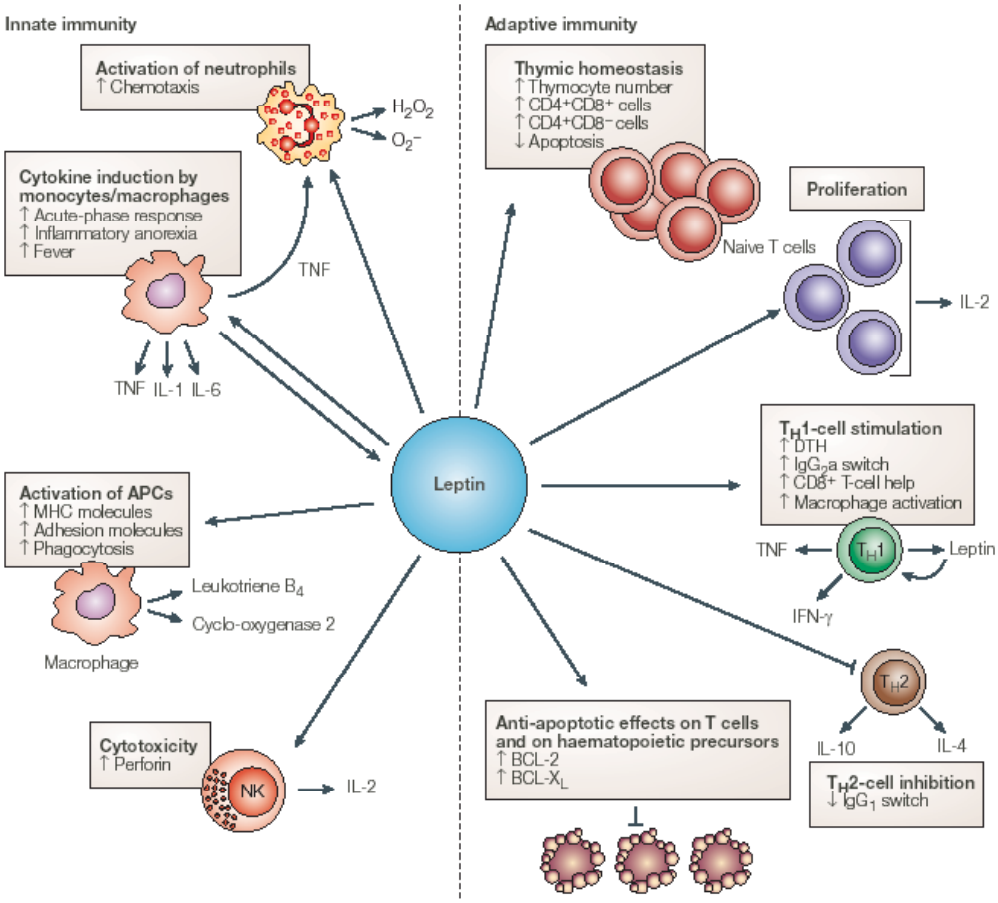


Figure 2: Schematic representation of the effects of leptin on both innate and adaptive immunity.

In this context, it is worth mentioning that other long-chain helical cytokines similar to leptin (such as IL-3, IL-7, and IL-15) are important in promoting the uptake and metabolism of glucose (52). In innate immunity, leptin seems to promote activation of and phagocytosis by monocytes/macrophages and their secretion of leukotriene B4 (LTB4), cyclooxygenase 2 (COX2), NO, and proinflammatory cytokines (53). The products of the inducible form of COX2—PGs and LTs (also known as eicosanoids)—as well as NO are involved in the regulation of inflammation, chemotaxis, and cytokine production and therefore, markedly impact the immune response. Moreover, leptin can induce chemotaxis of neutrophils and the release of oxygen radicals (such as superoxide anion and hydrogen peroxide) (53). 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 (54,55). Leptin also affects NK cell development and activation in vitro and in vivo (56-59). 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 STAT3 and the transcription of genes encoding IL-2 and perforin. Last but not least, it has recently been shown that leptin can stimulate the production of the growth hormone by PBMCs through protein kinase C- and NO-dependent pathways (59). This effect of leptin on the production of the growth hormone might be important in immune homeostasis, given the fact that this cytokine-like hormone has marked influences on immune responses by controlling the survival and proliferation of immune cells.

Multiple Sclerosis

Multiple sclerosis (MS) is one of the most common chronic and disabling disorders of the central nervous system (CNS), affecting 0.05–0.15% of Caucasians (60). The disease usually begins in young adulthood and affects women more frequently than men (2:1). In 80–90% of cases, MS starts with a relapsing–remitting course (RR-MS).

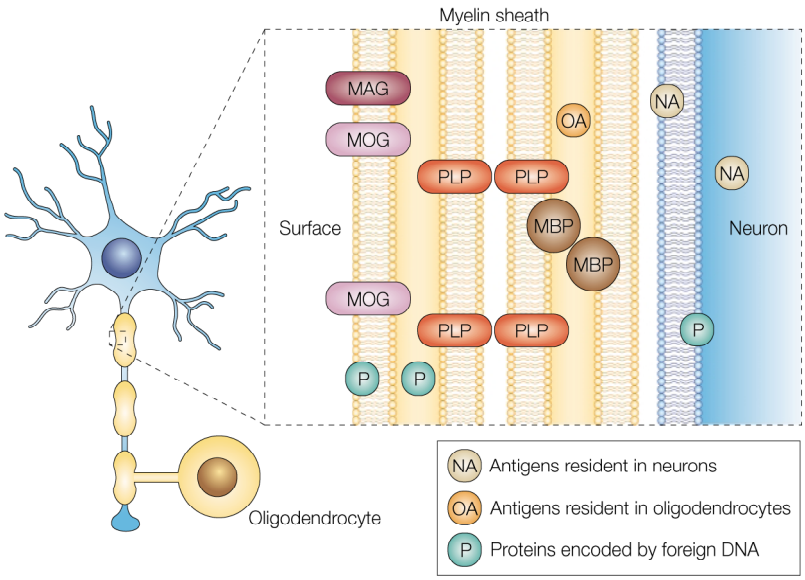


Figure 3. Possible target antigens in the white matter. Proteins of the myelin sheath, oligodendrocytes and neurons are possible targets of the immune response in multiple sclerosis. Among the candidates are myelin and neuronal antigens, and also proteins that are introduced into those cells by infectious agents. MAG, myelin-associated glycoprotein; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein.

Over time, the number of relapses decreases, but most patients develop progressive neurological deficits that occur independently of relapses (the so-called secondary progressive phase). In 10–20% of patients, MS begins with a primary progressive course (PP-MS) without acute relapses. In general, the progression rate in RR-MS is comparable to that of PP-MS as soon as the patients enter the secondary progressive phase (61).

Imaging studies have revealed differences between RR-MS and PP-MS. In patients that suffer from RR-MS, acute CNS lesions with spontaneous

resolution are frequently detected, even in the absence of clinical attacks (62). These lesions are usually located in areas of white matter, and are often characterized by a disturbance of the blood–brain barrier, local oedema and demyelination — features that are compatible with an inflammatory process. By contrast, when progressing to the secondary phase and in patients with PP-MS, such inflammatory activity is much less conspicuous (62). Global brain atrophy, however, is more dominant in the progressive stage and seems to correlate with disability (63,64). These findings indicate that early in the disease, ongoing inflammatory activity is present in most patients and is responsible for the relapsing–remitting course, whereas a distinct process might be operative in the progressive phase of the disease, when inflammatory activity diminishes despite faster evolution of disability.

Patients disability can be measured using disability scales. In MS the most frequently used is Kurtzke's Expanded Disability Status Scale (EDSS) (65). This scale was developed from the Disability Status Scale and is observer-(usually neurologist-) rated. EDSS steps 1.0 to 4.5 refer to people with MS who are fully ambulatory. EDSS steps 5.0 to 9.5 are defined by the impairment to ambulation. EDSS step 10 refers to death due to MS.

The prevalence of MS varies significantly depending on the genetic background of the patient (66). MS is highly prevalent in Caucasians, but only rarely observed in Asians or Africans. Moreover, the risk of developing the disease is significantly higher in family members of patients with MS (67). By contrast, the prevalence in spouses and adopted family members does not differ from that of the general population. These findings argue for a strong genetic predisposition to MS, and have prompted a large number of linkage and association studies to identify disease loci and alleles. The results of genomic screens in MS indicate that a considerable number of different genes, each having a relatively small contribution, are involved in the susceptibility to MS (68,69). So far, only the human leukocyte antigen (HLA) class II alleles DR15/DQw6 (HLA-DRB1*1501; HLA-DQB1*0601), which code for molecules that participate in antigen recognition by T lymphocytes, have been consistently associated with MS in Caucasians (70). 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 (71).

