Beta Interferon restricts the inflammatory potential of $CD4^+$ cells through the boost of the Th2 phenotype, the inhibition of Th17 response and the prevalence of naturally occurring T regulatory cells

Francisco M. Martín-Saavedra, Coral González-García, Beatriz Bravo, and Sara Ballester*
Gene Regulation Unit, Microbiology National Center, Instituto de Salud Carlos III, Madrid, Spain

*: Corresponding author. Tel.: +34 91 8223922; fax: +34 91 8097919.
e-mail address: sballes@isciii.es

Abbreviations used in this paper: MS, multiple sclerosis; MBP, myelin basic protein; MBP-EAE, experimental autoimmune encephalomyelitis induced by MBP; p.i., post-immunization; nTreg, natural T regulatory cells; Foxp3, forkhead box P3; GITR, glucocorticoid-induced TNF-related receptor; LNC, lymph node cells.

Beta-interferon (IFN-β) is a valuable therapy for Multiple Sclerosis (MS) which is also effective in the animal model of experimental autoimmune encephalomyelitis (EAE). However, the accurate mechanisms to explain its anti-inflammatory activity in the disease are not fully revealed. Available data support that T lymphocytes are among the main cell targets of IFN-β. We have found that *in vitro* anti-CD3 stimulation of uncommitted murine *naïve* T cells under IFN-β treatment results in skewing the T cell differentiation process towards the T2 phenotype, in a prevention from apoptosis of naturally occurring $CD4^+$ T regulatory cells (nTreg) in correlation with an increase in Bcl-x_L expression, and in a decrease of IL-17 expression. Elimination of nTreg from the primary culture of *naïve* $CD4^+$ cells abolished the down-regulation of IL-17 driven by IFN-β, what suggests the interaction between Th17 and nTreg subsets. Experiments in EAE induced in SJL mice, showed *in vivo* evidence for the accumulation of spleen $CD4^+CD25^+GITR^+Foxp3^+$ cells after IFN-β treatment. On the other hand, treated animals showed a striking decrease of IL-17 expression by peripheral $CD4^+$ cells (Th17) and MBP-specific spinal cord cells. Both the *in vivo* and *in vitro* results point out new targets through which IFN-β could exert its therapeutic action.

Keywords: IFN-β, EAE, IL-4, IL-17, Foxp3, nTreg

1. Introduction

The stability of the immune homeostasis depends on the adequate balance between the initiation and the restraint of the immune response, where $CD4^+$ T cells [T helper (Th)] play an essential role as mediators. Once the equilibrium is broken, distortions of the homeostasis may lead to chronic inflammation and autoimmunity. MS and its animal model, EAE, are considered as T cell-mediated autoimmune processes, where pro-inflammatory damage in the central nervous system has traditionally been ascribed to the establishment of a strong Th1 response leaded by IFN-γ. However, the loss of IFN-γ signalling in mice deficient in IFN-γ or in the IFN-γ receptor does not confer any resistance to autoimmunity; moreover, such animals are even more susceptible (Krakowski and Owens, 1996; Tran *et al.*, 2000; Willenborg *et al.*, 1996; Willenborg *et al.*, 1999). Those findings suggested the existence of an additional T cell subset, distinct from IFN-γ producing Th1 cells, capable of inducing tissue inflammation and autoimmunity. This led to the identification of IL-17 producing cells (Th17), a $CD4^+$ subset that has not only proved a closely involvement in the pathogenesis of murine autoimmune diseases as EAE (Batten *et al.*, 2006; Fitzgerald *et al.*, 2007; Gutcher *et al.*, 2006; Hofstetter *et al.*, 2005; Komiyama *et al.*, 2006; Kroenke and Segal, 2007; Langrish *et al.*, 2005; Park *et al.*, 2005) or rat adjuvant-induced arthritis (Bush *et al.*, 2002), but also shown an active role on human autoimmune disorders (Moseley *et al.*, 2003). Another $CD4^+$ subset that plays a critical role in the development of autoimmune diseases is represented by T regulatory cells (Treg), distinguished by the expression of the forkhead box P3 (Foxp3) transcription factor (Fontenot *et al.*, 2003) and capable of controlling T cell responses both *in vitro* and *in vivo* (Baecher-Allan and Hafler, 2006; Dieckmann *et al.*, 2001; Jonuleit *et al.*, 2001; Thornton and Shevach, 1998; Wing *et al.*, 2003). Data in favor of an involvement of Treg cells in EAE has been widely reported (Fernandez-Martin *et al.*, 2006; Kohm *et al.*, 2002; Liu *et al.*, 2006; Mann *et al.*, 2007; McGeachy *et al.*, 2005). Attending to their origin, Treg cells are usually classified as naturally occurring (nTreg) when they proceed directly from a thymic precursor (Sakaguchi, 2000), or adaptive (aTreg), after they differentiate from peripheral T helper precursors through the action of cytokines as TGF-β (Faria and Weiner, 2006). Evidences like the mutually exclusive skewing of murine uncommitted (*naïve*) $CD4^+$ T cells towards Th17 or aTreg points to a close interaction between the activity of both novel characterized T $CD4^+$ subsets (Bettelli *et al.*, 2006; Mangan *et al.*, 2006; Veldhoen *et al.*, 2006).

The effect of some immunomodulatory drugs, as beta-interferon (IFN-β), has proved to exert a notable therapeutic effect in MS (Comi *et al.*, 2001; Jacobs *et al.*, 1996; PRIMS and sclerosis., 1998; The IFNbeta Multiple Sclerosis Study Group, 1995). As it has been reported, the treatment of EAE with murine IFN-β delays the emergence of the clinical symptoms and reduces the severity of the disease (Floris *et al.*, 2002; Jaini *et al.*, 2006; Martin-Saavedra *et al.*, 2007; Tuohy *et al.*, 2000; Wender *et al.*, 2001; Yasuda *et al.*, 1999; Yu *et al.*, 1996). Several of these studies attribute some protective properties of IFN-β to a control of the T cell activity in the disease. However, the precise interactions of IFN-β with the biology of the T cell populations to achieve the prevention of EAE remain unsolved. We previously have proved that IFN-β treatment of MBP-EAE promotes the inhibition of NFκB activation and IL-17 expression, simultaneously to enhance the anti-inflammatory response via the increase of Stat6 and IL-4 activity (Martin-Saavedra *et al.*, 2007). Here, we expose evidences of that the *in vitro* treatment of $CD4^+$ T cells with IFN-β preserves nTreg subset from cell death related to an increase in Bcl-x_L expression, exerts a negative control of IL-17 expression, and unbalances the Th1/Th2 polarization process of naïve T cells towards the Th2 lineage. We also have found *in vivo* results in MBP-EAE in agreement with such IFN-β outcomes on $CD4^+$ cells with consequences for IL-17 expression in the target tissue.

2. Materials and methods

2.1. Animals, EAE induction and IFN-β treatment

All experiments were conducted according to the institutional ethical and safety guidelines. SJL (H-2^s) and C3H mice were used for the EAE experiments and to obtain T cells for *in vitro* assays, respectively. Both strains were purchased from Charles River Spain. Induction of EAE and IFN-β treatment were performed as previously described (Martin-Saavedra *et al.*, 2007).

