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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

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ABSTRACT

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.

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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. Multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (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, 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

Abbreviations: 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.

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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; PRISMS, 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 post-immunization (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 37 °C 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 3000 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 4th 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 1×10^6 cells/ml with 10 μM of CFSE (Cell Trace, Invitrogen) in PBS containing 0.1% BSA. After 15 min at 37 °C, washing procedures were performed as advised by manufacturer previous to cell culture. For MTT assays, 2×10^5 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 72 h. For suppression assays, the proliferation stimulus was soluble anti-CD3 (20 μg/ml) plus spleen Mitomycin C (MMC)-treated spleen cells from C3H mice (1×10^6 cells/ml) as antigen-presenting cells (APC) and *naïve* spleen CD4+ cells were used as responders (5×10^5 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, 2 mM EDTA, pH 7.2), 5×10^5 cells were incubated for 15 min at 4 °C with saturating amounts of antibodies. FITC-anti-CD4, PE-anti-CD25, and APC-anti-GITR were purchased from Miltenyi Biotec; 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 (2×10^6/ml) were incubated with 70% PBS–ethanol for 2 h at 4 °C, washed with PBS, and incubated with permeabilization and DNA staining solution (0.1% Triton X-100, 200 μg/ml of DNAse-free RNAse A, 20 μg/ml of propidium iodide) for 20 min at room temperature.

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