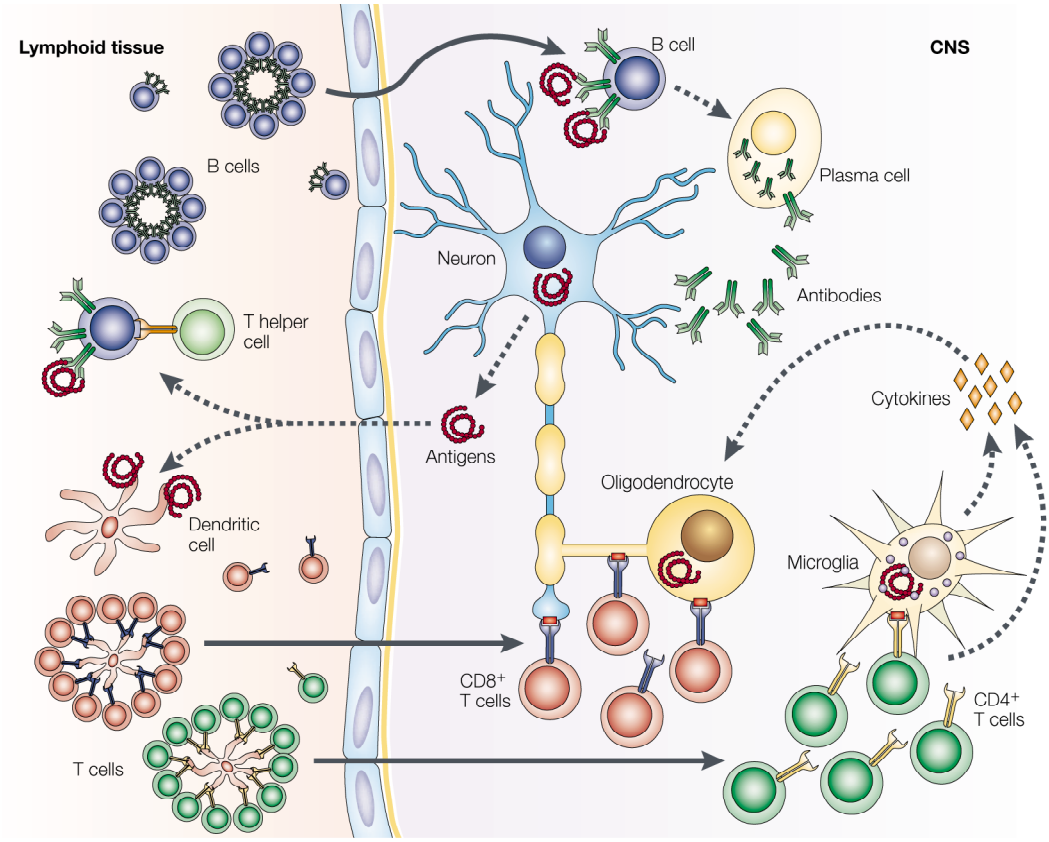


Figure 4. Immune responses in multiple sclerosis. Hypothetical view of immune responses in acute multiple sclerosis lesions. Independent of the causative event, two steps are required to induce an immune response in the central nervous system (CNS): a pro-inflammatory milieu in the CNS, leading to upregulation of major histocompatibility complex (MHC) molecules, co-stimulatory receptors and inflammatory cytokines and an antigen-driven acquired immune response. T- and B-cell responses are primed in the peripheral lymphoid tissue by antigens that are released from the CNS or by cross-reactive foreign antigens. Dendritic cells that present neural antigens are strong stimulators of T-cell responses. After clonal expansion, T and B cells infiltrate the CNS. Clonally expanded B cells re-encounter their specific antigen, mature to plasma cells and release large amounts of immunoglobulin-γ (IgG) antibodies. These antibodies bind soluble or membrane-bound antigen on expressing cells. Clonally expanded $CD8^+$ T cells also invade the brain and could encounter their specific peptide ligand, presented by glial or neuronal cells on MHC class I molecules. The recognition of specific MHC–peptide

complexes on these cells prompts direct damage to expressing cells. $CD4^+$ T cells migrate into the CNS and encounter antigens that are presented by microglial cells on MHC class II molecules. Reactivation of these cells leads to heightened production of inflammatory cytokines. These cytokines attract other immune cells, such as macrophages, which contribute to inflammation through the release of injurious immune mediators and direct phagocytic attack on the myelin sheath.

The leptin connection: regulatory T cells and multiple sclerosis

It has been shown that leptin has an important role in controlling the anergy and hyporesponsiveness of regulatory T cells. A clue that leptin might be involved in the homeostasis of regulatory T cells came from a study showing that leptin is higher in serum of multiple sclerosis patients and correlated with reduced numbers of regulatory T cells (72). Indeed it has been reported that the secretion of leptin is increased in both serum and cerebrospinal fluid (CSF) of naive-to-treatment patients with MS, an aspect that positively correlates with the secretion of IFN-γ in the CSF and inversely correlates with the percentage of circulating Treg cells. Of note, the number of peripheral Tregs in patients with MS inversely correlates with the serum levels of leptin, suggesting a link between the number of Treg cells and leptin secretion. This observation was supported by another paper showing that reduction of Treg cells are likely a direct consequence of leptin binding to receptors on the regulatory T cells (73). Regulatory T cells are normally anergic and hyporesponsive to T cell receptor (TCR) signalling, but De Rosa et al. showed that specific neutralization of leptin combined with TCR signaling reversed anergy and hyporesponsiveness of this cellular subset. Proliferation was dependent on IL-2 but appeared to operate through a distinct mechanism. Interestingly, an important source of leptin was the regulatory T cells themselves, which both secreted leptin and expressed leptin receptors. Thus, leptin can mediate a negative autocrine loop in regulatory T cells even in the absence of exogenous leptin. The reversal of anergy by leptin neutralization was associated with phosphorylation of ERK1/2 kinase and rapid degradation of the cell-cycle inhibitor p27kip1 (73). Importantly, regulatory T cells that were expanded in the presence of leptin antibody retained their immunosuppressive phenotype for up to 8 days in culture. In vivo results supported a role for leptin-receptor signaling in

impaired regulatory T cell proliferation. Mice with genetic deficiency of leptin (*ob/ob*) had higher percentages and absolute numbers of circulating regulatory T cells (74). Furthermore, wild-type regulatory T cells adoptively transferred into leptin-deficient mice expanded substantially more than those transferred into wild-type mice. Thus, regulatory T cells proliferated better in a leptin-poor environment. Consistent with that conclusion, treatment of wild-type mice with leptin-neutralizing antibody produced an expansion of regulatory T cells. In contrast, administration of leptin reduced the numbers of regulatory T cells in leptin-deficient mice to amounts equivalent to those in wild type mice (72). Given the proven role of regulatory T cells in protection from autoimmune diseases, it appears that at least part of the resistance to autoimmune disease in leptin-deficient and leptin-receptor-deficient mice may be due to increased numbers of regulatory T cells. De Rosa et al. also showed that although leptin had a negative impact on regulatory T cell proliferation, it had the opposite effect on conventional CD4+ T cells. In contrast to results from regulatory T cells, in vitro expansion of conventional CD4+CD25$^-$ T cells by stimulation with CD3 and CD28 antibodies was markedly less effective on cells lacking the leptin receptor than on wild-type cells. Furthermore, neutralizing leptin reduced the proliferation of conventional CD4+CD25$^-$ T cells in vitro. These effects were not peculiar to anti-CD3 and anti-CD28 stimulation because similar findings were made when stimulation was done with a pancreatic autoantigen in the nonobesediabetic-mouse model coupled with a leptin-receptor mutation. Thus, the increased resistance to autoimmune disease and susceptibility to infection in leptin-deficient (*ob/ob*) and leptin-receptor deficient (*db/db*) mice is likely a combined effect from enhancement of the immune system regulatory arm with a concomitant impairment of the effector arm. Moreover it has been shown that leptin is involved in the induction and in the progression of EAE the animal model of MS (74,75). Leptin-deficient mice are resistant to induction of active and adoptively transferred EAE. This protection is reversed by leptin administration and associates with a switch from Th2- to Th1-type responses and a IgG1-to-IgG2a isotype switch. Similarly, in susceptible wild-type C57BL/6J mice, leptin worsens disease by increasing IFN-γ release and IgG2a production (75). Importantly, a surge of serum leptin anticipates the onset of clinical manifestations of EAE. The peak

of serum leptin correlates with inflammatory anorexia, weight loss, and the development of pathogenic T cell responses against myelin. Lymphomononuclear infiltrates in the CNS of EAE mice indicate in situ production of leptin in active, inflammatory lesions, thus representing a significant local source of leptin (75). Systemic and/or in situ leptin secretion were instead lacking in EAE-resistant mice. Taken together, these data suggest an involvement of leptin in CNS inflammation in the EAE model of MS probably correlated to the modulation of Treg cells function.