2.2. Cell isolation, cell lines and culture

For phenotype polarization and proliferation experiments spleen cells from C3H mice were used. For analysis of *in vivo* IFNβ effects, lymph nodes or spleens were removed from EAE animals on day 9 p.i. Spleen cells were treated with Red Blood Cell Lysing buffer (Sigma) according manufacturer's instructions to remove erythrocytes. All samples were homogenized-through a 30-μm nylon mesh (Millipore). Cells from each group of EAE animals were pooled before processing. $CD4^+$ or $CD8^+$ T cells were magnetically sorted (Miltenyi Biotech) to 90-95 % purity. Primary T cells were washed and suspended in Click's medium (Peck and Bach, 1973) before *in vitro* treatment. IFN-β (ICN) was used for *in vitro* treatments at 1000 or 2500 U/ml, indicated in each case. For CNS cell isolation the animals were sacrificed and perfused through the left ventricle with 15–20 ml of PBS to wash out leukocytes present within the blood vessels. Spinal cord were removed and pooled from each group of EAE animals. Tissue was carefully homogenized through a 100-μm pore size strainer before enzymatic digestion for 60 min at 37ºC in EBSS (Gibco) with collagenase IV (2 mg/ml; Sigma-Aldrich). After 10 min of centrifugation the pellet obtained was dissolved in 30% Percoll (Amersham). Subsequently, the 30% Percoll homogenate mix was layered over 80% Percoll. Leukocytes were collected from the 30%-80% interface after centrifugation at 3.000 rpm for 25 min at room temperature. Cells were stimulated with PMA and Ionomycin during 18 h before RNA extraction and IL-17 mRNA analysis. For MBP-stimulated IL-17 expression in total spinal cord, cells were cultured 18 h in the presence of MBP (10 μg/ml) without percoll gradient purification.

2.3. T cell phenotype polarization

Freshly isolated spleen $CD4^+$ T cells were *in vitro* stimulated by coated-plate anti-CD3 (YCD3-1, 50 μg/ml) (Portolés *et al.*, 1989) in the presence of 10 ng/ml of IL-12 (Prepotech) and 25 μg/ml of anti-IL-4 (11B11; ATCC HB188) for T1 phenotype induction; or 500 u/ml of IL-4 (Prepotech) and 25 μg/ml of anti-IFN-γ (R46A2; ATCC HB 170) for T2 polarization. For T0 samples only anti-CD3 was used as supplement to the medium. After 24 h, all cultures were supplemented with 50 u/ml IL-2 (Prepotech). For cultures longer than 4 days, on the fourth day after stimulation, cells were expanded in the absence of anti-CD3 antibody but in the continued presence of cytokines and antibodies. After a total of 7 days of culture, cells were harvested, extensively washed, and re-stimulated as indicated for each experiment. T1 or T2 phenotype establishment was checked to every assay by measurement of mRNA expression and protein production of IFN-γ and IL-4 after the second round of anti-CD3 stimulation.

2.4. T cell proliferation and suppressor assays

For CFSE staining, magnetically isolated $CD4^+$ T lymphocytes were incubated at $1x10^6$ cells/ml with 10μM of CFSE (*Cell Trace*, Invitrogen) in PBS containing 0.1% BSA. After 15 minutes at 37ºC, washing procedures were performed as advised by manufacturer previous to cell culture. For MTT assays, $2x10^5$ cells were split on plate-bound anti-CD3 p-96 microtiter wells. Each sample was assayed in triplicate, and cell growth was measured after 72 h of culture by the colorimetric assay as described in (Mosman, 1983) for 72h. For suppression assays, the proliferation stimulus was soluble anti-CD3 (20 μg/ml) plus spleen Mitomycin C (MMC)-treated spleen cells from C3H mice ($1x10^6$ cells/ml) as antigen presenting cells (APC) and *naïve* spleen $CD4^+$ cells were used as responders ($5x10^5$ cells/ml). A control for suppressor activity was supplied by $CD4^+CD25^+$ fraction of spleen cells. Subsequently, cells were exhaustively washed with Click's medium and set up in culture.

2.5. Surface and intracellular protein staining for flow cytometry analysis

After washing in staining buffer (PBS containing 0.5% BSA, 2mM EDTA, pH 7.2), $5x10^5$ cells were incubated for 15 minutes at 4ºC with saturating amounts of antibodies. FITC-anti-CD4, PE-anti-CD25, and APC-anti-GITR were purchased from Miltenyi Biotec; and APC-anti-CD4, PE-Cy7-anti-CD25 were from BD Pharmigen. For Foxp3 intracellular staining, cells were permeabilized and fixed with *Fix-Perm* buffer (eBioscience), and subsequently stained with PE-anti-Foxp3 (eBioscience), according to the manufacturer instructions.

2.6. Cell cycle assay

Primary $CD4^+$ cells ($2x10^6$/ml) were incubated with 70% PBS-Ethanol for 2 hours at 4ºC, washed with PBS, and incubated with permeabilization & DNA staining solution (0.1% Triton X-100, 200 μg/ml of DNAse-free RNAse A, 20 μg/ml of Propidium Iodide) for 20 minutes at room temperature.

2.7. Cell Sorting

$CD4^+$ T lymphocytes were magnetically separated and stained with FITC-anti-CD4 and APC-anti-CD25 (Miltenyi Biotec). 1μg/ml Propidium Iodide (Sigma) was added to discard non viable cells. T cells were sorted in *FACS Vantage SE* (Becton-Dickinson) and both fractions $CD4^+CD25^-$ and $CD4^+CD25^+$ were collected to 97-99% purity. Those fractions were cultured with Click's medium and exposed to the indicated conditions.

2.8. Annexin-V staining

$2x10^5$ cells were resuspended in 90 µl of Annexin Binding Buffer (10mM Hepes/NaOH pH7.4, 140mM NaCl, 2.5mM $CaCl_2$) and incubated with 10 µl of Annexin-V-FITC (Immunostep) for 15' at room temperature according to manufacturer's instructions.

2.9. Flow cytometry data management

Data were acquired on *FACSCanto* (Becton-Dickinson) or *FACSCalibur* (Becton-Dickinson) flow cytometers and analyzed by FACSDiva (Becton-Dickinson), Cell Quest Pro (Becton-Dickinson) and FlowJo (Tree star) softwares.

2.10. ELISA

For IL-4 detection, both 11B11 (ATCC HB188) and BVD6-24G2 (Becton-Dickinson) were used as detecting and capture anti-IL-4 antibodies, respectively. For IL-17 amount determinations, the Biolink mouse IL-17 ELISA system was applied. Supernatants of $2x10^5$ cells stimulated by plate-bound anti-CD3 for 48 h were tested in serial dilutions to IL content as previously described (Martin-Saavedra *et al.*, 2007).

2.11. Measurement of mRNA expression

Retrotranscription assay and quantitative real-time PCR conditions for IL-4, IFN-γ and IL-17 were as in (Martin-Saavedra *et al.*, 2007). Primers used for Foxp3 and Bcl-x_L amplification were: 5′-ACCACCTTCTGCTGCCACTG-3′, as Foxp3 forward, and 5′-TGCTGTCTT TCCTGGGTGTACC-3′, as Foxp3 reverse; and 5´-ACAGCAGCAGTTTGGATGC-3´ as Bcl-x_L forward, and 5´-AACTTGCAATCCGACTCACC-3´ as Bcl-x_L reverse. The PCR products quality was checked by a melting curve analysis. Each result was normalized by the housekeeping β-actin gene expression. Relative quantification of gene expression analysis was performed using the Pfaffl method (Pfaffl, 2001).

2.12. Statistics

Statistical analysis was performed with Graph Pad Prism version 4.02 (Graph Pad software, Inc). The t-test with Welch's correction was used for unpaired data. Statistical significance was established at $p<0.05$ (*) and $p<0.01$ (**).

3. Results

3.1. Influence of IFN-β on the T2 phenotype polarization process

Some of the therapeutic properties of IFN-β could be related to our previous findings, which showed that IFN-β treatment contributes to the strengthening of an anti-inflammatory response via the increase of IL-4 expression and Stat6 activity (Martin-Saavedra *et al.*, 2007). Since IL-4 is the trigger of the T2 cytokine profile, we wanted to know if IFN-β influences the T2 phenotype polarization process from *naïve* T lymphocytes.

