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Costimulatory Molecule Expression on Leukocytes from Mice with Experimental Autoimmune Encephalomyelitis Treated with IFN-β

DANNIE E.M. HALLAL,[1] ALESSANDRO S. FARIAS,[1] ELAINE C. OLIVEIRA,[1] BLANCA MARIA DIAZ-BARDALES,[1] CARLOS OTAVIO BRANDÃO,[1] GUSTAVO G. PROTTI,[1] FERNANDA G. PEREIRA,[2] IRENE L. METZE,[2] and LEONILDA M.B. SANTOS[1]

ABSTRACT

Interferon-β (IFN-β) is of benefit in the treatment of multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE), but the mechanisms by which it exerts this beneficial effect remain uncertain. The present data demonstrate that IFN-β therapy impairs the proliferative response to concanavalin A (ConA) and myelin basic protein (MBP), decreases expression of the CD80 molecule on leukocytes of treated mice, and may thereby impede the Th1 cell activation-promoting anergy in EAE. Moreover, IFN-β therapy increases expression of the CTLA4 molecule, which induces a counterregulatory Th2 response. The reduction of CD80 expression with concomitant increase of CTLA4 expression alters the course of EAE and may be useful as a monitor in therapy with IFN-β.

INTRODUCTION

Interferon-β (IFN-β) has been shown to reduce the frequency of clinical attacks, the activity of MRI lesions, and the evolution of the disease in patients with multiple sclerosis (MS).[1,2] Moreover, there is evidence that IFN-β directly modulates the immune response and reduces the severity of experimental autoimmune encephalomyelitis (EAE),[3,4] which is a demyelinating diseases of the central nervous system (CNS) that is mediated by Th1 type $CD4^+$ lymphocytes and serves as a model for MS.[5] The progression of MS and EAE correlates with increasing levels of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IFN-γ, and interleukin-12 (IL-12), as well as a decrease in the levels of anti-inflammatory (Th2 type) cytokines, such as IL-10, IL-4, and transforming growth factor-β (TGF-β).[6] There is also evidence of an effect of IFN-β on cytokine production. Levels of IFN-γ,[7] TNF-α,[8] and IL-12[9] decreased in patients with MS during treatment with IFN-β as well as in the experimental model. On the other hand, IFN-β enhanced the production of anti-inflammatory cytokines, such as IL-10[10,11] and TGF-β,[10,12] in patients with MS and in the EAE model.[3]

Two signals are needed for T cell activation. Binding of the T cell receptor by a peptide/MHC complex provides the first, and the second is provided by cytokines such as IL-2 and costimulatory proteins such as CD80 and CD86 expressed on antigen-presenting cells (APCs).[13] In the EAE model, the $CD80^+$ cells activate Th1 lymphocytes, and CD86 activates the Th2 cells.[14] Moreover, cytotoxic T lymphocyte-associated antigen 4 (CTLA4), a counterreceptor in addition to CD28 for the B7 family of costimulatory molecules, is a negative regulator of T cell activation.[15,16]

The present study investigates whether IFN-β affects the expression of costimulatory molecules (CD28, CD80, CD86, and CTLA4), causing the consequent induction of anergy in EAE. Because the EAE model presents many clinical and histologic similarities with MS, this approach underlines the importance of studies in animal models exploring the effects of IFN-β immunotherapy.

MATERIALS AND METHODS

Animals

Six-to-eight-week-old female SJL mice, which are highly susceptible to the induction of EAE,[3,4] were obtained from The Harlan Sprague Dawley Laboratory (Indianapolis, IN). The

[1]Neuroimmunology Unit–Department of Microbiology and Immunology, and [2]Laboratório Marcadores Celulares, University of Campinas (UNICAMP), Campinas-SP, Brazil.

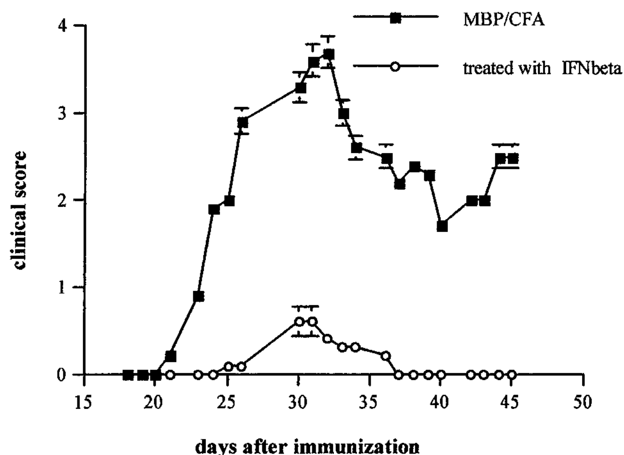

FIG. 1. Clinical scores of EAE in mice treated *in vivo* with recombinant mouse IFN-β (rMuIFN-β). Treatment with 10,000 IU/day was given on days −7, −5, and −2, followed by immunization with MBP/CFA on day 0.

animals were housed and maintained pathogen free in the university animal facility. All procedures were carried out in accordance with the guidelines proposed by the Brazilian Council on Animal Care (COBEA) and approved by the Ethical Committee on Animal Experimentation (CEEA/UNICAMP).

Treatment with IFN-β

The mice were divided into two groups of 10. One group received no IFN-β treatment (controls), and the other received IFN-β (10,000 IU mouse/day, every other day, for a total of 30,000 IU on days −7, −5, and −2. On day 0, all mice were immunized with myelin basic protein (MBP)/complete Freund's adjuvant (CFA).

