

IL-10 suppressor activity and *ex vivo* Tr1 cell function are impaired in multiple sclerosis

Ivan Martinez-Forero^{1,2}, Ricardo Garcia-Munoz³, Sara Martinez-Pasamar², Susana Inoges³, Ascension Lopez-Diaz de Cerio³, Ricardo Palacios¹, Jorge Sepulcre¹, Beatriz Moreno¹, Zaira Gonzalez¹, Begoña Fernandez-Diez¹, Ignacio Melero³, Maurizio Bendandi³ and Pablo Villoslada¹

¹ Department of Neurology, University of Navarra, Pamplona, Spain

² Department of Immunology, University of Navarra, Pamplona, Spain

³ Cell Therapy Area, Clinica Universitaria de Navarra and Center for Applied Medical Research, University of Navarra, Pamplona, Spain

T regulatory cells type 1 (Tr1 cells) are excellent candidates for cell therapy in multiple sclerosis (MS). The aim of our study was to assess the functional state of Tr1 cells and IL-10R signaling in patients with MS. Tr1 cells were induced *in vitro* by activation with anti-CD46 antibodies in controls and patients with MS. Cells were phenotyped by cytometry and suppression assays, and the expression of cytokines and transcription factors was evaluated by real-time PCR, ELISA, cytometry and Western blotting. We found that the activity of Tr1 cells and IL-10R signaling is impaired in MS patients since Tr1 cells isolated from MS patients produced less IL-10 than those obtained from controls. Indeed, the supernatants from Tr1 cells from controls did not suppress the proliferation of stimulated CD4⁺ cells from patients with MS. Furthermore, the IL-10R signaling pathway was not fully active in CD4⁺ cells from MS patients and these cells had higher baseline levels of SOCS3 transcripts than controls. Indeed, after *in vitro* IL-10 stimulation, the expression levels of the STAT1, STAT3 and IL-10RA genes were higher in MS patients than in controls. Moreover, Stat-3 phosphorylation was lower in controls than in patients after IL-10 stimulation. These results indicate that IL-10 regulatory function is impaired in patients with MS.

Received 14/3/07

Revised 29/10/07

Accepted 7/12/07

[DOI 10.1002/eji.200737271]

Key words:
CD46 · IL-10 · MS
· Stat-3 · Tr1 cells

Introduction

Despite the fact that the immune repertoire of healthy individuals contains autoreactive T cells and natural antibodies, under normal circumstances autoimmune diseases do not develop. For example, in the repertoire of healthy humans [1] and non-human primates [2]

myelin oligodendrocyte glycoprotein-reactive T cells exist that can induce autoimmune encephalomyelitis in marmosets when activated *in vitro* [2, 3]. Hence, despite their silencing, these autoreactive T cells clearly maintain the ability to mediate autoimmune disease. Thus, as well as central tolerance other mechanisms must be active to prevent autoimmune diseases. One of the most important factors capable of preventing autoimmune diseases is the activity of regulatory T cells (Treg). Over the past few years, several subsets of Treg have been identified, including natural CD4⁺CD25⁺Foxp3⁺ T cells and acquired regulatory T cells such as regulatory type 1 cells (Tr1) and Th3 cells [4]. Nevertheless, in order to unravel the pathogenesis of autoimmune conditions, we must still better understand how Treg cells function in autoimmune disease,

Correspondence: Dr. Pablo Villoslada, Neuroimmunology lab 2.05, Center for Applied Medical Research, Pio XII 55, Pamplona 31008, Spain
Fax: +34-948-194715
e-mail: p.villoslada@unav.es

Abbreviations: **c(t):** cycle threshold · **HC:** healthy control · **MNE:** mean normalized expression · **MS:** multiple sclerosis · **SOCS:** suppressor of cytokine signaling · **Tr1 cell:** T regulatory cell type 1

particularly since Treg cell transfer might be exploited as a tool in immunotherapy for autoimmune diseases [5].

Tr1 cells are defined by their ability to produce high levels of IL-10, moderate amounts of TGF- β and IFN- γ , and no IL-2 or IL-4. In addition, they also have a poor proliferative capacity [6, 7]. Moreover, Tr1 cells display a memory phenotype, and they can suppress naive and memory Th1 or Th2 responses through the production of IL-10. The suppressive effects of Tr1 cells differentiated *in vitro* are remarkably potent, and fewer than 4000 Tr1 cells are capable of suppressing the proliferation of 50 000 CD4 $^{+}$ T cells by 50% [8]. Tr1 cells participate in the maintenance of peripheral tolerance by suppressing the activation of autoreactive T cells [9, 10]. Tr1 cells can be generated *in vitro* from CD4 $^{+}$ cells by stimulation with high doses of IL-10, alone or in conjunction with IFN- α [11, 12], by adding vitamin D and dexamethasone [13] or by stimulating CD46 on CD4 $^{+}$ cells with specific monoclonal antibodies [14, 15]. By contrast, generation of Tr1 cells is inhibited by OX40L [16].

Multiple sclerosis (MS) is a chronic inflammatory disease in which activated T and B cells infiltrate the central nervous system, provoking demyelination and axonal damage, which produces significant neurological disability [17]. To date, the primary antigens against which the immune response is directed in MS remain unknown, but immunological dysfunction has been identified in patients with MS [18]. Impairment of suppressor cell function has classically been demonstrated in patients with MS [19], and a few years ago, the functional impairment of natural CD4 $^{+}$ CD25 $^{+}$ Foxp3 $^{+}$ Treg cells has also been demonstrated [20]. Recently, Astier *et al.* [21] demonstrated that Tr1 function is also impaired in patients with MS. Moreover, the expression of the immunosuppressor cytokine IL-10 is diminished in MS patients [22, 23], in accordance with previous studies describing fewer IL-10-secreting peripheral blood mononuclear cells (PBMC). Interestingly, there is a reduction in the expression of IL-10 but elevated numbers of PBMC expressing IL-10 transcripts before clinical relapse in MS [24], and there is a correlation between IL-10 levels and IgG in the cerebrospinal fluid of MS patients [25]. Engineered Th2/Tr1 cells that produce high levels of IL-10 prevent epitope spreading and ameliorate the disease course in animal models of MS [26]. However, it is unknown whether the functional activity of IL-10 in MS is preserved, and attempts to treat MS patients with IL-10 have not been successful to date.

In order to explore the functionality of Tr1 regulatory function in MS, we assessed the *ex vivo* induction of the Tr1 phenotype and the response of CD4 $^{+}$ cells to the IL-10 produced by these cells in patients with MS. We found that CD4 $^{+}$ cells from MS patients were less disposed to differentiate into a Tr1 cell phenotype *ex vivo* and that, in addition, these cells produce less IL-10.

Importantly, CD4 $^{+}$ cells from MS patients were more resistant to suppression by IL-10 because the IL-10R signaling cascade is defective in MS patients.