Murine *naïve* spleen cells (Sc) were treated with anti-CD3 in the presence of IL-12 or IL-4 to promote polarization to phenotypes T1 and T2, respectively. The culture condition without specific polarizer pressure is referred as T0. The resulting populations are named as Sc1, Sc2 and Sc0 accordingly the culture condition. After seven days, cells were subjected to a second round of anti-CD3 stimulation to measure the expression levels of IL-4 mRNA. Irrespective of the polarization condition, cells exposed to IFN-β showed increased levels of IL-4 mRNA, while the IFN-γ mRNA expression profile was not significantly altered by the IFN-β treatment (Figure 1A).

This trend to T2 phenotype under IFN-β treatment could be due to a direct effect of IFN-β on the T cell population. Alternatively, it could be a secondary consequence resulting from an effect of IFN-β on any other spleen cell type. To explore these possibilities, we purified individually and co-cultured the $CD4^+$ and $CD8^+$ T cell subsets at the same ratio found in the spleen (7/3 respectively), under T0 polarization condition. In this case, the resulting population was named as Tm0. Similarly to Sc, after seven days of culture, Tm0 population showed a significant increase of IL-4 mRNA expression after had been exposed to IFN-β (Figure 1B). This increase of IL-4 mRNA correlated with higher protein production mediated by IFN-β, as it was verified by ELISA for IL-4 protein detection in the culture media. The IL-4 production detected in the Tm population can be ascribed to $CD4^+$ cells since IL-4 mRNA and protein levels were undetectable in a Tc0 population ($CD8^+$) irrespective of the presence of IFN-β (not shown).

3.2. IFN-β effect on $CD4^+$ cell activation

To investigate the proliferative ability of T cells defined under IFN-β treatment, we checked the response of $CD4^+$ and $CD8^+$ populations to the second round of anti-CD3 stimulation in the presence of IFN-β. Proliferation of the $CD8^+$ population (Tc0) was not affected by IFN-β. On the contrary, $CD4^+$ cells (Th0) treated with IFN-β showed a significant lower proliferative response than the untreated sample in a dose-dependent way (Figure 2A). The same difference between $CD4^+$ and $CD8^+$ cell types was observed for the phenotype definition conditions T1 and T2 (not shown). The IFN-β-mediated inhibition of the anti-CD3 proliferative response could be also detected in $CD4^+$ *naïve* cells. Freshly obtained $CD4^+$ cells were subjected to staining with CFSE probe and analyzed by flow cytometry. Results showed that the kinetics of cell division was delayed by a complete cell generation in the IFN-β treated population after 48h of TCR stimulation (Figure 2B). To know if this antiproliferative effect could be consequence of a cell cycle arrest mediated by IFN-β, we performed a cell cycle distribution assay by Propidium Iodide staining and flow cytometry analysis. Results showed that there was not significant difference between untreated and IFN-β-treated cultures in the cell percentages for each cycle phase (Figure 2C).

As $CD4^+$ proliferation is markedly influenced by the presence on membrane of the α chain constituent of the IL-2 receptor, CD25, we studied if the inhibition of $CD4^+$ proliferation by IFN-β was related to a down-regulation of this T cell activation marker. Unexpectedly, quantification by FACS of CD25 expression on the recently Th0 defined population showed a clear increase promoted by IFN-β in a dose-dependent way (Figure 2Di). To follow this difference between untreated and the IFN-β-treated cells throughout TCR activation, we quantified the presence of CD25 on cell membrane at different stages of the process. The initial up-regulation of CD25 expression promoted by anti-CD3 activation is not noticeably modified by IFN-β treatment. However, at later stages, while the levels of CD25 are progressively decreased in the control culture, cells in the IFN-β-treated population keep high levels of this surface marker (Figure 2Dii).

3.3. IFN-β effect on the survival of nTreg

We addressed if the IFN-β-mediated deficiency in $CD4^+$ proliferation was a transient or a permanent effect. The inhibition of the proliferative response of Th0 cells defined in the presence of IFN-β was detected even if the drug was absent during the second round of activation (Figure 3A), signifying that it is an enduring feature established in the cell population through IFN-β action.

It is well known that stimulation of $CD4^+$ cells with immobilized anti-CD3 in the absence of costimulation leads to an anergic state. Thus, such cell population fails in the proliferative response to a close secondary full stimulation of TCR, and exhibits a suppressor activity on $CD4^+$ *naïve* cell proliferation (Chai *et al.*, 1999; Chai and Lechler, 1997). To determine if these attributes are modified by IFN-β, cells stimulated by plate-coated anti-CD3 were exposed as soon as after 72 h to a re-challenge to soluble anti-CD3 in the presence of MMC pre-treated APC. The IFN-β treatment during the first anti-CD3 round did not change the lack of proliferative response to the second round of stimulation (Figure 3Bi). We also analyzed if IFN-β could modify the suppressor activity of the population resulting from immobilized anti-CD3 stimulus. For that, we set up co-cultures of *naïve* $CD4^+$ (responder cells) and cells stimulated by one anti-CD3 round (suppressor cells). A freshly obtained *naïve* $CD4^+$ population enriched in nTreg (> 70%) was used to test the adequate susceptibility of responder cells to a well categorized suppression activity. The proliferation of $CD4^+$ *naïve* cells in co-culture with the different suppressor populations was lower than in the control culture in all cases. Attending to samples in which cells pre-activated with plate bound anti-CD3 were used as suppressors, the remaining percentages of proliferation showed that the suppressor activity was increased by treatment with IFN-β during pre-activation (Figure 3Bii).

To find out if the suppression ability of the pre-activated populations can be assigned to Treg cells present in these samples, we analyzed the repression of proliferation when the starting population is depleted of nTreg cells. In conditions of partial depletion (by about 2/3) of the $CD4^+CD25^+$ subset, the difference between IFN-β treated and untreated cells is maintained, showing higher suppressor activity when IFN-β had been present during pre-activation. On the contrary, if $CD4^+CD25^+$ cells are completely depleted by FACS-sorting, the suppression ability is nearly abolished irrespective of the IFN-β presence (Figure 3Biii).

To address if these data could be related to a higher presence of nTreg cells in the total $CD4^+$ population when it is treated with the drug, we quantified the mRNA of the nTreg marker Foxp3 along pre-activation with anti-CD3. In spite of the fact that Foxp3 expression levels decreased along the time of culture whether IFN-β is added or not, the mRNA levels of this gene were clearly higher in the population treated with IFN-β than in the untreated culture at any time analyzed (Figure 4A). This result was corroborated by Foxp3 intracellular staining which showed that the number of $Foxp3^+$ cells severely decreased after 72 hours of stimulation with anti-CD3 at any condition assayed (Figure 4B). However, population exposed to IFN-β shows a less pronounced decrease in the number of $CD4^+Foxp3^+$ cells when compared with untreated sample (Figure 4B, upper). Similar results were observed for partial nTreg depletion from the starting population (Figure 4B, middle). Nevertheless, the complete elimination of $CD25^+$ cells revealed that no $Foxp3^+$ cells could be detected in the $CD4^+$ culture after 72 h of IFN-β *in vitro* treatment (Figure 4B, lower), excluding a possible role of IFN-β as promoter of a $CD4^+Foxp3^+$ adaptive subset.

The well known tendency to programmed death cell of nTreg subset prompted us to analyze a possible effect of IFN-β on nTreg apoptosis. AnnexinV-FITC staining of a pure nTreg population treated with IFN-β demonstrated that the high propensity to apoptosis of nTreg cells is significantly decreased by the drug treatment (Figure 5Ai). This result correlated with an increase in the expression of the anti-apoptotic protein $Bcl\text{-}x_L$ by the $CD4^+CD25^+$ population exposed to IFN-β (Figure 5Aii). The protective role against apoptosis was not derived from a general effect of IFN-β on $CD4^+$ cell apoptosis, as deduced by the result of AnnexinV-FITC analysis performed on total $CD4^+$. In this case, IFN-β did not lead to an overall decrease of the apoptotic rate, but even mediated an increase of the AnnexinV staining (Figure 5Bi) and a progressive drop in the percentage of cells with the size and complexity corresponding to viable lymphocytes (Figure 5Bii).