Aim of the study

Multiple sclerosis is an autoimmune disease characterized by chronic inflammation of the central nervous system. The pathology of MS is largely attributed to autoreactive effector T cells that penetrate the blood-brain barrier and damage CNS myelin. Active suppression by regulatory T (Treg) cells plays a key role in the control of self-antigen-reactive T cells and the induction of peripheral tolerance in vivo. In particular, the importance of antigen-specific Treg cells in conferring genetic resistance to organ-specific autoimmunity and in limiting autoimmune tissue damage has been documented in many disease models including MS.

In this study, we aimed at evaluating the proliferative potential of Treg cells in MS patients, to determine whether during MS there could be an alteration in regulatory T cells homeostatic processes. Since it has been shown that proliferation of Treg cells is inhibited by the adipocytokine leptin and *in vitro* leptin neutralization results in Treg cells proliferation, we investigated the capacity of Treg cells, purified from RR-MS patients, to expand upon leptin-neutralization and we compared the proliferation rate of these cells with that observed in healthy controls.

Despite intensive studies to understand the alterations occurring during autoimmune diseases, so far there are no surrogate markers of immune tolerance either capable of "measuring" self-antigen tolerance in MS or to anticipate clinical progression of the disease. In this report, we evaluated the relationship between the proliferative potential of Treg cells and clinical progression in MS to determine whether this marker could be related with the state of immune tolerance in MS patients.

More in detail, we analyzed the relationship between the *in vitro* proliferative capacity of Treg cells and the EDSS score of MS patients to understand whether the major or minor proliferation of Treg cells could be related with a better or worse progression of disease curse. This assay was named "TregsAssay", and our findings suggest the use of the *in vitro* Treg cells proliferation index as marker for evaluation of immunological tolerance in MS and autoimmunity.

Material and methods

Subjects

All MS patients and controls were recruited at the Università di Napoli "Federico II." For Treg proliferation assay we included in the study 71 patients with MS defined according to the criteria of McDonald *et al* (76) and 40 healthy controls. We additional included for hMBP-specific short-term T cells lines 52 patients and 30 healthy controls. All MS patients had RR-MS and an expanded disability status score of ≤ 7.0. All blood samples from patients and controls were collected at 9.00 am in heparinised vacutainers (BD Biosciences, Milan, Italy) and processed within the following 3 hours.

Regulatory T cell Purification, Cultures, and Proliferation Assays

Human $CD4^+CD25^{hi}$ $CD127^-$ Regulatory T cells were isolated from MS patients and from healthy controls. Human $CD4^+CD25^{hi}CD127^-$ and $CD4^+CD25^-$ T cells were purified from PBMCs from buffy coats of human healthy donors or from peripheral blood of MS patients by high-performance cell sorting (MoFlo, Dako) after staining with FITC anti-human CD4 (BD PharMingen, clone RPA-T4), PE anti-human CD25 (BD PharMingen, clone M-A251), APC anti-human CD127 (R&D Systems, clone 40131), or magnetic cell separation with Dynabeads Regulatory T Cell Kit (Invitrogen). Soon after isolation, $CD4^+CD25^{hi}CD127^-$ cells were rapidly cleaned with Detach reagent (Invitrogen) to remove surface-bound CD25 mAb and beads. Antibody/bead-free cells were 95%–98% pure by FACS analysis, and >95% expressed FoxP3. Cells were cultured (1×10^4 cells/well) in round-bottom 96-well plates (Becton-Dikinson Falcon,) with RPMI medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies Inc) and either 5% patients autologus serum or 5% AB human serum (Sigma-Aldrich). Cells were stimulated for 3 days in the presence of anti-CD3/CD28 Dynabeads (0.1 bead/cell) (Invitrogen). For in vitro blocking experiments, human leptin-neutralizing mAb (R&D system) was used at a final concentration of 20 mg/ml. On the last day, [^{3}H]thymidine (0.5 μCi/well) (Amersham-Pharmacia Biotech) was added to the cultures and cells harvested after 12 hr. Radioactivity was measured with a β-cell-plate scintillation counter (Wallac).

IL-2 Measurement

IL-2 measurement was done with a human IL-2-specific ELISA kit purchased from BD-bioscience and measurements was performed according to the manufacturer's instructions. All serum samples from MS patients and controls were collected and stored at −80 °C before the analysis. Supernatants were collected from each experimental point 24 hours after starting stimulation.

Leptin measurement

A human leptin ELISA kit (R&D Systems, Minneapolis) were used according to the manufacturer's instructions.

Ki67 expression

Freshly isolated PBMCs by Ficoll Hypaque gradient centrifugation from MS patients and healthy controls were immunostained with the anti-CD4-Pe-Cy5 and anti-CD25-APC monoclonal antibodies from BD Biosciences. Thereafter cells were washed, fixed, permeabilized (fixation-permeabilization buffer, eBioscience) and stained with anti-Foxp3-PE (eBioscience) and anti-Ki67-FITC (BD Biosciences) monoclonal antibodies. Cells were analyzed employing the Diva software and a FACSCanto (Becton-Dickinson).

Human Myelin Basic Protein (hMBP) short-term T cell lines.

hMBP-specific short-term T-cell lines were generated from PBMC from MS patients and healthy controls. PBMC were separated by Ficoll Hypaque gradient centrifugation and were cultured (1 x 10^6 cells/well) for 7 days in 48-well plates with RPMI medium supplemented with 100 U/ml pennicillin, 100 mg/ml streptomycin and either 5% autologous serum for patients or 5% AB human serum for controls. Cells were stimulated for 7 days in presence of hMBP (25 mg/ml) alone or in presence of hMBP plus human leptin-neutralizing antibody (20 mg/ml) or in the presence of 50 U/ml of human recombinant interleukin-2 (r-IL-2). On the last day FACS analysis was performed. FITC-anti-human-Ki67 and PeCy5-anti-human-CD4 were from BD Biosciences; the anti-human Foxp3 staining set was from eBiosciences; APC-anti-human-CD25 was from Miltenyi.

Statistical analysis

Comparisons between MS patients and healthy controls were evaluated using a non-parametric Mann-Whitney test. Comparisons between patients were evaluated using a matched paired t test. These statistical analyses were performed using the program GraphPad InStat3 (Abacus Concepts, Cary, NC). The difference in the Treg cells fold increase in proliferation values among patients classified according to their clinical status (EDSS) has been evaluated using the non parametric Kruskal-Wallis procedure followed by the Mann-Whitney U test with Bonferroni correction. In order to evaluate the relationship between the dependent variable (EDSS score) and the fold increase in proliferation of Treg cells upon leptin neutralization, a quantile regression model was fitted. To investigate the biological correlations between the different variables analyzed in this study, the Spearman correlation coefficient was computed. Due to the large number of correlations involved and in order to control the familywise error rate at level alpha, the adaptive Bonferroni procedure described in Guo W. (77) was used. $P < 0.05$ was considered statistically significant.

Results

Treg cells from MS patients show a reduced *in vitro* hyporesponsiveness.

$CD4^+CD25^{high}CD127^-$ regulatory T cell (Treg) were purified from human healthy donors and from MS patients. Firstly we evaluated the *in vitro* anergic state of Treg cells purified from healthy controls and MS patients upon anti-CD3 and anti-CD28 stimulation. As expected, Treg cells purified from healthy donors were completely hyporesponsive to in vitro TCR mediated stimulation. On the contrary, Treg cells from MS patients showed a reduced *in vitro* hyporesponsiveness resulting in a partial cell proliferation (Fig 5A). This phenomenon associated with low albeit significant IL-2 secretion when compared with controls (Fig 5B). Interestingly no significant differences in the proliferation and IL-2 production of effectors T cells ($CD4^+CD25^-$) were detected between controls group and MS patients (Fig 8A and B).