Results

In vitro generation of CD46-induced Tr1 cells is impaired in MS patients

In healthy controls ($n = 8$), CD4 $^{+}$ cells stimulated *in vitro* with anti-CD46 + anti-CD3 and anti-CD28 antibodies for 3 days acquired a Tr1 phenotype (Fig. 1). The presence of surface CD25 and CD45RO in these cells increased by up to 68 and 63%, respectively (Fig. 1A), while the intracellular levels of IL-10 rose up to 27% and the cells secreted up to 3566 pg/mL IL-10 protein (Fig. 1B; $p < 0.05$ in all cases). Unstimulated cells did not augment the expression of these markers of activation and they did not produce higher levels of IL-10 after 3 days in culture (data not shown). Indeed, stimulation of CD4 $^{+}$ cells increased the expression of IL-10 and IFN- γ RNA ($p < 0.05$ in both cases) but not that of IL-2 (Fig. 1C). Moreover, we assessed whether such CD4 $^{+}$ T cell cultures remain stable over time and whether Tr1 cells released large amounts of IL-10 upon further stimulation. The same CD46-induced Tr1 induction protocol was applied to cells from five healthy control (HC) individuals and a second stimulation was applied 10 days later with an anti-CD3 rather than the anti-CD46 antibody. When the Tr1 phenotype was assessed at different intervals, we found that after the first stimulation the majority of the cells had developed an activation and memory phenotype. Moreover, they increased the release of IL-10, the production of which peaked at day 3 and decreased over the following 10 days. Over the next 7 days, the Tr1 cells maintained the activation and memory phenotype, but they produced less IL-10. After a second stimulation 10 days later, the majority of the cells showed a CD25 $^{+}$ CD45RO $^{+}$ activation/memory phenotype (65%; Fig. 1D) and they also augmented the levels of both intracellular (Fig. 1D) and secreted IL-10 protein (Fig. 1E; $p < 0.05$ in all cases). Finally, we assessed whether Tr1 cells acted as regulatory T cells, by assessing the capacity of the secreted cytokines (mainly IL-10) to suppress the proliferation of CD4 $^{+}$ cells stimulated with anti-CD3 and anti-CD28 antibodies. We found that the supernatants from Tr1 cell cultures, containing high levels of IL-10 (in the range of 5580–7533 pg/mL), as well as recombinant IL-10, were able to suppress the proliferation of CD4 $^{+}$ T cells (Fig. 1F; $p < 0.001$). This suppression was impaired in the presence of the anti-IL-10 antibody, confirming that the suppression was mediated by IL-10 (Fig. 1F). Thus, in accordance with earlier data [14], we confirm that CD46

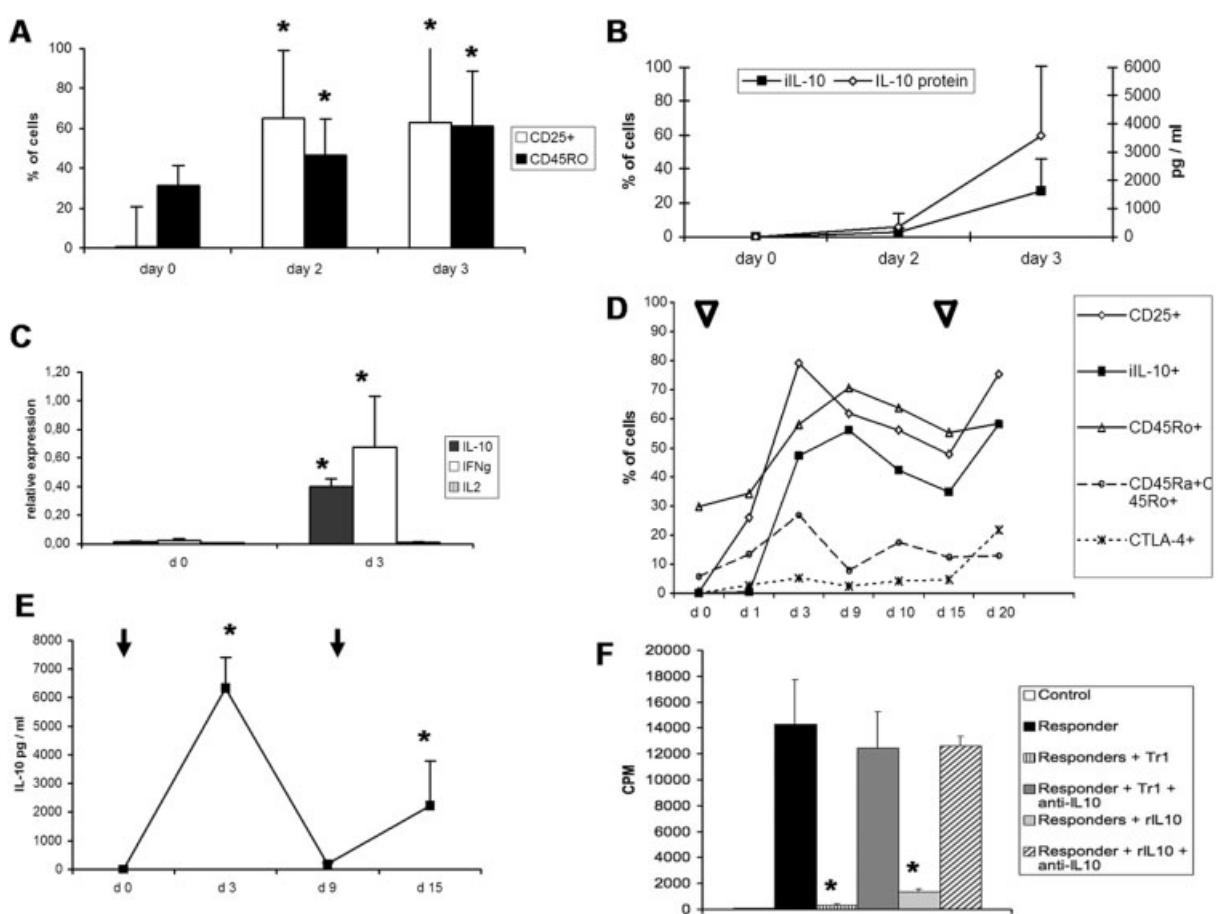


Figure 1. Induction of Tr1 phenotype in CD4 $^{+}$ from healthy controls ($n = 8$). (A) Stimulated CD4 $^{+}$ cells expressed significantly higher levels of CD25, CD45RO, and both intracellular and secreted IL-10 (B), at days 2 and 3 than at baseline (day 0), indicated by *. The results are expressed as the percentage of cells displaying each marker in terms of flow cytometry, or in pg/mL in the case of secreted IL-10 protein. (C) IL-10, IFN γ and IL-2, increased in Tr1 cultures; * indicates significant differences between day 0 and day 3. (D) Kinetics of Tr1 cultures induction. The results are expressed as the percentage of cells displaying each marker in flow cytometry assays. (E) Levels of IL-10 protein in the supernatant of Tr1 cultures assessed by ELISA. Arrows indicate the antibody stimulation (see above) and the results are the summary of three different experiments on PBMC from eight HC individuals. * indicates significant differences from day 0 to days 3 and 15. (F) Tr1 cultures suppression. In order to confirm the involvement of IL-10 in the suppressive activity, blocking antibodies against IL-10 were added. Proliferation was assessed on the basis of [3 H]thymidine incorporation and the results are expressed as the mean \pm standard deviation of the cpm for each group. Non-responder: unstimulated CD4 $^{+}$ cells; responder cells + Tr1: responder cells incubated with supernatant from CD46-induced Tr1-like cells. Results are expressed as the mean \pm standard deviation. Differences between groups were assessed with the Mann-Whitney U test and differences along time were assessed with the Friedman test. * $p < 0.05$.

stimulation of CD4 $^{+}$ cells induces a Tr1-like phenotype and that this phenotype is stable in culture for 20 days, although IL-10 production is dependent on further TCR stimulation.

When the same CD46-induced Tr1 induction protocol was applied to CD4 $^{+}$ cells from untreated patients with MS ($n = 11$), we found that there was a similar increase in the expression of the CD25 and CD45RO activation and memory markers, as well as in intracellular and secreted IFN- γ (Fig. 2A, B). However, both the intracellular and secreted levels of IL-10 produced by Tr1 cells from MS patients were significantly lower at day 3 than those from HC (Fig. 2A, C; $p < 0.05$ in both cases). Because CD46 have a pleiotropic activity in the immune

system that strongly influences T cell activation and function [27], we compared the levels of CD46 in cells isolated from MS patients and HC. No differences were found in the levels of CD46 expression between MS patients and HC (HC: 70.5%; MS: 72.3%) (Fig. 2A, Table 1). Thus, our results suggest that Tr1 activity is impaired in MS patients.