3.4. Influence of IFN-β on IL-17 levels expression

To know if the IFN-β effect on *in vitro* $CD4^+$ cell cultures has any consequence on the levels of proinflammatory effector cytokines, we analyzed the expression of IFN-γ and IL-17 by $CD4^+$ cells when IFN-β is present during the first round of anti-CD3 activation. The analysis was performed at days three and seven from the onset of TCR stimulation. At day seven, both cytokines were expressed at low-level, likely due to a cell state closer to resting than at day three. IFN-β did not influence the IFN-γ mRNA production (Figure 6A). On the contrary, IL-17 mRNA and protein expression was dramatically

decreased as a result of IFN-β *in vitro* treatment (Figure 6B and 6C). A direct correlation with the presence of nTreg could be deduced by complete removal of $CD25^+$ cells from the starting population. In this case, similar levels of IL-17 expression were found for IFN-β-treated and untreated cells (Figure 6D).

As we previously described (Martin-Saavedra *et al.*, 2007), IFN-β treatment of MBP-EAE in SJL mice promotes the down-modulation of IL-17 in total LNC (lymph node cells), concurring with a significant attenuation of the clinical score in the diseased animals, without effect on IFNγ mRNA levels. To find out if the IL-17 down-modulation is extensive to other peripheral organs, we analyzed the IL-17 mRNA levels in spleen cells from EAE animals, where we also found a notable decrease of IL17 transcripts driven by IFN-β *in vivo* treatment (Figure 7A). In addition, the number of spleen $CD4^+CD25^+$ cells was significantly higher in IFN-β treated EAE animals than in the untreated controls. Similar increase was found by an nTreg specific labelling performed by intracellular staining of $CD4^+$ cells with Foxp3 antibody simultaneous to surface staining for GITR and CD25 markers (Figure 7B). On the other hand, the IFNβ-mediated decrease in the IL-17 expression could be also detected in the CNS of EAE animals. This result was found for both total mononuclear cells and MBP recognizer cells isolated from spinal cord (Figure 7C).

4. Discussion

IFN-β is nowadays the main therapy used for MS. The widespread thinking is that the therapeutic function of IFN-β on the disease is achieved through its anti-inflammatory properties, but the accurate mechanisms for such beneficial role are not fully understood thus far. The results from the present study can shed some light on the action of IFN-β to control the inflammatory response directed by $CD4^+$ cells. Three noteworthy effects on $CD4^+$ T cells by *in vitro* treatment with IFN-β are described here: i) an unbalance in the T1/T2 polarization process of the *naïve* T cells in favor of the T2 phenotype, ii) a negative control on IL-17 expression, and iii) a protective role against apoptosis of nTreg cells.

Our results demonstrate that the presence of IFN-β throughout the process of definition of T phenotype leads to an increase of the IL-4 production (Figure 1). This finding is according to our previous data showing that IFN-β treatment of MBP-EAE can enhance the IL-4 expression in LNC. Both *in vivo* e *in vitro* IL-4 increase mediated by IFN-β can be related to the effect of mRNA stabilization of IL-4 transcripts displayed by the drug on the T cell clone D10.G4.1 (Martin-Saavedra *et al.*, 2007). Due to the potent role of IL-4 as inductor agent of the T2 phenotype, such increase could in turn cooperate to the T2 definition process.

Results concerning IL-17 are in agreement to our previous report showing a fall of IL-17 expression by total lymph node cells after *in vivo* IFN-β treatment (Martin-Saavedra *et al.*, 2007). Another recent report proposes that signalling of endogenous type I IFN increases the IL-27 production during the innate immune response through macrophages and dendritic cells with a negative influence on the IL-17 expression (Guo *et al.*, 2008). However, the results described here show that IFN-β reduces IL-17 expression even on isolated $CD4^+$ cells cultured *in vitro* (Figure 6), establishing that the IL-17 decrease does not occur through any intermediate effect of IFN-β on other cell type target, but it is a direct effect of the drug on the $CD4^+$ population. Another significant remark drawn from our *in vitro* results is that after removal of the $CD25^+$ subset from the pool of $CD4^+$ cells, IL-17 mRNA levels remain unchanged after IFN-β exposure. This suggests that the down-modulation of IL-17 expression promoted by IFN-β could be mediated by nTreg cells present in the total $CD4^+$ population. Thus, this would mean that in addition to the regulation of IL-17 production through the innate immune response described by (Guo *et al.*, 2008), IFNβ could modulate IL-17 expression through a memory response involving nTreg cells. In fact, an increase in the number of $CD4^+CD25^+Foxp3^+$, and a delay in the decline of Foxp3 mRNA expression, was found in the total $CD4^+$ population when it was cultured in the presence of IFN-β (Figure 4). This *in vitro* observation is in agreement with the correlation found *in vivo* between the increase of nTreg and the decrease of IL-17 expression in spleen of EAE mice treated with the drug (Figure 7B). Furthermore, although Foxp3 mRNA levels in CNS were not enough to be detected, we could confirm a decrease of IL-17 expression in spinal cord after the treatment of EAE with IFNβ (Figure 7C).

Besides, suppressive function on *naïve* cells displayed by the $CD4^+$ population pre-activated by immobilized anti-CD3 was significantly intensified by IFN-β treatment during pre-activation (Figure 3Bii), indicating a relationship with the higher amount of nTreg found in these cultures. Moreover, an unequivocal connection between both findings was demonstrated in experiments of $CD25^+$ subset depletion, circumstance in which the difference in suppressive activity between IFN-β-treated and untreated cells was abolished (Figure 3Biii).

It is noteworthy the contrasting anti-proliferative, even pro-apoptotic, and anti-apoptotic roles played by IFN-β on T lymphocytes under different situations, as age, differentiation state or activation status (Akbar *et al.*, 2000). Some evidence has been reported about the involvement of Stat1, or Stat3 and Stat5, respectively, as different IFN-β signal mediators in each of these opposite responses (Tanabe *et al.*, 2005). Our results show differential action of IFN-β on different T cell subsets. Indeed, while the cytokine protects $CD4^+CD25^+Foxp3^+$ cells from the spontaneous apoptosis suffered in culture by this cell type, it is not able to rescue from apoptosis other $CD4^+$ cells, as deduced from data obtained by AnnexineV test for the total $CD4^+$ population (Figure 5B). In fact, the presence of IFN-β in total $CD4^+$ cultures leads to certain increase of apoptotic cells. The decay in the content of $CD4^+CD25^+Foxp3^+$ and Foxp3 mRNA during *in vitro* cultures of $CD4^+$ cells (Figure 4) is in agreement with the well known propensity to

apoptosis of nTreg cells (Taams *et al.*, 2001). The endurance of this cell subset mediated by IFN-β, and the retrieval of $CD4^+CD25^+GITR^+Foxp3^+$ cells from apoptosis, indicate a role for IFN-β as a survival factor for nTreg cells. A support for this suggestion is the reported prevention of human $CD4^+CD25^+$ cell apoptosis by fibroblast-conditioned medium (FCM), a common source of type-I IFN (Taams *et al.*, 2001). Taking into account the anergic status of the $CD4^+CD25^+Foxp3^+$ subset, the mechanism whereby IFN-β rescues nTreg cells from apoptosis could be similar to the proposed for $CD4^+$ cells induced to anergy by T:T peptide presentation (Lombardi *et al.*, 2000). These authors suggested that death cell prevention by IFN-α/β was mediated by the reversion of the translocation from the cytoplasm to the nucleus of PKC-δ. This mechanism together the previously reported ability of IFN-α/β to inhibit caspase-3 activation and to induce the expression of the anti-apoptotic protein Bcl-x_L (Gombert *et al.*, 1996; Pilling *et al.*, 1999; Scheel-Toellner *et al.*, 1999), reveals the interference of type-I IFNs with the induction, commitment and execution phases of apoptosis. Our results concerning to the up-regulation of Bcl-x_L in $CD4^+CD25^+$ cells (Figure 5Aii) could explain partially the survival enhancement of nTreg driven by IFN-β that we described here.