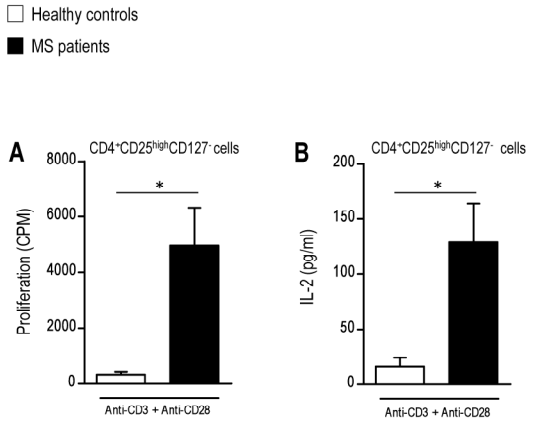


Figure 5. Treg cells from MS patients show a reduced *in vitro* hyporesponsiveness. (**A**) Proliferation of $CD4^+CD25^{high}CD127^-$ T cells from healthy controls and MS patients stimulated with anti-CD3 and anti-CD28. (*p = 0.0012). (**B**) IL-2 secretion by $CD4^+CD25^{high}CD127^-$ Treg cells from healthy controls and MS patients stimulated with anti-CD3 and anti-CD28. (*p = 0.003). The data are shown as mean ± SEM.

Treg cells from MS patients show a reduced anti-leptin-induced proliferation and IL-2 secretion.

To address the effect of leptin neutralization on Treg cells purified from MS patients, we compared proliferation of Treg cells purified from MS patients with proliferative potential of Treg cells from healthy controls. Leptin neutralization resulted in proliferation of Treg cells purified from both healthy subjects and patients (Fig 7A). However, Treg cells from MS patients showed a significantly lower proliferative capacity upon leptin neutralization. Indeed Treg cells from MS patients showed a lower fold increase in proliferation upon leptin neutralization when compared with healthy controls (Fig 7B). Since leptin-mAb-induced Treg cells proliferation is IL-2 dependent (75), to test whether the reduced expanding capacity of Treg cells from MS patients could be ascribed to a decrease in IL-2 production, we measured Treg cells IL-2 secretion upon leptin neutralization and found that also IL-2 secretion was impaired in MS patients (Fig 7C).

Finally, in agreement with previous findings (74) we found that leptin production was significantly increased in serum of MS patients (Figure 6A). Moreover we confirmed that Treg cells from healthy controls showed higher leptin production than did $CD4^{+}CD25^{-}$ effector T cells during anti-CD3 and anti-CD28 stimulation (Figure 6B) and we showed that the amount of leptin secreted by Treg cells from MS patients was similar to that observed in healthy controls (Figure 6B). Addition of neutralizing leptin monoclonal antibody (mAb) to Treg cells cultures stimulated with anti-CD3 and anti-CD28, reverses the hyporesponsiveness and promotes Treg cells proliferation in a dose-dependent fashion (75).

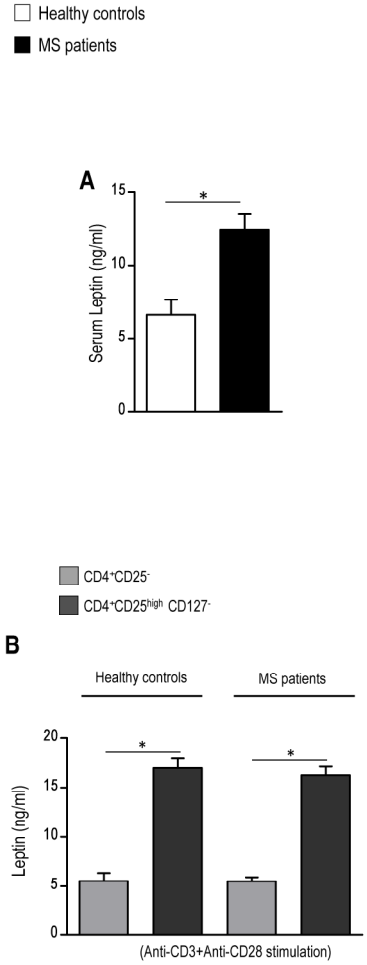


Figure 6. Serum leptin level and Treg cells leptin production in healthy controls and MS patients. (A) Serum Leptin level in healthy controls and MS patients. The data are shown as mean±SEM (*p=0.0006). **(B)** Regulatory T cells secrete an higher amount of leptin with respect to effector T cells in both controls and MS patients. Leptin secretion by Treg cells and effector T cells, stimulated with anti-CD3 and anti-CD28, from Healthy controls and MS patients. The data are shown as mean ± SEM (*p < 0.0001).

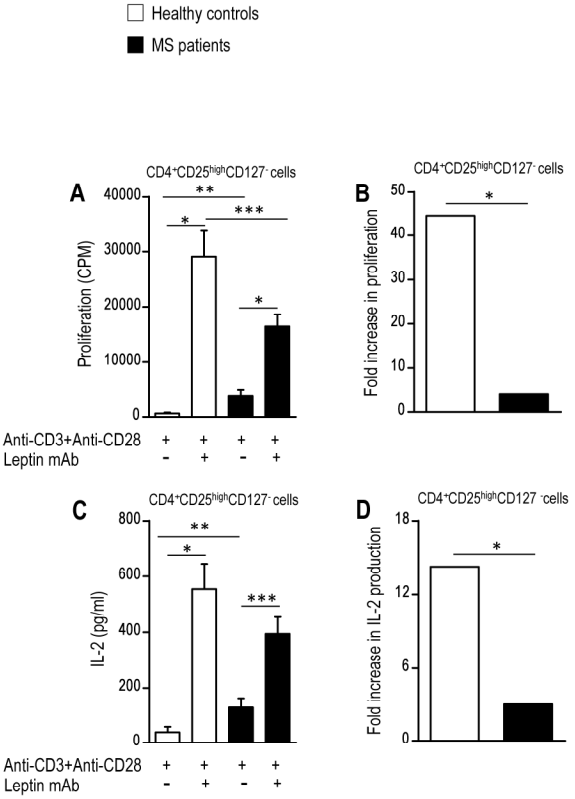


Figure 7. Regulatory T cells from MS patients exhibit reduced proliferation and IL-2 production upon leptin neutralization. (**A**) Proliferation of $CD4^{+}CD25^{high}CD127^{-}$ Treg cells from healthy controls and MS patients stimulated with anti-CD3 and anti-CD28 in the presence or absence of leptin mAb. The data are shown as mean ± SEM (*$p < 0.0001$, **$p = 0.0012$, ***$p = 0.021$). (**B**) Fold increase in proliferation of $CD4^{+}CD25^{high}CD127^{-}$ Treg cells from healthy controls and MS patients upon leptin neutralization. The graph shows the mean of proliferation of Treg cells upon leptin neutralization with respect to the mean of proliferation of Treg cells without leptin neutralization. (**C**) IL-2 secretion by Treg cells from healthy controls and MS patients stimulated with anti-CD3 and anti-CD28 in the presence or absence of leptin mAb. The data are shown as mean ± SEM (*$p = 0.0008$, **$p = 0.003$, ***$p < 0.0001$). (**D**) Fold increase in IL-2 secretion by Treg cells from healthy controls and MS patients upon leptin neutralization. The graph shows the mean of IL-2 secretion by Treg cells upon leptin neutralization with respect to the mean of IL-2 secretion by Treg cells without leptin neutralization.

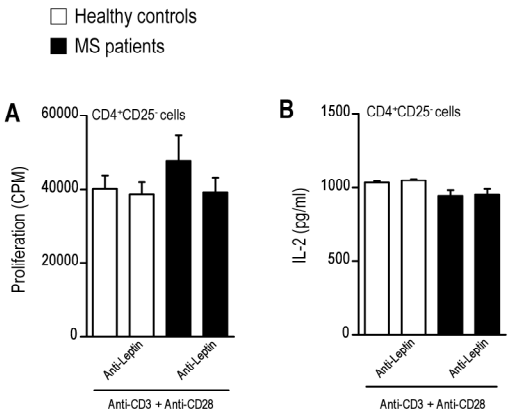


Figure 8. Effector T cells proliferation and IL-2 production. (**A**) Proliferation of $CD4^+CD25^-$ effector T cells from healthy controls and MS patients stimulated with anti-CD3 and anti-CD28 in the presence or absence of leptin mAb. (**B**) IL-2 secretion by $CD4^+CD25^-$ effector T cells from healthy controls and MS patients stimulated with anti-CD3 and anti-CD28 in the presence or absence of leptin mAb. The data are shown as mean ± SEM.

Loss of linear correlation between anti-leptin induced Treg cells proliferation and IL-2 secretion in MS patients.