CD4 $^{+}$ cells from MS patients are hyporesponsive to the suppressive activity of IL-10

Because IL-10 is the mediator of Tr1 cell immunomodulatory activity, we assessed whether the Tr1 activity contributed to peripheral tolerance in MS patients to the

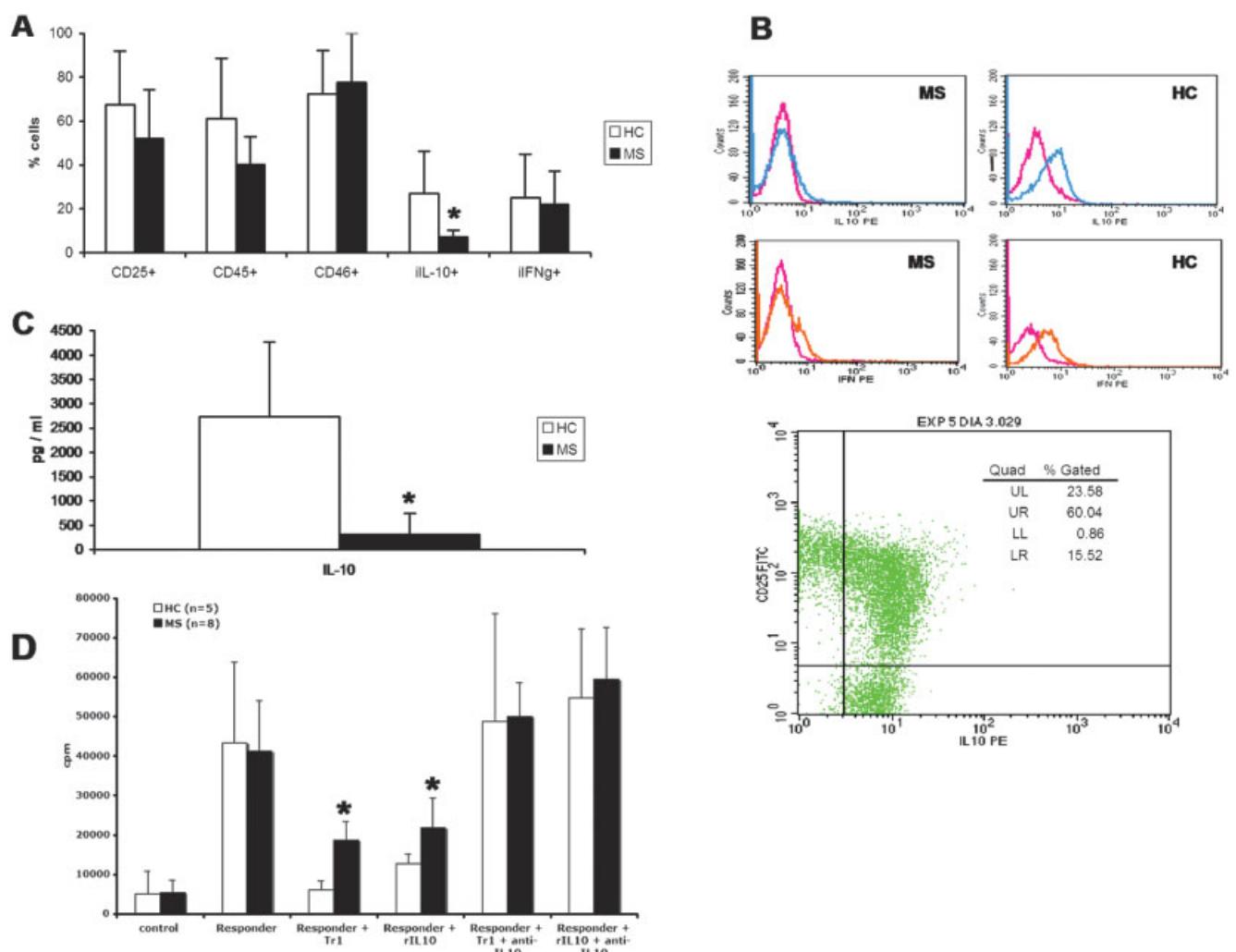


Figure 2. Differences in the induction of the CD46-induced Tr1 phenotype between untreated MS patients ($n = 11$) and HC ($n = 8$) after stimulation of CD4⁺ cells with anti-CD46, anti-CD3 and anti-CD28 antibody for 3 days. (A) Expression of CD25⁺ and CD45RO, and intracellular cytokines by day 3. (B) Representative histograms from flow cytometry studies showing the levels of the intracellular IL-10 (top) and IFN- γ (middle) staining 3 days after CD46 stimulation in patients with MS and HC. A representative IL-10 flow cytometry stain from an HC is shown (bottom) (y axis: anti-CD25; x axis: anti-IL-10). (C) Levels of IL-10 secreted protein. (D) Suppression assays in CD4-stimulated cultures using Tr1 cell culture supernatants containing large amounts of IL-10 or recombinant IL-10, with or without adding the blocking anti-IL-10 antibody. Results are the summary of three different experiments in HC ($n = 8$) and MS patients ($n = 11$). Results are expressed as the mean cpm + standard deviation. Differences between groups were assessed with the Mann-Whitney U test. * indicates significant differences between HC and MS patient cpm in Tr1 supernatant and rIL-10 assays, $p < 0.05$.

same extent as in HC. We studied the response of CD4⁺ cells from MS patients to IL-10 produced by Tr1 cells *in vitro*. We performed suppression assays on CD4⁺ cells stimulated with anti-CD3 antibodies using the supernatant from Tr1 from HC or recombinant IL-10. We found that in contrast to CD4⁺ from HC, proliferation of CD4⁺ from MS patients was not inhibited by the supernatant conditioned by Tr1 cells or recombinant IL-10 (Fig. 2D). Thus, these results suggest that the immunosuppressive activity of IL-10 is defective in MS patients. Moreover, our findings help to explain why clinical trials with IL-10 in MS patients have failed to show an effect in preventing disease activity [28].

Defective regulation of IL-10 signaling in MS patients

Since we found that CD4⁺ cells from MS patients were more resistant to the immunosuppressive activity of IL-10, we studied the IL-10 signaling pathway to identify at which point signaling was impaired. We first measured the *ex vivo* expression of *IL-10*, *IL-10RA*, *STAT1*, *STAT3* and *SOCS3* in PBMC from HC and MS patients, and we found that PBMC from MS patients expressed higher levels of *SOCS3* transcripts than HC (Fig. 3A, $p = 0.008$). Suppressor of cytokine signaling (Socs)-3 is a potent suppressor of cytokine production

Table 1. Baseline phenotypic analysis in MS patients and HC by flow cytometry^{a)}

	MS	HC	p value
CD4 ⁺	29.65 (11.33)	36.20 (16.03)	ns
CD25 ⁺	10.99 (8.91)	17.37 (15.18)	ns
CD4 ⁺ CD25 ⁺	8.40 (6.13)	14.39 (13.46)	ns
CD46 ⁺	77.48 (22.71)	72.52 (19.77)	ns
CD4 ⁺ CD46 ⁺	29.51 (11.30)	36.00 (15.93)	ns
CD46 ⁺ CD25 ⁺	11.84 (9.07)	16.89 (14.69)	ns
CD45RA ⁺	57.40 (19.75)	45.49 (13.67)	ns
CD45RO ⁺	20.17 (7.89)	33.20 (20.25)	ns
CD4 ⁺ CD45RA ⁺	18.57 (11.95)	17.15 (3.67)	ns
CD4 ⁺ CD45RO ⁺	14.22 (5.59)	22.22 (16.82)	ns
CD45RA ⁺ RO ⁺	2.82 (2.45)	6.07 (3.90)	ns

^{a)} We studied PBMC from MS patients ($n = 11$) and HC ($n = 8$) and found no differences between either group for any of the markers tested. The results are expressed as mean (standard deviation) of the percentage of cells expressing each marker, and each group was compared by means of the Mann-Whitney U test. In addition, no differences were found in phenotype when compared between MS disease subtypes (data not shown).

that regulates the negative feedback on IL-10 production after IL-10R stimulation [28]. Hence, the increased levels of *SOCS3* transcripts might inhibit IL-10 production. Accordingly, we investigated the kinetics of gene expression in CD4⁺ cells stimulated with IL-10 for 12–24 h. We found a significant increase in the expression of mRNA encoding IL-10RA ($p = 0.022$), Stat-1 ($p = 0.03$), and Stat-3 ($p = 0.034$) in PBMC from MS patients but not from HC 24 h after IL-10 stimulation. Again, *SOCS3* transcripts were also augmented in MS patients when compared to the baseline levels of expression in HC. However, the kinetics of expression in PBMC from both MS and HC groups was similar (Fig. 3B; $p = 0.1$).