Another differential effect of IFN-β on different T cell types found here is related to $CD4^+$ versus $CD8^+$ subtypes. We demonstrate that the anti-proliferative effect of IFN-β on T cells rather acts on $CD4^+$ than on $CD8^+$ cell type (Figure 2). It could be conclude that this anti-proliferative effect on the $CD4^+$ population is neither related to a cell cycle arrest nor to a deficiency in IL-2Rα expression promoted by the drug. Furthermore, $CD4^+$ cells treated with IFN-β showed a deficient proliferation even if the drug is removed from the culture medium before a second round of stimulation (Figure 3A). This implies that the effect of IFN-β to reduce proliferation ability is due to a lasting effect introduced in the $CD4^+$ population. We propose that after immobilized anti-CD3 signal, remaining nTreg cells are able to partially inhibit the effector cells present in the same population to respond to a second round of TCR stimulation. Since an increased number of $CD25^+Foxp3^+$ regulatory cells are able to survive in $CD4^+$ samples subjected to pre-treatment with IFN-β, proliferation of effector cells should be more efficiently suppressed in these cultures than in the control. Under this proposal, it may be reasoned that the different susceptibility to IFN-β between $CD4^+$ and $CD8^+$ proliferation might be due to the absence of $CD4^+CD25^+Foxp3^+$ regulator cells in the $CD8^+$ population.

Each of the three abilities described here for IFN-β to modulate $CD4^+$ T cell activity could be related to the therapeutic effect on EAE and multiple sclerosis. It is common knowledge that resistance to, or recovery from, EAE can be mediated through T2 cells producing IL-4 and IL-10 (Chitnis and Khoury, 2003). Data in the present work, together with our previous results (Martin-Saavedra *et al.*, 2007), points out the influence of IFN-β on IL-4 expression and hence on the favoritism to T2 phenotype definition from *naïve* cells. On the other hand, our results show for the first time that recombinant IFN-β promotes specifically the accumulation of the nTreg subset on a $CD4^+$ primary culture, most likely through a rescue of this cell subset from apoptosis. The prevention from apoptosis of nTreg cells by IFN-β observed *in vitro* could be also involved in an *in vivo* control of damaging effector cells. Increasing information about the role of Treg in EAE is accumulating (Fernandez-Martin *et al.*, 2006; Kohm *et al.*, 2002; Liu *et al.*, 2006; Mann *et al.*, 2007; McGeachy *et al.*, 2005), and some evidence in relation to an involvement in human multiple sclerosis begins to be drawn (Haas *et al.*, 2005; Hong *et al.*, 2005; Matarese *et al.*, 2005; Viglietta *et al.*, 2004). Although no differences in $CD4^+CD25^+$ cell number in lymph nodes were detected after the treatment of EAE with IFN-β (Martin-Saavedra *et al.*, 2007), an increase in the percentage of cells expressing surface markers of nTreg was found in spleen from IFN-β-treated animals (Figure 7B). This fact correlates with a decrease in IL-17 expression (Figure 7A), which could as well collaborate to the therapeutic effect of IFN-β. Expression of IL-17 has extensively shown to exert a pathogenic function in EAE (Batten *et al.*, 2006; Fitzgerald *et al.*, 2007; Gutcher *et al.*, 2006; Hofstetter *et al.*, 2005; Komiyama *et al.*, 2006; Kroenke and Segal, 2007; Langrish *et al.*, 2005; Park *et al.*, 2005), and some data in favor of active immunization against IL-17 as a therapeutic approach for inflammatory diseases as EAE has been reported (Rohn *et al.*, 2006). In multiple sclerosis only few contributions have reported a possible association of IL-17 expression with the disease (Lock *et al.*, 2002; Matusevicius *et al.*, 1999). However, in the light of the growing information gained about the critical role exerted by IL-17 in inflammation disorders as EAE, it can be expected that future research will produce much more data about the function of this cytokine in human multiple sclerosis, and about the opportunity of its counteraction through nTreg cells.

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Figure Legends

Figure 1. IFN-β *in vitro* treatment improves the T2 phenotype polarization. Total spleen cells were subjected to T0/T1/T2 polarization conditions as indicate in "Materials and methods" (A). Freshly isolated spleen $CD4^+$ and $CD8^+$ cells were co-cultured in a 7:3 ratio (Tm) under T0 condition (B). IFN-β was used at the concentrations indicated on the top. After seven days, cells were exposed to a second round of anti-CD3 for 72h before analysis of cytokine expression. The mRNA and protein levels were measured by real time RT-PCR and ELISA, respectively. Results for mRNA expression were normalized with values obtained for β-actin. Values shown are representative of three independent experiments in which each sample was assayed in triplicate. **: $p<0.01$.

Figure 2. IFN-β *in vitro* treatment reduces the $CD4^+$ proliferative response to secondary CD3 stimulation round without cell cycle arrest or inhibition of IL2Rα expression. (A) Freshly isolated $CD4^+$ and $CD8^+$ cells were exposed to T0 polarization condition in the presence of IFN-β at the doses indicated. After seven days of culture, Th0 ($CD4^+$) and Tc0 ($CD8^+$) populations were re-stimulated during 72 h by plate-bound anti-CD3 (αCD3) maintaining the IFN-β doses. Samples were assayed for proliferation by MTT colorimetric method. Values are means of three independent experiments in which each sample was assayed in triplicate. **: ($p<0.01$). (B) $CD4^+$ *naïve* cells were stained with CFSE and exposed to plate bound anti-CD3 antibody alone or in the presence of IFN-β. After 48h, fluorescence was analyzed by flow cytometry to determine the number of cell divisions. (C) $CD4^+$ *naïve* cells were stimulated as in (B) without CFSE pre-treatment. After 72 h, cells were incubated with DNAse-Free RNAse and Propidium Iodide. Fluorescence was measured by flow cytometry to cell cycle distribution analysis. (D) $CD4^+$ *naïve* cells were subjected to T0 condition in the presence of the indicated IFN-β dosage for seven days and CD25 was quantified by flow cytometry analysis after the staining of the samples with PE- anti-CD25 antibody. Sub-panel Di shows the result after the whole period of treatment. Sub-panel Dii shows the switches in CD25 levels on the cell surface along successive phases of the polarization process in samples untreated or treated with 2500 u/ml. The day of analysis from the onset of culture is indicated in the different sub-panels as D1-D7. The results of one of three independent experiments are shown.