We next compared Treg cells anti-leptin-induced proliferation with anti-leptin-induced IL-2 production in both controls and patients. The fold increase in proliferation and the fold increase in IL-2 production of Treg cell, upon leptin neutralization, were evaluated. In healthy controls there was a linear correlation between Treg cells anti-leptin induced proliferation and anti-leptin induced IL-2 production (Fig 9A, right panel), indicating that IL-2 production supports their own anti-leptin-induced proliferation. In MS patients there was a loss of this correlation (Fig 9A, left panel) and thus Treg cells fold increase in proliferation upon leptin neutralization was not correlated with a concomitant increase in IL-2 production, suggesting a disruption of this axis in MS We next evaluated IL-2 serum level in both controls and MS patients. MS patients showed a lower IL-2 concentration when compared to healthy controls (Fig 9B).

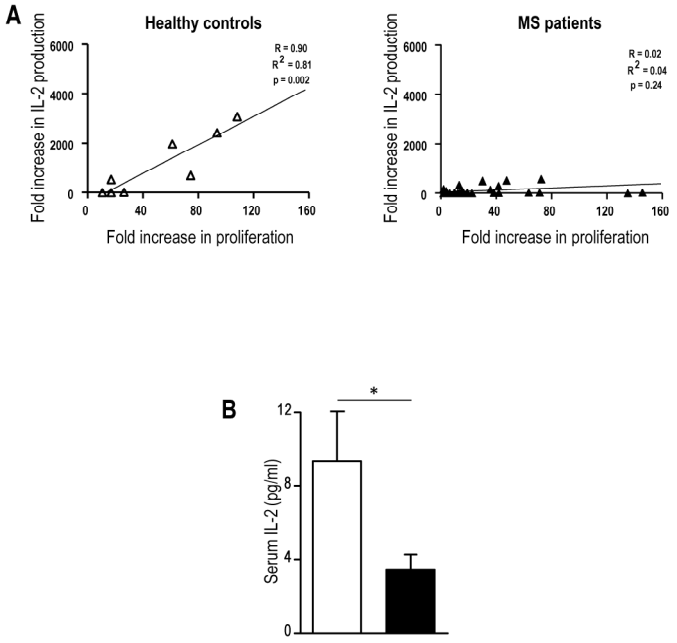


Figure 9. Anti-leptin induced proliferation of Treg cells from MS patients is not correlated with anti-leptin induced IL-2 production (**A**) The graphs show the correlation between the fold increase in proliferation of Treg cells and the fold increase in IL-2 secretion by Treg cells upon leptin neutralization in healthy controls (left panel) and MS patients (right panel). (**B**) Serum IL-2 levels in healthy controls and MS patients. The data are shown as mean ± SEM (*p = 0.02).

Inverse correlation between Treg cells Expansion Index and MS clinical severity.

We next evaluated the relation between the proliferative capacity of Treg cells isolated from MS patients upon leptin neutralization and the clinical status of MS patients. Disease severity of each patient was assessed clinically using the Expanded Disability Status Scale (EDSS), a method of quantifying disability in multiple sclerosis (67). We divided all patients in three groups on the basis of their EDSS value. We observed that MS patients with a lower EDSS displayed an higher proliferative capacity of Treg upon leptin neutralization when compared with patients whose clinical score was much more severe (Fig. 10, left panel) indicating that the progression of pathology compromises the proliferative potential of Treg cells or that impaired Treg cells expansion could be associated with a worse clinical progression of MS. In order to evaluate the relationship between the EDSS score (dependent variable) and the fold

increase in proliferation of Treg cells upon leptin neutralization, a quantile regression model was fitted. The estimated quantile regression coefficients for the different quantiles of the EDSS distribution, along with the 95% confidence bands, are shown in Figure 10 (Right panel). The results confirm that Treg cells expansion upon leptin neutralization (fold increase) is negatively associated with EDSS score. This analysis also reveals that this association is not constant across the different quantiles of the EDSS. In particular the association is not significant until the 0.4 quantile (which corresponds in our data to an EDSS score of 1) and become stronger in the right tail of the EDSS distribution. For instance, in subjects with an EDSS score corresponding to the 0.5 quantile (median), a 10% decrease of the Treg cells fold increase determines a 0.1 point increase in the corresponding quantile (i.e. the median value of the EDSS distribution raise by 0.1 point). An analogous 10% decrease of the Treg fold increase in proliferation will instead cause an increase of 0.3 point in the 80th percentile of the EDSS distribution.

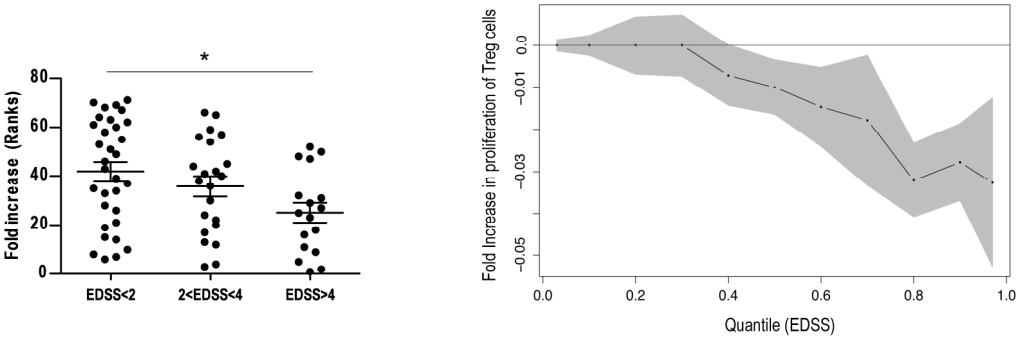


Figure 10. MS patients regulatory T cells expansion inversely correlates with patients clinical status. (Left panel) The graph shows the fold increase in proliferation (expressed in ranks) of $CD4^{+}CD25^{high}CD127^{-}$ T cells purified from MS patients upon leptin neutralization, in patients with an EDSS<1, 2≤EDSS≤4 and EDSS>4. **(Right panel)** Estimated parameters (dots) with 95% confidence bands (gray area) in the quantile regression of EDSS score by Treg cells fold increase in proliferation upon leptin neutralization. The vertical axis shows the values of the estimated parameter (fold increase in proliferation of Treg cells) in correspondence of the different quantiles of EDSS that are reported on the horizontal axis. Points are connected by dashes to visualize trends by outcome percentile.

Treg cells from MS patients showed an impaired *in vivo* and *in vitro* antigen-specific proliferation.

Human Treg cells are anergic to *in vitro* anti-CD3 plus anti-CD28 stimulation but are highly proliferative *in vivo* (33). The proliferating index *in vivo* of the Treg cells was determined by assessing Ki67 expression. Treg cell from MS patients showed a reduced *in vivo* proliferation, when compared with healthy controls, confirmed by the lower expression of Ki67 (Fig. 11A and B). No differences in proliferation rate of effector T cells were observed between controls and MS patients (Fig. 12A and B).

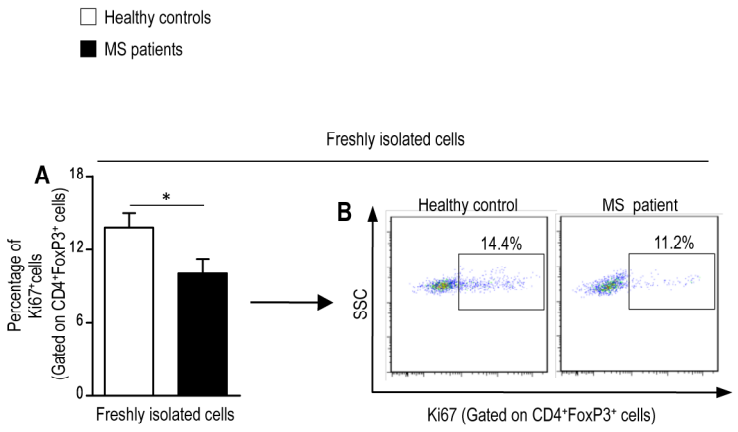


Figure 11. Regulatory T cells from MS patients display a reduced proliferation *in vivo*. (**A**) Ki67 expression in freshly isolated $CD4^{+}Foxp3^{+}$ cells from healthy controls and MS patients. The data are shown as mean ± SEM (*p = 0.04). (**B**) Representative flow cytometry plots of Ki67 expression in freshly isolated $CD4^{+}Foxp3^{+}$ cells from healthy control and MS patient.