Protein phosphorylation is a critical process in IL-10 signaling [28, 29]. For this reason, we evaluated the phosphorylation of Stat-1, Stat-3 and Socs-3 in PBMC from MS patients and HC by flow cytometry. In HC we found that, after stimulation of PBMC with IL-10, the phosphorylation levels of Stat-1, Stat-3 and Socs-3 rise above baseline levels (Fig. 4A). However, in MS patients we found that Stat-3 showed a different kinetics, with lower levels of Stat-3 phosphorylation upon IL-10 stimulation (Fig. 4A; $p = 0.03$). Indeed, we analyzed which were the cell subtypes responsible for such behavior. We found that both CD4⁺ and CD8⁺ cells but not CD56⁺, CD14⁺ and CD19⁺ cell populations showed an impaired phosphorylation of Stat-3 (data not shown). By Western blot, we confirmed the decreased phosphor-

ylation of Stat-3 in PBMC from MS patients stimulated with IL-10 (Fig. 4B). Finally, we performed an IL-10 dose-response by analyzing the phosphorylation of Stat-1, Stat-3 and Socs-3 after stimulation with increasing doses of IL-10. We found that patients and controls display a similar Stat-1 and Socs-3 phosphorylation kinetics when using different doses of IL-10, but we found a trend for a different kinetics in Stat-3 phosphorylation, showing a decreased response to IL-10 stimulation in MS patients (Fig. 4C; $p = 0.06$). In summary, our findings suggest that IL-10 signaling is defective in patients with MS.

The most common drug used to treat MS is IFN- β [17]. It has been shown that IFN- β therapy increases the levels of IL-10 in MS patients [30], suggesting that one of the mechanisms of action of IFN- β treatment may be to promote the activity of immunosuppressive cytokines. Because we found that the response to IL-10 is impaired in MS patients, we assessed whether IFN- β might revert such a status and in this way become a useful adjuvant for Tr1 cell immunotherapy. We initially measured the *ex vivo* expression of *IL-10RA*, *STAT1*, *STAT3* and *SOCS3* in MS patients treated with IFN- β . We found that patients treated with IFN- β have higher levels of *STAT1* and *STAT3* transcripts than HC (Fig. 3A; $p = 0.019$ and 0.038, respectively), and that there was a trend towards higher levels of *IL-10* transcripts when compared to HC (Fig. 3A; $p = 0.06$). Indeed, MS patients treated with IFN- β displayed higher levels of *IL-10RA* transcripts than untreated patients (Fig. 3A; $p = 0.015$). The rise in *IL-10* transcripts in PBMC from MS patients treated with IFN- β has been related to the fact that IFN- β therapy inhibits the proliferation of myelin-reactive T cells, increasing the frequency of other T cell populations, including Treg [30]. In the kinetic analysis, we found that patients treated with IFN- β did not differ significantly from untreated patients (Fig. 3B). Moreover, we analyzed the effect of IFN- β therapy on the IL-10 signaling cascade by flow cytometry. We found that phosphorylation of Stat-1, Stat-3 and Socs-3 was similar in IFN- β -treated and untreated patients, although we found a non-significant difference in levels of Stat-3 phosphorylation at 60 min. These results suggest that IFN- β therapy is unable to recover the response to IL-10 *in vitro* (Fig. 4D).

Discussion

Recently, Astier *et al.* reported the impairment of Tr1 cell function in patients with MS [21]. They found that Tr1 cells from MS patients produce lower amounts of IL-10 but that they retain their capacity to proliferate and to produce IFN- γ . They found this defective response both in IFN- β -treated and untreated MS patients. In our study, we found similar findings because we also

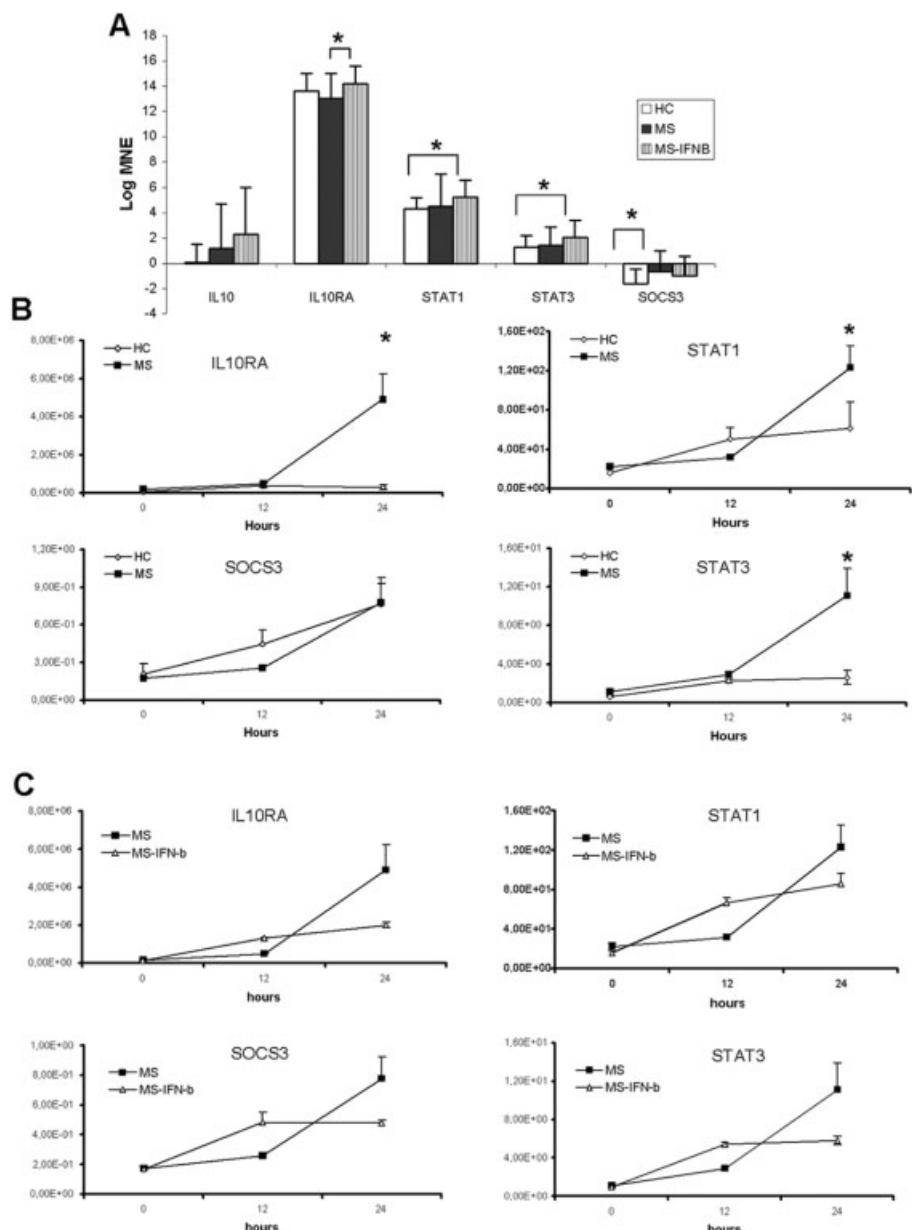


Figure 3. Gene expression analysis of IL-10, IL-10RA, STAT1, STAT3 and SOCS3 in HC ($n = 21$), untreated MS patients ($n = 20$), and MS patients treated with IFN- β (MS-IFNB, $n = 19$). Total RNA was reverse transcribed and the cDNA obtained was amplified using TaqMan probes. Each sample was run in triplicate and in each plate the target and the endogenous control were amplified in different wells. The expression of the different genes tested was quantified relatively to the level of the housekeeping gene GAPDH. Results are expressed as the logarithm of the MNE \pm standard deviation. (A) Gene expression was assessed ex vivo in PBMC RNA. (B) Kinetics of IL-10RA, STAT1, STAT3 and SOCS3 gene expression in PBMC from HC and MS patients stimulated in vitro with IL-10 for up to 24 h. (C) Kinetics of IL-10RA, STAT1, STAT3 and SOCS3 gene expression in PBMC from untreated and IFN- β -treated MS patients stimulated in vitro with IL-10 for up to 24 h. Differences between groups were assessed with the Mann-Whitney U test and differences along time were assessed with the Friedman test. * $p < 0.05$.