Figure 3. The proliferation defect introduced by IFN-β in $CD4^+$ cultures is related to suppressor activity of double positive $CD4^+CD25^+$. (A) Removal of IFN-β during the second round of TCR stimulation does not restore proliferative ability of $CD4^+$ cells. $CD4^+$ *naïve* T cells were subjected to T0 conditions at the indicated doses of IFN-β. After seven days, cells were re-stimulated by plate bound anti-CD3 (αCD3) in the absence of IFN-β for 72 h and proliferation was determined by MTT assay. (B) IFN-β treatment of anergic $CD4^+$ cells increases its suppressor activity on *naïve* T cell proliferation. (Bi) Response to complete stimulation (soluble anti-CD3 and APC) of $CD4^+$ cells *naïve* or 72 h pre-stimulated by plate-bound anti-CD3 with or without IFN-β (2500 u/ml). (Bii) Suppression assay of *naïve* $CD4^+$ cells proliferation by total $CD4^+$ cells pre-stimulated by plate-bound anti-CD3 with or without IFN-β (2500 u/ml). A freshly population enriched in $CD4^+CD25^+$ nTreg (>70%) was used as control of suppressor cells (nTreg). (Biii) $CD4^+$ *naïve* T cells, partially (2/3) or fully depleted of $CD25^+$ cells, were stimulated with plate-bound anti-CD3 in the presence of IFN-β (2500 u/ml). After 72h, suppression assays were performed with a responder:suppressor ratio of 8:1. Proliferation was determined by the MTT assay. Values for each assay in each panel are representative of three independent experiments where samples were assayed in triplicate. *: $p<0.05$, **: $p<0.01$.

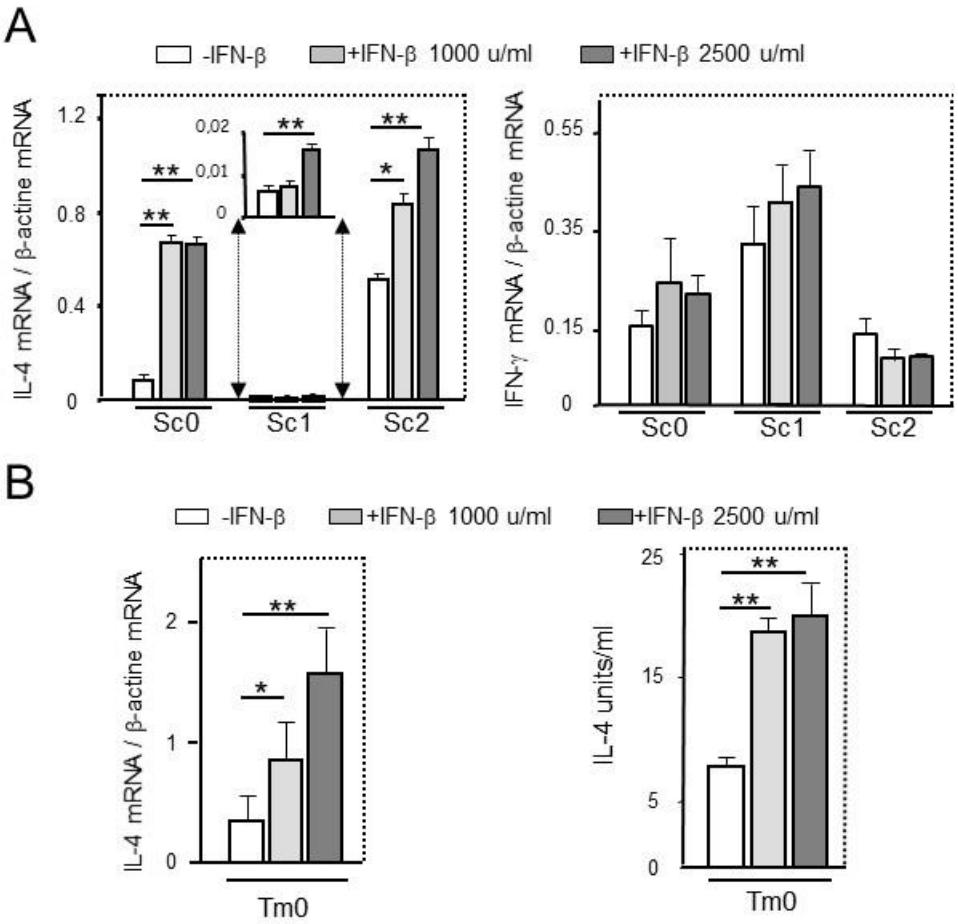
Figure 4. The decay of $CD4^+Foxp3^+$ cells in primary $CD4^+$ *in vitro* cultures is reduced by IFN-β. (A) $CD4^+$ *naïve* cells were stimulated with anti-CD3 alone or combined with IFN-β (2500 u/ml). Foxp3 mRNA levels were measured by real time RT-PCR at the indicated times. Results for each sample were normalized with values obtained for β-actin. (B) Percentages of $CD4^+CD25^+Foxp3^+$ cells in cultures of $CD4^+$ cells after 72 h of anti-CD3 (αCD3) stimulation with or without IFN-β (right). The starting population (left) was total $CD4^+$ cells (upper), or $CD4^+$ population partially (middle) or totally (lower) depleted of $CD4^+CD25^+$ fraction. Each starting population was subjected to double surface staining with FITC-anti-CD4 and APC-anti-CD25. Numbers in each sub-panel indicate the percentage of $Foxp3^+$ cells determined by intracellular staining. The results of one of three independent experiments are shown. *: $p<0.05$, **: $p<0.01$.

Figure 5. IFN-β treatment rescues nTreg from spontaneous apoptosis. $CD4^+CD25^+$ fraction (A) or total $CD4^+$ *naïve* population (B) were cultured in the presence of anti-CD3 and 2500 u/ml of IFN-β. After 24-48 h, cells were stained with AnnexinV-FITC and Propidium Iodide and the number of AnnexinV-FITC positive cells was determined by flow cytometry analysis (Ai and Bi), and Bcl-x_L mRNA was quantified by real time RT-PCR (Aii). The number of $CD4^+$ cells with size and complexity corresponding to viable T lymphocytes (Bii) was calculated by forward-scatter analysis of the samples by flow cytometry. Resulting values were obtained from three independent experiments where each sample was assayed in duplicated. **: $p<0.01$.

Figure 6. IFN-β down-modulates IL-17 expression in $CD4^+$ cells depending on the presence of $CD25^+$ cells. Total $CD4^+$ *naïve* cells (A, B and C), or the $CD4^+CD25^-$ fraction (D), were stimulated with plate-bound anti-CD3 antibody alone or in the presence of IFN-β (2500 u/ml). After three (D3) or seven (D7) days from the onset of culture, the levels of IFN-γ mRNA (A), IL-17 mRNA (B and D), or IL-17 protein (C) were measured by real time RT-PCR and ELISA, respectively. Results for IL-17 mRNA were normalized with values obtained for β-actin in each sample. The results were obtained from three independent experiments where each sample was assayed in duplicate. **: $p<0.01$.

Figure 7. IFN-β *in vivo* treatment of MBP-EAE decreases the expression of IL-17 mRNA in periphery and CNS tissues and promotes accumulation of nTreg in spleen. Groups of SJL/J mice were established by vehicle (control), MBP (EAE) and MBP+IFN-β (EAE/IFNβ). Spleen (A, B) or inflammatory spinal cord (C) cells from animals sacrificed on day 9 p.i. were pooled in suspensions corresponding to individual groups. IL-17 expression was quantified by real time RT-PCR in $CD4^+$ spleen cells (A), total mononuclear cells (MNCs) or MBP-reactive cells obtained from spinal cord (C). Spleen cells were processed to determination of nTreg cells content (B), cell percentages were determined by double surface staining with APC-anti-CD4 and PECy7-anti-CD25 (left), and by four-color staining with surface FITC-anti-CD4, PECy7-anti-CD25 and APC-anti-GITR antibodies and intracellular PE-anti-Foxp3 antibody (right). Values shown are the mean of three independent experiments where each sample was assayed in duplicate. **: $p<0.01$.