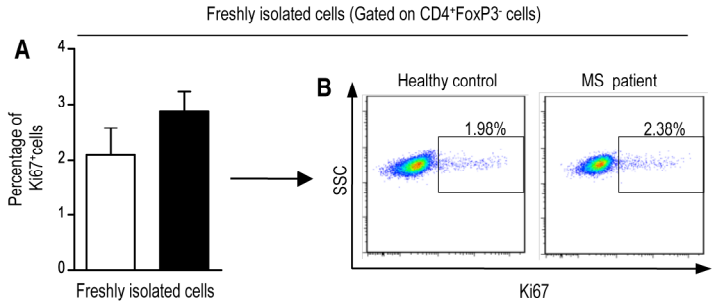
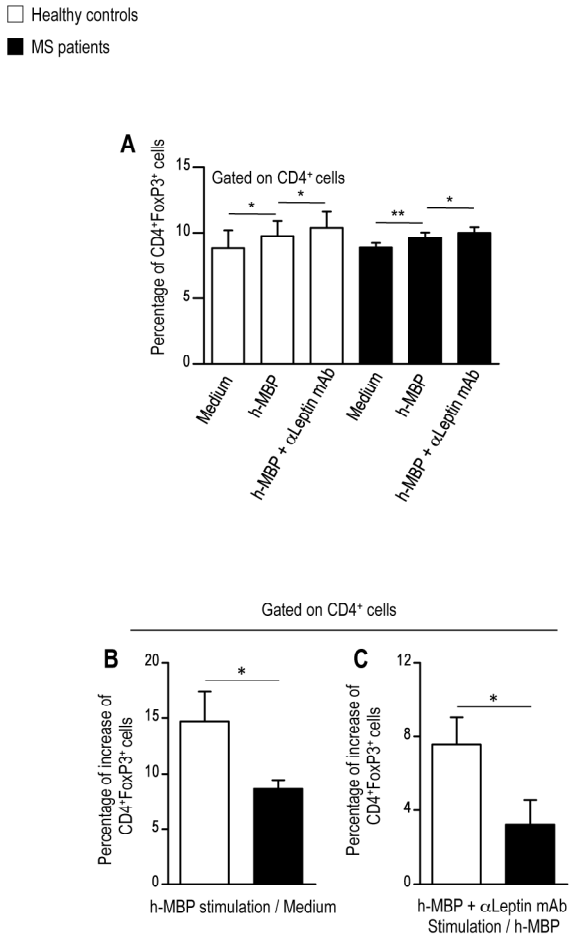


Figure 12. No differences in proliferation rate *in vivo* of effector T cells. (**A**) Ki67 expression in freshly isolated $CD4^{+}Foxp3^{-}$ cells from healthy controls and MS patients. The data are shown as mean ± SEM. (**B**) Representative flow cytometry plots of Ki67 expression in freshly isolated $CD4^{+}Foxp3^{-}$ cells from healthy control and MS patient.

To evaluate the anti-mielin-specific proliferation of Treg cells, hMBP-specific short-term T-cell lines were generated. We measured the proliferative response against hMBP on Treg cells from MS patients and controls, moreover we added an anti-leptin blocking antibody to the culture medium. We observed a significant lower hMBP-specific response of Treg cells from MS patients when compared with healthy controls (Fig 13 A and B). Moreover Treg cells from MS patients showed an impaired expanding capacity even in presence of neutralizing leptin monoclonal antibody with respect to healthy controls (Fig 13 C). We analyzed whether there could be differences in the proliferation rate against hMBP and hMBP plus leptin neutralizing antibody of effector T cells between controls and patients. The responses of effector T cells to h-MBP and to leptin neutralization were similar between controls and MS patients (Fig 13 D). These data suggest that this is a Tregs specific defect in MS patients.



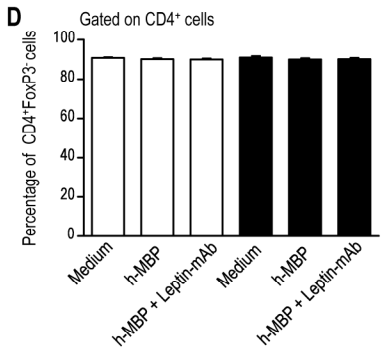


Figure 13. h-MBP specific proliferation of regulatory T cells from healthy controls and MS patients. (A) Percentage of $CD4^+FoxP3^+$ cells gated on $CD4^+$ cells from healthy controls and MS patients stimulated with h-MBP in the presence or absence of leptin mAb. (*p = 0.03, **p = 0.004). (**B**) Percentage of increase of $CD4^+FoxP3^+$cells from healthy controls and MS patients upon h-MBP stimulation. The graph shows the mean of the percentage of $CD4^+FoxP3^+$ cells upon 7 days of h-MBP stimulation with respect to the percentage of $CD4^+FoxP3^+$ cells without stimulation (*p = 0.004). (**C**) Percentage of increase of $CD4^+FoxP3^+$cells from healthy controls and MS patients upon h-MBP + anti-Leptin neutralizing antibody stimulation. The graph shows the mean of the percentage of $CD4^+FoxP3^+$ cells upon 7 days of h-MBP + anti-Leptin stimulation with respect to the percentage of $CD4^+FoxP3^+$ cells upon h-MBP stimulation alone (*p = 0.03) The data are shown as mean ± SEM. **(D)** Percentage of $CD4^+FoxP3^-$ cells gated on $CD4^+$ cells from healthy controls and MS patients stimulated for 7 days with h-MBP in the presence or absence of leptin mAb.

Inverse correlation between Tregs h-MBP-specific Expansion index and MS clinical severity.

We next evaluated the relation between the myelin-specific proliferative capacity of Treg cells and the clinical status of MS patients. Disease severity of each patient was assessed clinically using the EDSS score, a method of quantifying disability in multiple sclerosis (67). We divided all patients in three groups on the basis of their EDSS value. We firstly evaluated the fold of increase in KI67 expression of Treg cells upon h-MBP stimulation to understand whether there could be a different h-MBP-specific proliferation of Treg cells in patients with a different EDSS. All three patient groups analyzed showed a similar antigen-specific Treg cells proliferation, indicating that the progression of pathology was not correlated with an altered h-MBP-specific Treg cells response (Fig 14A). We next evaluated the fold increase in KI67 expression of Treg cells stimulated with h-MBP plus leptin neutralizing antibody with respect to Treg cells stimulated with h-MBP alone. On the contrary, in this case, we observed that MS patients with a lower EDSS

displayed an higher proliferative capacity of Treg, in response to h-MBP stimulation plus leptin neutralization when compared with patients whose clinical score was much more severe (Fig. 14B). Indeed patients with a lower EDSS score, showed a higher fold increase in KI67 expression when compared with patients with a more severe disease course (Fig. 14B).

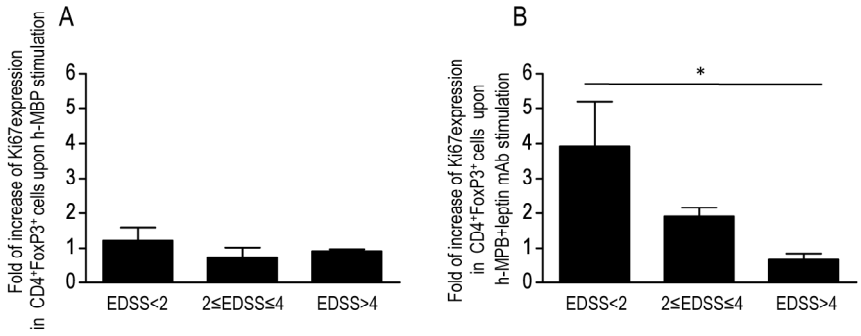


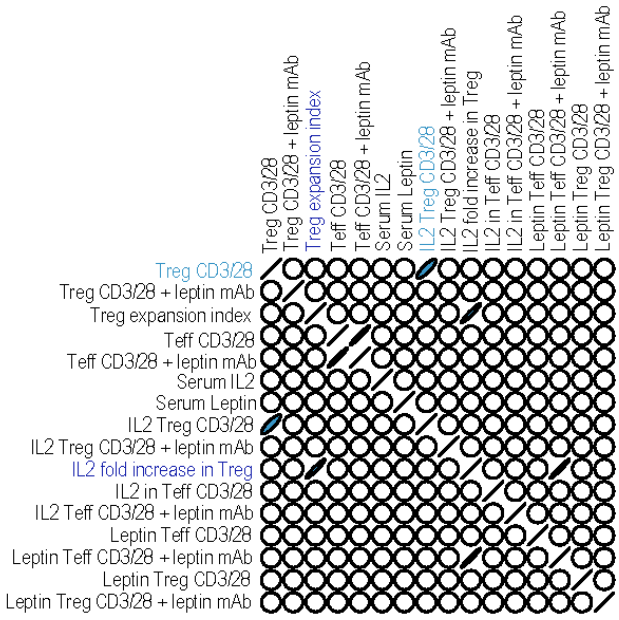
Figure 14. MS patients regulatory T cells antigen-specific expansion inversely correlates with patients clinical status. (A) The graph shows the fold increase in Ki67 expression of $CD4^+$ $FoxP3^+$ T cells purified from MS patients upon h-MBP stimulation , in patients with an EDSS<1, 2≤EDSS≤4 and EDSS>4. The graph shows the mean of Ki67 expression of $CD4^+FoxP3^+$ cells upon 7 days of h-MBP stimulation with respect to the mean of Ki67 expression of $CD4^+FoxP3^+$ cells without stimulation. **(B)** The graph shows the fold increase in Ki67 expression of $CD4^+$ $FoxP3^+$ T cells purified from MS patients upon h-MBP + leptin mAb stimulation, in patients with an EDSS<1, 2≤EDSS≤4 and EDSS>4. The graph shows the mean of Ki67 expression of $CD4^+FoxP3^+$ cells upon 7 days of h-MBP leptin mAb stimulation with respect to the mean of Ki67 expression of $CD4^+FoxP3^+$ cells upon h-MBP stimulation alone (*p = 0.04).