demonstrate that after CD46 activation, although Tr1 cells from patients with MS display an activated phenotype, they produce a lower amount of IL-10 and high amounts of IFN- γ , both at the RNA and protein level. Indeed, our study and Astier's study showed that the levels of CD46 receptors were similar between patients and controls. Our result and the findings in the study by Astier *et al.* indicate that stimulation with high doses of anti-CD3 antibody or IL-2 did not recover the IL-10 production by Tr1 cells, indicating that differences in the surface levels of this receptor were not the cause of the loss of function. Astier *et al.* found that this impaired regulatory phenotype was associated with the presence of an alternative splicing in the CD46 receptor that promotes a pro-inflammatory response. We further explored the implications of the defective Tr1 cell

function by assessing the IL-10R signaling in patients with MS. We found that IL-10R signaling is abnormal in MS patients, contributing to the impairment of Tr1 function because CD4 $^{+}$ from such patients will be less susceptible to the action of IL-10 produced by Tr1 cells. Although we did not assess the presence of the CD46 splicing in patients with MS, the Tr1 cell phenotypes in both studies are quite similar, indicating a consistent defect in this regulatory T cell function in patients with MS.

CD4 cells from patients with MS were significantly less responsive to suppression when cultured with supernatants from Tr1 cell cultures from HC or recombinant IL-10. This finding suggests that effector cells in MS patients may be less amenable to suppression. Although such differences might be attributable to

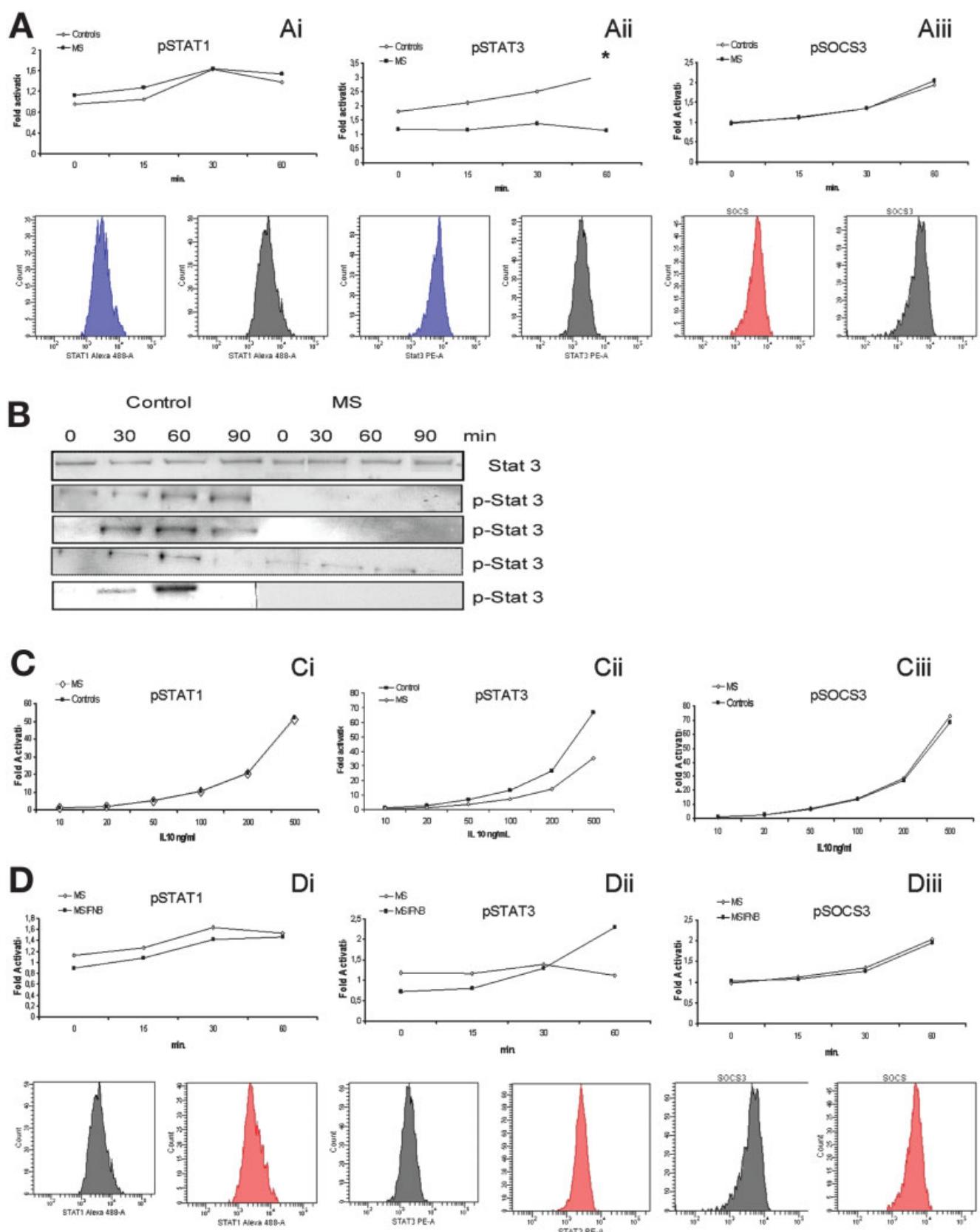


Figure 4. IL-10 signaling response. Kinase activity time courses were assessed by measuring Stat-1, Stat-3 and Socs-3 phosphorylation after stimulating PBMC from controls ($n = 6$) and MS patients ($n = 6$) in vitro with IL-10 (20 ng/mL) by flow cytometry and Western blot. (A) Kinase activity time courses in controls and MS patients: (Ai) pStat-1, (Aii) pStat-3, and (Aiii) pSocs-3 phosphorylation, at 0, 10, 30 and 60 min post stimulation with IL-10. Phosphorylation levels are plotted as the mean relative activation normalized to the activity of the unstimulated control at zero minutes (fold activation). Representative histograms of every phosphoprotein at 60 min are shown below each graph for controls (left) and MS (right). (B) Western blot analysis of the kinetics of Stat-3 phosphorylation after stimulation of PBMC from MS patients and HC with IL-10. Representative phospho-Stat-3 (pStat-3) stains are shown from four controls (left) and four untreated MS patients (right). Stat-3 (total protein, first row) is shown for load control from one control and one patient. (C) Dose-response curve for IL-10 signaling pathway: PBMC were stimulated with increasing concentrations of IL-10 (10, 20, 50, 200 and 500 ng/mL) and phosphorylation activity of Stat-1 (Ci), Stat-3 (Cii) and Socs-3 (Ciii) was analyzed 30 min after stimulation. (D) Kinase activity time courses in INF- β -treated ($n = 8$) and untreated MS patients ($n = 6$): (Di) pStat-1, (Dii) pStat-3, and (Diii) pSocs-3 levels at 0, 10, 30 and 60 min post stimulation with IL-10. Representative histograms of every phosphoprotein at 60 min are shown below each graph for controls (left) and MS (right). Differences between each pair of curves was evaluated by two-way ANOVA; * indicates significant differences ($p < 0.05$) along the curve.

differences in the composition of populations taken from the MS and HC groups, we found no differences in the CD4 $^{+}$ phenotype assessed by cytometry between MS and HC patients at the baseline. Because we did not measure the phenotypic characteristics of the different subpopulations of T cells and the levels of IL-10RB, and we did not sort for CD45RA cells, we could not rule out some other mechanisms explaining the poor response to IL-10 in MS patients.