Figure 1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B
-IFN-β
+IFN-β 1000 u/ml
+IFN-β 2500 u/ml
IL-4 mRNA / β-actine mRNA
2
1
0
**
*
Tm0
IL-4 units/ml
25
15
5
0
**
**
Tm0

Figure 2

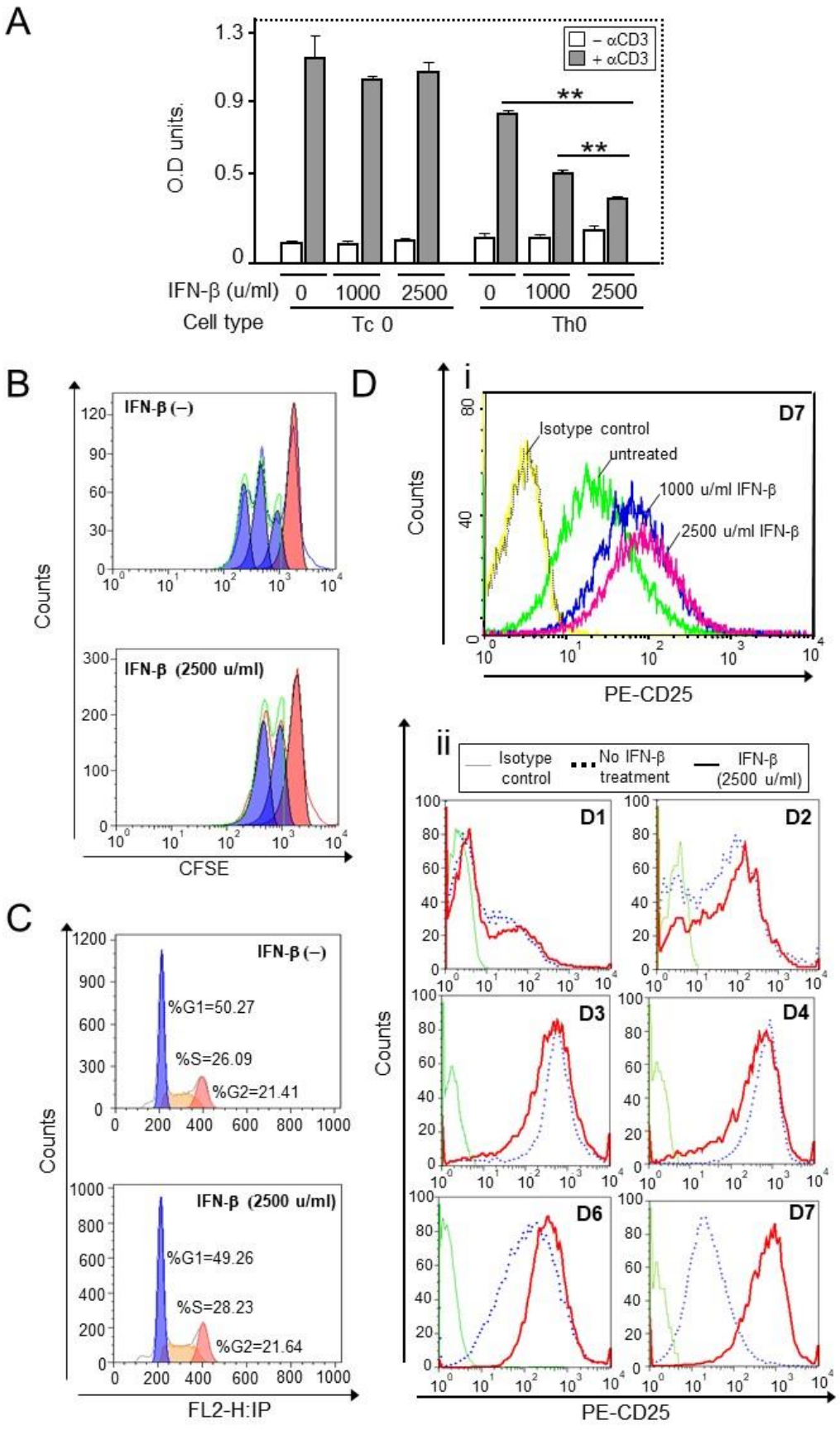
A
O.D units.
1.3
0.9
0.5
0
– αCD3
+ αCD3
**
**
IFN-β (u/ml) 0 1000 2500 0 1000 2500
Cell type
Tc 0
Th0
B
IFN-β (–)
IFN-β (2500 u/ml)
Counts
CFSE
C
IFN-β (–)
%G1=50.27
%S=26.09
%G2=21.41
IFN-β (2500 u/ml)
%G1=49.26
%S=28.23
%G2=21.64
Counts
FL2-H:IP
D
i
D7
Isotype control
untreated
1000 u/ml IFN-β
2500 u/ml IFN-β
Counts
PE-CD25
ii
Isotype control
No IFN-β treatment
IFN-β (2500 u/ml)
D1
D2
D3
D4
D6
D7
Counts
PE-CD25

Figure 3

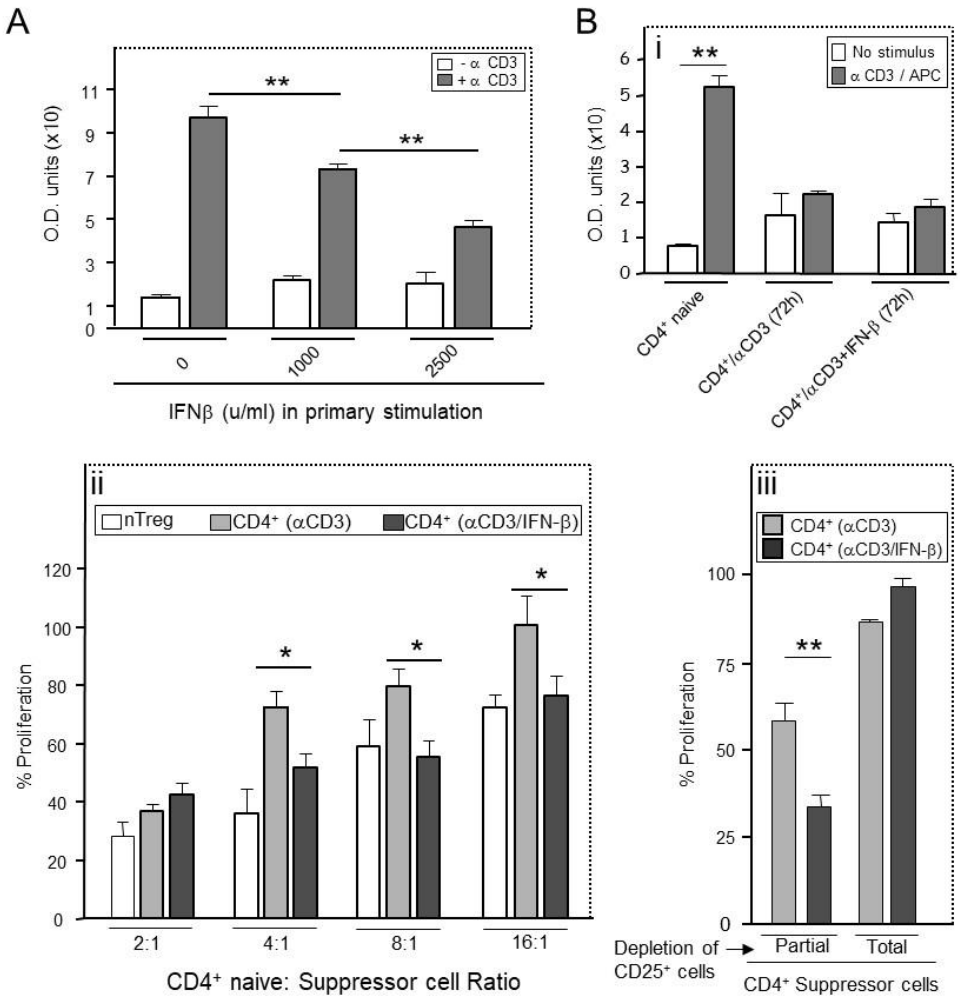
A
- α CD3
+ α CD3
O.D. units (x10)
**
**
0
1000
2500
IFNβ (u/ml) in primary stimulation
B
i
No stimulus
α CD3 / APC
O.D. units (x10)
**
CD4+ naive
CD4+/αCD3 (72h)
CD4+/αCD3+IFN-β (72h)
ii
nTreg
CD4+ (αCD3)
CD4+ (αCD3/IFN-β)
% Proliferation
*
*
*
2:1
4:1
8:1
16:1
CD4+ naive: Suppressor cell Ratio
iii
CD4+ (αCD3)
CD4+ (αCD3/IFN-β)
% Proliferation
**
Depletion of CD25+ cells
Partial
Total
CD4+ Suppressor cells