Multiple correlation matrix of molecular variables in controls and MS patients.

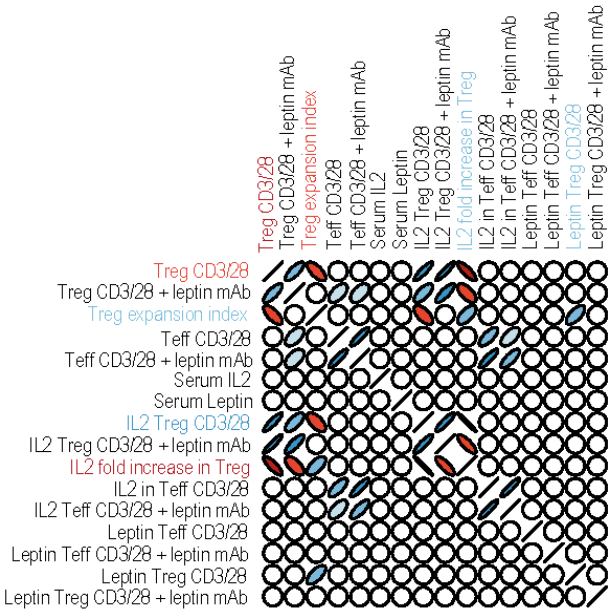
We performed a sophisticated mathematical model of data craft correlation matrix (Spearman rho non parametric correlation) among the molecular variables analyzed in the study in MS patients group stratified on the basis of EDSS score versus healthy controls. The results are presented in Fig.15. The controls group and patients groups showed significant differences in most of

variables analyzed. The results of the analysis showed that disease aggravation was correlated with a loss of a series of mechanisms of control. More specifically, we observed that, in controls group there was a strong positive correlation between the fold increase in proliferation of Treg cells upon leptin neutralization and the fold increase in IL-2 production (rho = 0.95, p= 0.001), in the same experimental condition (Fig. 15A). This correlation is less strong in patients with an EDSS minor than 2 (rho = 0.62, p = 0.007) (Fig. 15B) and is completely lost in patients with more severe disease (Fig. 15 C and D).

A



B


Treg CD3/28
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Serum IL2
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IL2 Treg CD3/28 + leptin mAb
IL2 fold increase in Treg
IL2 in Teff CD3/28
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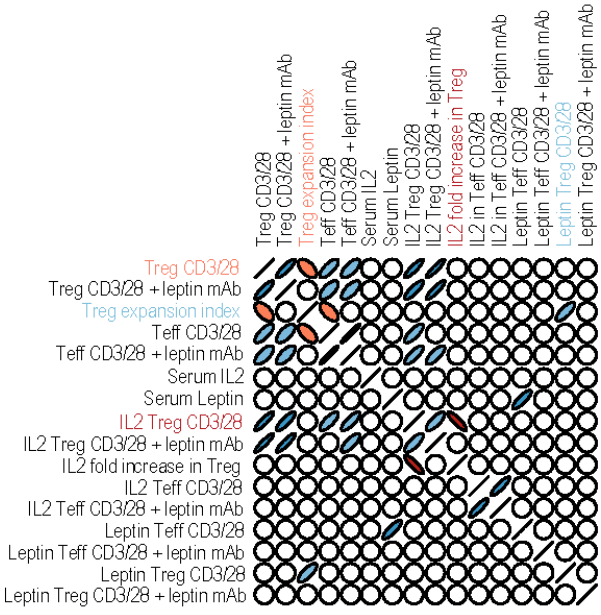
Leptin Treg CD3/28 + leptin mAb

C


Treg CD3/28
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Serum IL2
Serum Leptin
IL2 Treg CD3/28
IL2 Treg CD3/28 + leptin mAb
IL2 fold increase in Treg
IL2 Teff CD3/28
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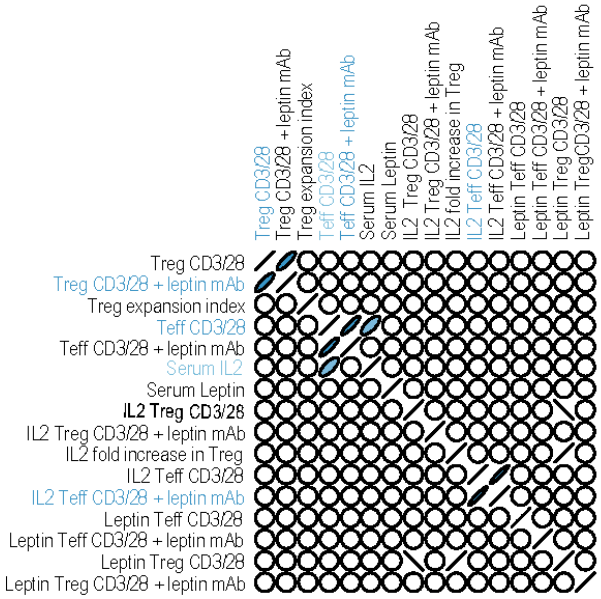

Figure 15. Graphical representation of the Spearman rho non parametric correlation matrix among the analyzed variables in the (A)control subjects, (B) patients with EDSS < 2, (C) patients with 2 ≤ EDSS ≤ 4, (D) **patients with EDSS > 4.**The presence of a significant correlation between two variables is expressed by means of a red (negative correlation) or blue (positive correlation) ellipse while an empty circle refers to a non significant correlation. The colour intensity and the thickness of each ellipse are proportional to the correlation values. The graphs showes correlations among: proliferation of Treg cells stimulated with anti-CD3/CD28 (indicated as Treg CD3/28); proliferation of Treg cells stimulated with anti-CD3/CD28 plus leptin mAb (indicated as Treg CD3/28 + leptin mAb); fold increase in proliferation of Treg cells upon leptin neutralization (indicated as Treg expansion index); proliferation of effector T cells stimulated with anti-CD3/CD28 (indicated as Teff CD3/28); proliferation of effector T cells stimulated with anti-CD3/CD28 plus leptin mAb (indicated as Teff CD3/28 + leptin mAb); serum IL-2 levels; serum leptin levels; Treg cell IL-2 secretion upon anti-CD3/CD28 stimulation (indicated as IL-2 Treg CD3/28); Treg cell IL-2 secretion upon anti-CD3/CD28 plus leptin mAb stimulation (indicated as IL-2 Treg CD3/28+ leptin mAb); Fold increase in IL-2 production upon leptin neutralization in Treg cells (indicated as IL-2 fold increase in Treg); Effector T cell IL-2 secretion upon anti-CD3/CD28 stimulation (indicated as IL-2 Teff CD3/28); Effector T cell IL-2 secretion upon anti-CD3/CD28 plus leptin mAb stimulation (indicated as IL-2 Teff CD3/28 + leptin mAb); Effector T cell leptin secretion upon anti-CD3/CD28 stimulation (indicated as leptin Teff CD3/28); Effector T cell leptin secretion upon anti-CD3/CD28 plus leptin mAb stimulation (indicated as leptin Teff CD3/28 + leptin mAb); Treg cell leptin secretion upon anti-CD3/CD28 stimulation (indicated as leptin Treg CD3/28); Treg cell leptin secretion upon anti-CD3/CD28 plus leptin mAb stimulation (indicated as leptin Treg CD3/28+ leptin mAb).