Our findings suggest that the critical step impairing the response to IL-10 in MS patients is the lack of phosphorylation of Stat-3. Stat-3 activation is the main mediator of the IL-10 response [28]. Suppression of Stat-3 activation inhibits the suppression of MHC class II expression mediated by IL-10 [31]. Because antigen presentation is a critical step in the generation of autoimmune responses, the failure of IL-10 in modulating this process might predispose to the generation of an autoimmune response. In addition, Stat-3 phosphorylation in macrophages is induced after vagus nerve stimulation through acetylcholine receptor signaling [32]. Thus, the impairment in the neuromodulatory activity of the parasympathetic system in patients with MS [33, 34] might prevent the activation of the IL-10 pathway in patients with MS. Finally, the lack of Stat-3 phosphorylation might impair the negative feedback of the constitutive high levels of Socs-3 that repress IL-10. However, the complexity of the regulatory network involved in IL-10 signaling will require further experiments to clarify the mechanistic basis of this finding.

Because T cells can be considered as minifactories that might release the right combinations of cytokines in the right place and time, and they are able to sense the tissue environment, they seem to be an ideal candidate for immunotherapy. In this respect, Tr1 cells might be a good candidate for cell immunotherapy for autoimmune diseases since they can be obtained in humans by stimulating CD4 $^{+}$ cells with different protocols. Moreover, their mechanism of action, mainly based on IL-10 secretion, is well enough defined in order to monitor the

response to therapy. Another advantage of using these cells is that the target antigen does not need to be known, as is indeed the case in most autoimmune diseases. However, our results and previous studies suggest that Tr1 function and IL-10R signaling are impaired in patients. The IL-10R signaling impairment would conceptually be expected to limit therapies that aim to merely increase IL-10 levels, and for this reason, Tr1 cell immunotherapy is not immediately advisable if such impairment is not reverted.

The lack of response to IL-10 in MS patients might be due to the impairment of IL-10 signaling, including the presence of increased levels of Socs-3 and the poorer phosphorylation of Stat-3 after IL-10 stimulation. The beneficial effects of IFN- β therapy might be due to the partial recovery of the response of CD4 $^{+}$ cells to IL-10. However, results from the study by Astier *et al.* [21] and

Table 2. Clinical characteristics of MS patients and HC

	MS	HC
n	41	20
Sex (male/female)	12/29	6/14
Age (years)	38.15 + 11.32	41.8 + 13.2
Disease duration (years)	8.93 + 8.64	–
EDSS ^{a)}	3.1 + 2.2 (0–7)	–
Disease subtype:		
RRMS ^{b)}	28	–
SPMS ^{c)}	6	–
PRMS ^{d)}	3	–
PPMS ^{e)}	4	–
IFN- β therapy (yes/no)	14/21	–

^{a)} EDSS: expanded disability status score

^{b)} RRMS: relapsing-remitting MS

^{c)} SPMS: secondary-progressive MS

^{d)} PRMS: progressive-relapsing MS

^{e)} PPMS: primary-progressive MS.

Table 3. Comparison between the three anti-CD46 antibodies in the induction of Tr1 cells^{a)}

Antibody	CD25 % of cells		CD45RO % of cells		iIL-10 % of cells		IL-10 pg/mL	
	Day 0	Day 3	Day 0	Day 3	Day 0	Day 3	Day 0	Day 3
GB24 ^{b)}	19.9	67.5	31.0	61.0	0.39	27.2	350	2731
Tra-2-10 ^{b)}	11.0	92.0	22.0	54.0	1.4	32	ND ^{d)}	915
J4-48 ^{c)}	13.0	80.5	33.5	61.0	0.94	50.0	ND ^{d)}	6561

^{a)} We found that each of the three antibodies tested efficiently induced a Tr1 phenotype in CD4⁺ cells from HC after 3 days of activation. The results show the change in the percentage of cells expressing markers of activation (either CD25 or CD45RO) or IL-10 [either intracellular (iIL-10) or secreted IL-10] from day 0 to day 3 after activation with each antibody.

^{b)} Generously provided by Drs. Kemper and Atkinson, Washington University.

^{c)} Purchased from Research Diagnostic Inc., US (catalogue number RDI-CBL488).

^{d)} ND: not done.

our study suggest that IFN- β is unable to recover Tr1 function. However, recent studies showed that a combination of dexamethasone and vitamin D3, a regimen that induces Tr1 cells *in vitro*, was able to revert the resistance to corticosteroid therapy in patients with asthma, and restoring levels of IL-10 [35], indicating that Tr1 dysfunction might be reversible, at least in patients with asthma. It still remains unclear whether IL-10R signaling and Tr1 dysfunction is either a primary or secondary phenomenon, as it has been suggested that autoimmune diseases might arise because of the deficiencies in Treg function [36]. Alternatively, during autoimmune diseases, the Treg activity may become exhausted. In either case, the reconstitution of Treg function in patients with autoimmune disease might improve the disease course. Further studies will be required to assess whether adoptive transfer of Tr1 cells in combination with immunomodulatory therapies may be a useful treatment for MS, as well as for other autoimmune diseases.

Methods and patients

Subjects

We studied 41 patients with MS [37] and 21 sex- and age-matched HC, all of whom provided their informed consent. The clinical data of the MS patients is shown in Table 2. The number of individuals used for each experiment is described in the Results section and in each figure legend. For the Tr1 induction experiments, patients were not receiving any immunomodulatory or immunosuppressive drug. This work was approved by the Ethical Committee of the University of Navarra and informed consent was obtained from all participating subjects.

Media and antibodies

Cells were maintained in RPMI medium with 10% heat-inactivated fetal calf serum and 200 mM L-glutamine, in the

presence or absence of recombinant human IL-2 (BioSource International). We assessed three different anti-CD46 antibodies: mAb GB24, mAb TRA-2-10 (both of them generously provided by C. Kemper and J. Atkinson, University of Washington), and the commercial anti-CD46 mAb (RDI-CBL488 from Research Diagnostics Inc., US). We found similar activity for each of these antibodies when analyzing the generation of CD46-induced Tr1-like cells (Table 3). The mAb against human CD3 (HIT3a) and CD28 (CD28.2), the neutralizing mAb to human IL-10 (JES3-9D7), IL-2 (MQ1-17H12), IL-12 (C8.6), the mAb against human CD4 (RPA-T4; PerCP-conjugated), CD25 (2A3; FITC-conjugated), CD45RA (HI100), CD45RO (UCHL1; PE-conjugated), IL-10 (PE-conjugated), and IFN- γ (PE-conjugated) were all obtained from Becton Dickinson (BD, San Diego, CA). The antibodies against Stat-3 and phospho-Stat-3 were obtained from Santa Cruz (Santa Cruz Biotechnology, USA), while that raised against actin was purchased from Sigma (Germany).

CD46-induced Tr1 cell stimulation

CD4⁺ lymphocytes were purified from whole blood by separating mononuclear cells with Ficoll (Amersham) and then applying negative selection using the Miltenyi CD4⁺ T cell isolation kit (Miltenyi Biotec, Germany). Only samples in which the selection process reached >95% CD4⁺ cells were used. *In vitro* primary stimulation was carried out overnight in 96-well culture plates coated with anti-CD46 (5 μ g/mL), anti-CD3 (10 μ g/mL), anti-CD28 (5 μ g/mL) and anti-IL-12 (5 μ g/mL) antibodies as described in Kemper *et al.* [14]. CD4⁺ cells (2×10^5 cells per well) were cultured for 3–20 days. Subsequently, the cells were washed and expanded for another 6 days in medium supplemented with 40 U/mL human IL-2 (R&D Systems). On the 7th day, the cells were subjected to secondary stimulation with anti-CD3 and anti-CD28 but not with anti-CD46 antibodies. All experiments were carried out at least three times, and each procedure for activation was analyzed in triplicate.