Figure 4

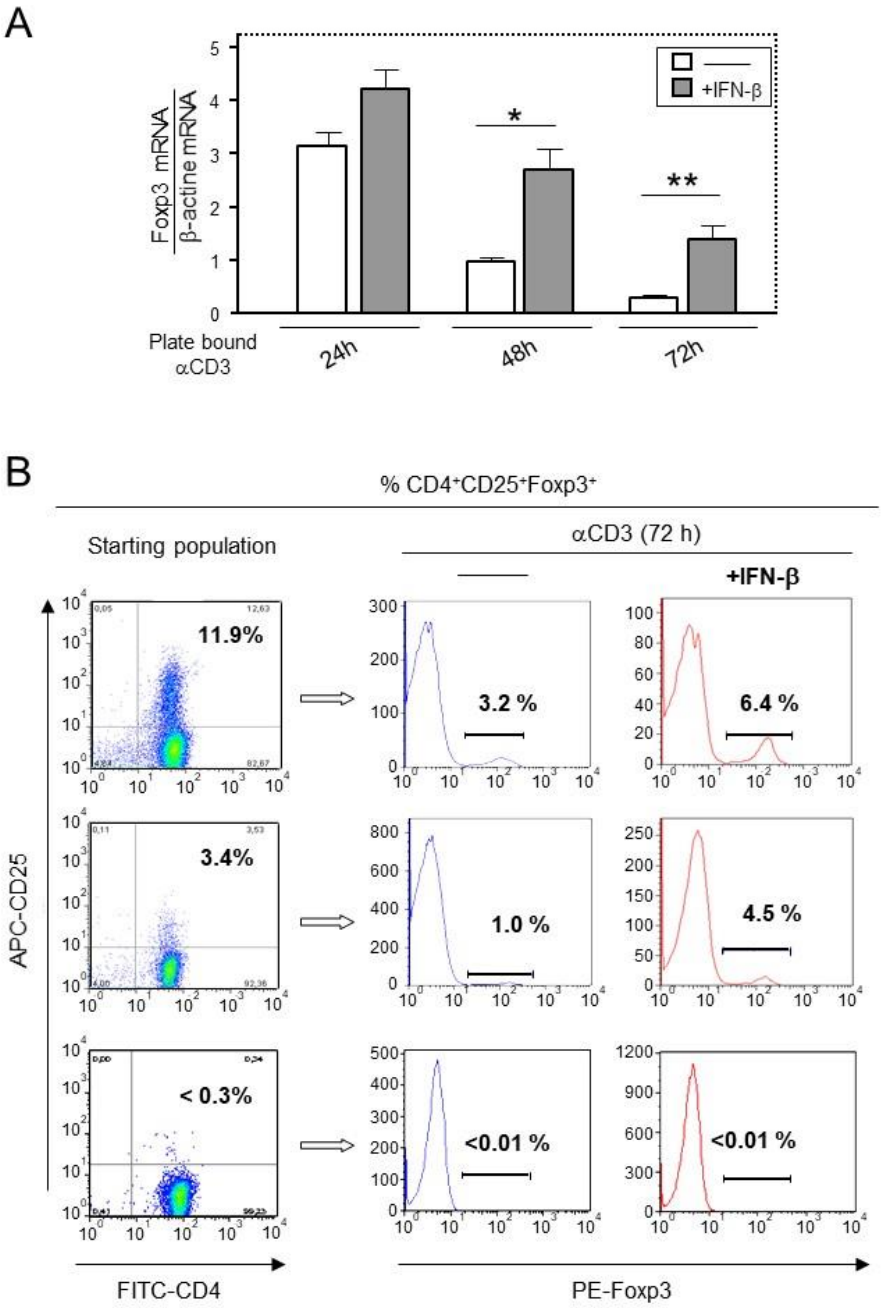
A
Foxp3 mRNA / β-actine mRNA
+IFN-β
*
**
Plate bound αCD3
24h
48h
72h
B
% CD4+CD25+Foxp3+
Starting population
αCD3 (72 h)
+IFN-β
11.9%
3.2 %
6.4 %
3.4%
1.0 %
4.5 %
< 0.3%
<0.01 %
<0.01 %
APC-CD25
FITC-CD4
PE-Foxp3

Figure 5

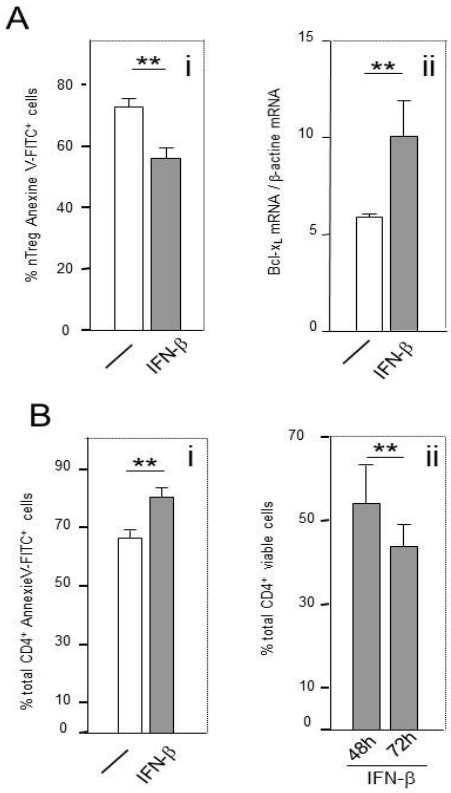

Figure 7

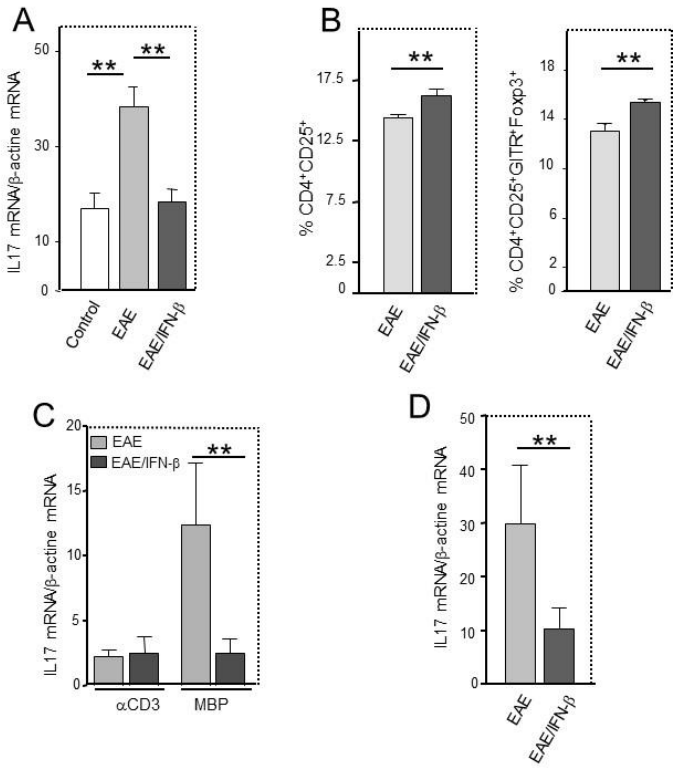

Figure 6