In addition we observed that in the group of patients that displayed an EDSS minor than 2, there was an inverse correlation between Treg cells proliferation upon anti-CD3/CD28 stimulation and the fold increase in proliferation of Treg cells upon leptin neutralization (rho = -0.68, $p < 0.001$) (Fig. 15 B). In other words, the fold increase in proliferation of Treg cells upon leptin neutralization, is major as Treg cells are more hyporesponsive upon anti-CD3/CD28 stimulation. This correlation is less strong in patients showed and EDSS between 2 and 4 (rho = -0.56, $p = 0.007$) (Fig. 15 C), and was completely lost in patients with EDSS higher than 4 (Fig. 15 D).

Discussion

Clonal deletion of self-reactive T cells in the thymus and induction of T-cell anergy alone do not explain the maintenance of immunologic self-tolerance, as potentially pathogenic autoreactive T cells are present in the periphery of healthy individuals (78,79). Thus, other regulatory mechanisms exist to prevent autoreactive T cells from causing immune disorders. Active suppression by regulatory T cells plays a key role in the control of self-antigen-reactive T cells and the induction of peripheral tolerance *in vivo* (80,81). Seminal experiments performed by Sakaguchi *et al*. (82) have shown that depletion of $CD4^+CD25^+$ suppressor cells results in the onset of systemic autoimmune diseases in mice. Furthermore, cotransfer of these cells with $CD4^+CD25^-$ cells prevents the development of experimentally induced autoimmune diseases such as colitis, gastritis, insulin-dependent autoimmune diabetes, and thyroiditis (83). Human $CD4^+CD25^{hi}$ T cells, similar to the mouse $CD4^+CD25^+$ suppressor cells, are anergic to *in vitro* antigenic stimulation and strongly suppress the proliferation of responder T cells upon co-culture (84). $CD4^+CD25^+$ T cells are among the best-characterized immune regulatory subsets shown to prevent activation and effector function of activated responder T cells (85). In these contests there are several studies performed to understand which is the alteration of this cellular subset occur during autoimmune diseases. However data from literature are a still controversial on the question whether qualitative more than quantitative alterations of Treg cells compartments, are responsible for the break of self-immune tolerance.

Indeed, previous studies have reported a significant decrease in the suppressive function of $CD4^+CD25^{hi}$ Treg cells from peripheral blood of patients with MS as compared with healthy donors but no differences in the frequency of $CD4^+CD25^{hi}$ Treg cells have been found between patients and healthy controls (86). On the other hand, other groups have shown that in patients with MS the compromised $CD4^+CD25^{high}$ regulatory T-cell function is correlated with a reduced frequency of FOXP3-positive cells (87) but in the same time, an increased frequency of $CD4^+CD25^+$ regulatory T cells in the cerebrospinal fluid MS patients has been observed (88). To complicate this scenario there are

data suggesting that patients with the MS have only a strikingly reduced number of $CD39^+$ and $CD4^+$ $CD25^+$ Treg cells in the peripheral blood (72,89).
Since previous studies have showed that cloned $CD4^+$ T cells could be induced to become regulatory if they were first rendered anergic and so that anergy of Treg cells correlates with suppressive function (90), in this study we firstly analyzed the anergic state of Treg cells from MS patients, with the attempt to find novel surrogate markers of immune tolerance. More specifically, we stimulated *in vitro* Treg cells purified from MS patients and healthy controls and we evaluated their proliferation after stimulation. Treg cells from MS patients showed a reduced *in vitro* hyporesponsiveness during TCR-induced stimulation resulting in a partial proliferation. This evidence was also supported by some low but consistent IL-2 secretion by Treg cells from MS patients in the same experimental conditions. This reduced *in vitro* hyporesponsiveness of Treg cells from MS patients could be partly responsible for the reduced *in vitro* suppressive function observed by other groups (86).
We confirmed that leptin concentration was higher in sera of MS patients and that freshly isolated human Treg cells produce higher amounts of leptin with respect to effector T cells. No significant difference, in terms of leptin production, between Treg cells from MS patients and healthy controls was measured.
We have reported that *in vitro* leptin neutralization results in proliferation of Treg cells purified from healthy controls during polyclonal anti-CD3/CD28 stimulation (73). To further address whether the proliferative potential of Treg cells could be altered during MS, we analyzed the effect of leptin neutralization on proliferative capacity of this cellular subset in MS patients. We found that Treg cells from MS patients showed an impaired proliferation upon leptin neutralization. Indeed the fold increase in proliferation, mediated by leptin neutralization, of Treg cells from MS patients, has been shown to be lower when compared to that observed in healthy controls. As previously mentioned, the amount of leptin produced by Treg cells was the same in patients and controls, thus suggesting that the impaired proliferative potential of Treg cells could not be ascribed to the amount of leptin produced by Treg cells but rather to an intrinsic alteration of proliferation of these cells during MS. Once again, these data are confirmed by IL-2 secretion. Indeed, we observed in Treg from

MS patients a lower fold increase in IL-2 production upon leptin neutralization when compared to that observed in healthy controls, supporting the hypothesis that Treg cells are characterized by an altered response to leptin neutralization during MS. It is important to note that no differences in effector T cells proliferation and IL-2 secretion between controls and patients where found, confirming that the break of self antigen tolerance is mediated by alterations regarding specifically Treg cells compartment.

It has been shown that Treg cells leptin-neutralization induced proliferation is IL-2 dependent (73). We evaluated the correlation between the fold increase in proliferation and the fold increase in IL-2 production of Treg cells upon leptin neutralization. In healthy controls there is a linear positive correlation between the two parameters analyzed, confirming the presence of an autocrine stimulatory loop in which IL-2 sustains the expansion of Treg cells. In MS patients there is a loss of this correlation indicating that the proliferation of Treg cells, although very mild, is not supported by an increase in IL-2 production. It is possible to speculate that in MS patients Treg cells are characterized by alteration in IL-2/IL-2R pathway. Further studies are being performed to address this point.

In spite of intensive studies to understand which is the alteration occurs during autoimmune diseases, so far there are not reliable surrogate markers of immune tolerance capable of detecting the loss of self-tolerance in autoimmunity and MS.

In this context, there is no means to identify *a priori* those MS patients who will have a good progression of disease (in terms of relapses and EDSS score) and those who will have a worst MS course. In other words we do not have either predictive markers able to predict the clinical progression in MS or the clinical response to classical therapeutic regimes. In this study, we also aimed at developing reliable and reproducible *in vitro* assay capable of detecting tolerance state in MS patients and possibly to anticipate clinical progression towards a worse disease. We observed that leptin-neutralization induced Treg cells proliferation is higher in patients with a better clinical score assessed using the Expanded Disability Status Scale (EDSS). We observed that MS patients with a lower EDSS displayed an higher expansion index of Treg cells upon leptin neutralization when compared with patients whose clinical score

was much more severe, indicating that the progression of pathology compromises the proliferative potential of Treg cells or that impaired Treg cells expansion could be associated with a worse clinical progression of MS. In other words Treg cells expansion upon leptin neutralization (fold increase) is negatively associated with EDSS score. These findings envision new possibilities of using the anti-leptin-based Treg cell expansion index in evaluation of immune tolerance and to predict clinical progression in MS patients. These data were confirmed not only in non-antigen specific conditions during CD3/CD28 stimulations but also in myelin-specific assays by using anti-h-MBP responses, thus suggesting that it is likely that in MS there is a reduced capacity of Tregs to respond towards h-MBP likely impairing their capacity to control CNS immune tolerance.

It is interesting to note that also Treg cells antigen-specific expansion index inversely correlated with patients clinical status, supporting the hypothesis that Treg cells proliferation rate (policlonal or antigen-specific) could be related with clinical progression of disease.

Finally, in order to evaluate the alterations occurring in MS patients and the differences characterizing MS patients with different EDSS score, we performed a multiple Spearman rho non parametric correlation matrix among the molecular variables analyzed in the study in both controls and patients. We found a marked difference between controls and patients indicating that a series of mechanisms of control are altered during disease compromising the correlation that are physiologically present in healthy individuals. Moreover the analysis reveals that the progression of disease, evaluated as EDSS score, is associated with a further alteration of these correlations. Indeed, there is a strong difference, in terms of correlations, among the three groups of patients (separated on the basis of EDSS score) analyzed.

Conclusions

We believe that the Treg cell expansion index can represent a valuable and repeatable assay to measure immune tolerance and disease progression in MS. We are putting all the effort to translate this assay in clinical practice and to measure immune tolerance also in other autoimmune diseases such as type 1 diabetes and intestinal bowel disease. Future investigations and prospective studies will provide sufficient evidence for the application of our results.

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