Suppression assays

CD4⁺ cell stimulation was carried out in 96-well culture plates coated with anti-CD3 (10 μ g/mL) and anti-CD28 (10 μ g/mL)

antibodies. CD4⁺ T cells (2×10^5 per well) were incubated in triplicate in the presence of CD46-induced Tr1 culture supernatants from HC, which contain high levels of IL-10 (5580–7533 pg/mL), rIL-10 (20 ng/mL; R&D Systems) and/or anti-IL-10 antibodies (BD Biosciences, San Jose, CA). The concentration of Tr1 supernatant was adjusted to obtain the same concentration as rIL-10 (20 ng/mL). After 7 days, 0.5 mCi of [³H]thymidine was added to each well, and 5 h later, the cells were harvested and the thymidine incorporated into the cells in each well was determined by scintillation counting. The suppression index was determined as cpm incorporated into culture with Tr1 cell culture supernatant/cpm incorporated into culture with media only.

IL-10 stimulation assays

PBMC (2×10^5 cells/well) were stimulated with IL-10 (20 ng/mL; R&D Systems) in 96-well culture plates for up to 24 h. Experiments were repeated at least three times, and each experimental condition was analyzed in triplicate.

Flow cytometry analysis

Cells were phenotyped by three-color flow cytometry (FACScan) according to the expression of CD25, CD45, CD46, CTLA4 and the intracellular cytokines iIL-10 and iIFN- γ . For intracellular cytokine staining, cells (2×10^5 cells/well) were activated with immobilized anti-CD3 antibodies over 24 or 72 h. Brefeldin A was added for the last 4–8 h in culture before the cells were fixed (Fix and Perm; Caltag), stained with intracellular antibodies (anti-iIL-10-PE or anti-iIFN- γ -PE) and analyzed by flow cytometry. The phenotypic analysis of PBMC before CD4⁺ cell purification (day 0) from the HC and MS patients studied is shown in Table 1.

Phosphorylation analysis by flow cytometry

PBMC were incubated at 37°C in RPMI 1640 at 10^6 cells/mL and stimulated at different times (15, 30, 60 min) or left unstimulated with 20 ng/mL human recombinant IL-10 (BD Biosciences, San Jose, CA). For the dose-response experiments, the cells were stimulated with increasing doses of rIL-10 (10, 20, 50, 100, 200, 500 ng/mL). Phosphorylated Stat-1, Stat-3 and Socs-3 were detected by flow cytometry as described by He *et al.* [38]. Briefly, the cells were fixed with paraformaldehyde, permeabilized with methanol and stained with PE-conjugated anti-pStat-1, Alexa-Fluor 488-conjugated anti-pStat-3 (BD Biosciences, San Jose, CA) and APC-conjugated anti-Socs-3 antibody (Santa Cruz Biotechnology) and then analyzed with a flow cytometer. Phosphorylation increase is plotted as the mean relative activation of two samples normalized to the activity of the unstimulated control at zero minutes.

ELISA

We assessed the secretion of IL-10 and IFN- γ in the supernatants of CD4⁺ cell cultures by ELISA (BD OpteiaTM ELISA kit), following the manufacturer's instructions.

Real-time quantitative PCR

PBMC were isolated using Ficoll-Paque (Pharmacia Biotech) and were immediately submerged in RNAlater RNA Stabilization Reagent (Qiagen) to preserve gene expression patterns. Total RNA was isolated using the RNeasy Mini Kit (Qiagen). During RNA purification, DNA was removed with a DNase treatment using the RNase-Free DNase Set (Qiagen). Synthesis of cDNA from total RNA was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems). Primer sequences and target-specific fluorescence-labeled TaqMan probes were purchased from Applied Biosystems (TaqMan Gene Expression Assays). Quantitative real-time PCR was performed with the DNA Engine Opticon2 (MJ Research). Each sample was run in triplicate, and in each plate the target and the endogenous control gene (GAPDH) were amplified and placed in different wells. Cycle threshold (c(t)) values were acquired with Opticon Monitor 2.01 software (MJ Research). The normalized gene expression was calculated using the Q-Gene software application [39]. The mean normalized expression (MNE) was given by calculating the average c(t) values of the triplicate of the target, and of the reference.

Western blot analysis

Western blots were performed as described previously [40] and the membranes were probed with both the corresponding anti-Stat-3 and anti-phosphorylated Stat-3 antibodies. Equal loading of gels and the specificity of the effects observed was demonstrated by hybridizing membranes with antibodies specific for Actin and Stat-3.

Statistical analysis

The differences between groups were assessed with the Mann-Whitney U test. Differences over time were assessed using the Friedman test and two-way analysis of variance (ANOVA). The significance of the difference between each pair of flow cytometry curves was calculated by two-way ANOVA. Because real-time PCR measurements are highly skewed, we performed a logarithmic transformation for the treatment of non-normality. Normality was assessed with the Kolmogorov-Smirnov test, and all statistical tests were performed using the SPSS 11.0 software.

Acknowledgements: We would like to thank Drs. Atkinson and Kemper, from the Division of Rheumatology, Washington University School of Medicine for generously providing the anti-CD46 antibodies. This work was supported by the University of Navarra Research Program (PIUNA), the Spanish Ministry of Health (FIS PI051201) and the Fundacion Uriach to P.V. J.S. was a fellow of the Spanish Ministry of Health (FIS CM#05/00222).

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

- 1 Diaz-Villoslada, P., Shih, A., Shao, L., Genain, C. P. and Hauser, S. L., Autoreactivity to myelin antigens: Myelin/oligodendrocyte glycoprotein is a prevalent autoantigen. *J. Neuroimmunol.* 1999. **99:** 36–43.
- 2 Villoslada, P., Abel, K., Heald, N., Goertsches, R., Hauser, S. L. and Genain, C. P., Frequency, heterogeneity and encephalitogenicity of T cells specific for myelin oligodendrocyte glycoprotein in naïve outbred primates. *Eur. J. Immunol.* 2001. **31:** 2942–2950.
- 3 't Hart, B. A., van Meurs, M., Brok, H. P., Massacesi, L., Bauer, J., Boon, L., Bontrop, R. E. and Laman, J. D., A new primate model for multiple sclerosis in the common marmoset. *Immunol. Today* 2000. **21:** 290–297.
- 4 Zou, W., Regulatory T cells, tumour immunity and immunotherapy. *Nat. Rev. Immunol.* 2006. **6:** 295–307.
- 5 Bluestone, J. A., Regulatory T-cell therapy: Is it ready for the clinic? *Nat. Rev. Immunol.* 2005. **5:** 343–349.
- 6 Battaglia, M., Gregori, S., Bacchetta, R. and Roncarolo, M. G., Tr1 cells: From discovery to their clinical application. *Semin. Immunol.* 2006. **18:** 120–127.
- 7 Meiffren, G., Flacher, M., Azocar, O., Rabourdin-Combe, C. and Faure, M., Cutting Edge: Abortive proliferation of CD46-induced Tr1-like cells due to a defective Akt/Survivin signaling pathway. *J. Immunol.* 2006. **177:** 4957–4961.
- 8 Levings, M. K., Sangregorio, R., Sartirana, C., Moschin, A. L., Battaglia, M., Orban, P. C. and Roncarolo, M. G., Human CD25⁺CD4⁺ T suppressor cell clones produce transforming growth factor beta, but not interleukin 10, and are distinct from type 1 T regulatory cells. *J. Exp. Med.* 2002. **196:** 1335–1346.
- 9 Wildbaum, G., Netzer, N. and Karin, N., Tr1 cell-dependent active tolerance blunts the pathogenic effects of determinant spreading. *J. Clin. Invest.* 2002. **110:** 701–710.
- 10 Gianfrani, C., Levings, M. K., Sartirana, C., Mazzarella, G., Barba, G., Zanzi, D., Camarca, A. et al., Gliadin-specific type 1 regulatory T cells from the intestinal mucosa of treated celiac patients inhibit pathogenic T cells. *J. Immunol.* 2006. **177:** 4178–4186.
- 11 Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J. E. and Roncarolo, M. G., A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997. **389:** 737–742.
- 12 Levings, M. K., Sangregorio, R., Galbiati, F., Squadrone, S., de Waal, M. R. and Roncarolo, M. G., IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells. *J. Immunol.* 2001. **166:** 5530–5539.
- 13 Barrat, F. J., Cua, D. J., Boonstra, A., Richards, D. F., Crain, C., Savelkoul, H. F., Waal-Malefyt, R. et al., In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J. Exp. Med.* 2002. **195:** 603–616.
- 14 Kemper, C., Chan, A. C., Green, J. M., Brett, K. A., Murphy, K. M. and Atkinson, J. P., Activation of human CD4⁺ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. *Nature* 2003. **421:** 388–392.
- 15 Barchet, W., Price, J. D., Celli, M., Colonna, M., MacMillan, S. K., Cobb, J. P., Thompson, P. A. et al., Complement-induced regulatory T cells suppress T-cell responses but allow for dendritic-cell maturation. *Blood* 2006. **107:** 1497–1504.
- 16 Ito, T., Wang, Y. H., Duramad, O., Hanabuchi, S., Perng, O. A., Gilliet, M., Qin, F. X. and Liu, Y. J., OX40 ligand shuts down IL-10-producing regulatory T cells. *Proc. Natl. Acad. Sci. USA* 2006. **103:** 13138–13143.
- 17 Steinman, L., Multiple sclerosis: A two-stage disease. *Nat. Immunol.* 2001. **2:** 762–764.
- 18 Sospedra, M. and Martin, R., Immunology of multiple sclerosis. *Annu. Rev. Immunol.* 2005. **23:** 683–747.
- 19 Antel, J. P., Richman, D. P., Medoff, M. E. and Arnason, B. G., Lymphocyte function and the role of regulator cells in multiple sclerosis. *Neurology* 1978. **28:** 106–110.
- 20 Viglietta, V., Baecher-Allan, C., Weiner, H. L. and Hafler, D. A., Loss of functional suppression by CD4⁺CD25⁺ regulatory T cells in patients with multiple sclerosis. *J. Exp. Med.* 2004. **199:** 971–979.
- 21 Astier, A. L., Meiffren, G., Freeman, S. and Hafler, D. A., Alterations in CD46-mediated Tr1 regulatory T cells in patients with multiple sclerosis. *J. Clin. Invest.* 2006. **116:** 3252–3257.
- 22 Soldan, S. S., Alvarez Retuerto, A. I., Sicotte, N. L. and Voskuhl, R. R., Dysregulation of IL-10 and IL-12p40 in secondary progressive multiple sclerosis. *J. Neuroimmunol.* 2004. **146:** 209–215.
- 23 Huang, W. X., Huang, P., Link, H. and Hillert, J., Cytokine analysis in multiple sclerosis by competitive RT-PCR: A decreased expression of IL-10 and an increased expression of TNF-alpha in chronic progression. *Mult. Scler.* 1999. **5:** 342–348.
- 24 Navikas, V., Link, J., Palasik, W., Soderstrom, M., Fredrikson, S., Olsson, T. and Link, H., Increased mRNA expression of IL-10 in mononuclear cells in multiple sclerosis and optic neuritis. *Scand. J. Immunol.* 1995. **41:** 171–178.
- 25 Nakashima, I., Fujihara, K., Misu, T., Okita, N., Takase, S. and Itoyama, Y., Significant correlation between IL-10 levels and IgG indices in the cerebrospinal fluid of patients with multiple sclerosis. *J. Neuroimmunol.* 2000. **111:** 64–67.
- 26 Yin, L., Yu, M., Edling, A. E., Kawczak, J. A., Mathisen, P. M., Nanavati, T., Johnson, J. M. and Tuohy, V. K., Pre-emptive targeting of the epitope spreading cascade with genetically modified regulatory T cells during autoimmune demyelinating disease. *J. Immunol.* 2001. **167:** 6105–6112.
- 27 Riley-Vargas, R. C., Gill, D. B., Kemper, C., Liszewski, M. K. and Atkinson, J. P., CD46: Expanding beyond complement regulation. *Trends Immunol.* 2004. **25:** 496–503.
- 28 Moore, K. W., de Waal, M. R., Coffman, R. L. and O'Garra, A., Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* 2001. **19:** 683–765.
- 29 Pestka, S., Krause, C. D., Sarkar, D., Walter, M. R., Shi, Y. and Fisher, P. B., Interleukin-10 and related cytokines and receptors. *Annu. Rev. Immunol.* 2004. **22:** 929–979.
- 30 Kozovska, M. E., Hong, J., Zang, Y. C., Li, S., Rivera, V. M., Killian, J. M. and Zhang, J. Z., Interferon beta induces T-helper 2 immune deviation in MS. *Neurology* 1999. **53:** 1692–1697.
- 31 Williams, L., Bradley, L., Smith, A. and Foxwell, B., Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. *J. Immunol.* 2004. **172:** 567–576.
- 32 de Jonge, W. J., van der Zanden, E. P., The, F. O., Bijlsma, M. F., van Westerloo, D. J., Bennink, R. J., Berthoud, H. R. et al., Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. *Nat. Immunol.* 2005. **6:** 844–851.
- 33 Giorelli, M., Livrea, P. and Trojano, M., Post-receptorial mechanisms underlie functional disregulation of beta2-adrenergic receptors in lymphocytes from multiple sclerosis patients. *J. Neuroimmunol.* 2004. **155:** 143–149.
- 34 Cosentino, M., Zaffaroni, M., Giorelli, M., Marino, F., Ferrari, M., Bombelli, R., Rasini, E. et al., Adrenergic mechanisms in multiple sclerosis: The neuro-immune connection? *Trends Pharmacol. Sci.* 2004. **25:** 350–351; author reply 351–352.
- 35 Xystrakis, E., Kusumakar, S., Boswell, S., Peek, E., Urry, Z., Richards, D. F., Adikibi, T. et al., Reversing the defective induction of IL-10-secreting regulatory T cells in glucocorticoid-resistant asthma patients. *J. Clin. Invest.* 2006. **116:** 146–155.
- 36 Baecher-Allan, C. and Hafler, D. A., Suppressor T cells in human diseases. *J. Exp. Med.* 2004. **200:** 273–276.
- 37 McDonald, W. I., Compston, A., Edan, G., Goodkin, D., Hartung, H. P., Lublin, F. D., McFarland, H. F. et al., Recommended diagnostic criteria for multiple sclerosis: Guidelines from the International Panel on the diagnosis of multiple sclerosis. *Ann. Neurol.* 2001. **50:** 121–127.
- 38 He, X. S., Ji, X., Hale, M. B., Cheung, R., Ahmed, A., Guo, Y., Nolan, G. P. et al., Global transcriptional response to interferon is a determinant of HCV treatment outcome and is modified by race. *Hepatology* 2006. **44:** 352–359.
- 39 Muller, P. Y., Janovjak, H., Miserez, A. R. and Dobbie, Z., Processing of gene expression data generated by quantitative real-time RT-PCR. *Biotechniques* 2002. **32:** 1372–1374.
- 40 Moreno, B., Hevia, H., Santamaria, M., Sepulcre, J., Munoz, J., Garcia-Trevijano, E. R., Berasain, C. et al., Methylthioadenosine reverses brain autoimmune disease. *Ann. Neurol.* 2006. **60:** 323–334.