Antigen, antibody, and recombinant cytokines

Mouse IFN-β (Cytimmune) was purchased from Lee Biomolecular Research Inc. (San Diego, CA). Monoclonal antibodies (mAbs) anti-CD4, anti-CD8, anti-CD28, anti-CD80, anti-CD86, and anti-CTLA4, conjugated to FITC or phycoerythrin (PE) were purchased from PharMingen (San Diego, CA). MBP was purified from guinea pig spinal cords as previously described.[17]

Immunization and induction of EAE

The mice were immunized with MBP. Each animal received an injection in the flank of 400 μg MBP in 0.10 ml phosphate-buffered saline (PBS) emulsified in an equal volume of CFA containing 4 mg/ml *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI), an immunization protocol that has been previously described.[18] Mice were evaluated daily for signs of disease and graded on the following scale: grade 1, limp tail; grade 2, hind limb weakness; grade 3, plegia of both hind limbs; grade 4, plegia of three or four limbs; grade 5, moribund.

Isolation of lymphocytes

Spleen and draining lymph node cells were minced through a steel sieve in Hank's buffer. Connective tissue fragments were allowed to settle for 10 min at 4°C, and lymphocytes were sedimented from the supernatant by low-speed centrifugation (170*g* for 10 min at 4°C).

Proliferation assay

For concanavalin A (ConA) or MBP stimulation, lymphocytes were cultured in RPMI 1640 with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 2% heat-inactivated fetal bovine serum (FBS), and 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO). The optimal concentration of mitogens had been determined in pilot experiments establishing the dose-response pattern, involving various concentrations of ConA and MBP, with the optimums fixed at 2.5 μg/ml for ConA and 25.0 μg/ml for MBP. The lymphocytes (2×10^5/well) were cultured for 48 h for ConA and 144 h for MBP stimulation. The cultures were pulsed with 1 μCi ^{3}H-thymidine (Amersham, Buckinghamshire, U.K.) per well during the last 16 h of culturing and then harvested (Cell Harvester, Cambridge Technology, Cambridge, MA). Thymidine uptake was measured in a scintillation counter (Beckman System, San Jose, CA).

Flow cytometry

Single cell suspensions (1×10^6 cells/ml) were stained using anti-CD4, anti-CD8, anti-CD28, anti-CD80, anti-CD86, and anti-CTLA4 mAbs conjugated to FITC or PE. The analysis was performed using a Becton Dickinson FACScan (Becton Dickinson, Mountain View, CA).

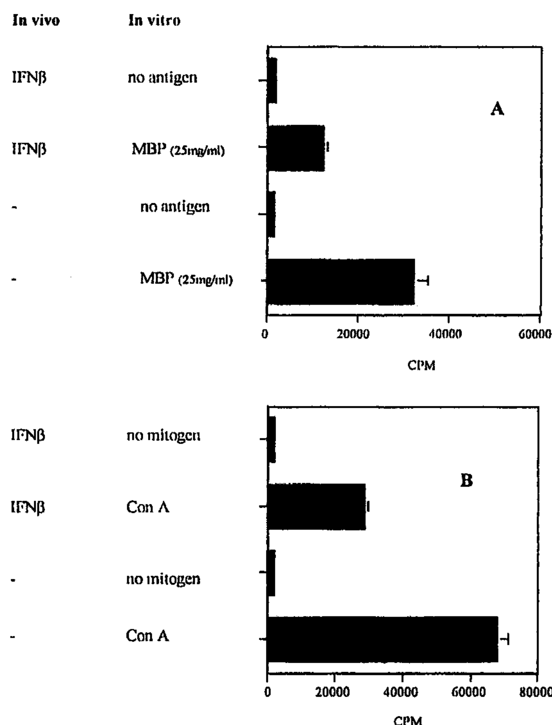

FIG. 2. Effect of IFN-β on proliferative response of lymph node cells stimulated with MBP and ConA. SJL mice treated or not with IFN-β *in vivo* were immunized, and lymph node cells were cultured and stimulated *in vitro* with MBP (**A**). Spleen cells from naive mice treated *in vivo* with IFN-β were stimulated *in vitro* with ConA (**B**).

Statistical analysis

The statistical significance of the data was determined by a Student's t-test and a two-tailed Wilcoxon rank sum test. A p value < 0.05 was considered significant.

RESULTS

Reduction of clinical signs of EAE by administration of IFN-β

To examine the effect of IFN-β on EAE, two groups of mice (10 per group) were immunized with MBP/CFA. One group was treated with 30,000 IU IFN-β/mouse in three doses, starting 7 days prior to immunization, and the other group served as the untreated control. Administration of IFN-β caused a delay in the onset of the disease and a much milder disease course (maximum clinical score: immunized group 3.8 ± 0.4 vs. 0.6 ± 0.1 for the treated group, $p < 0.001$) (Fig. 1). No significant difference in animal weight was noted between the IFN-β-treated group and the control group.

Inhibition of T cell proliferation by IFN-β

The effects of *in vivo* administration of IFN-β on the *in vitro* proliferative response to MBP of lymph nodes from MBP/CFA-immunized mice (Fig. 2A) and of ConA-stimulated spleen cells from nonimmunized mice are shown in Figure 2B. IFN-β administration effectively inhibited the T cell response to MBP ($27{,}620 \pm 1{,}600$ cpm) for nontreated group vs. $12{,}640 \pm 1{,}200$ cpm for the treated group ($p < 0.01$) (Fig. 2A). Administration of IFN-β also reduced the proliferative response of lymphocytes from nonimmunized mice stimulated with nonspecific mitogen ConA ($61{,}420 \pm 2{,}620$ cpm and $37{,}840 \pm 2{,}400$ cpm for the nontreated and treated group, $p < 0.01$) (Fig. 2B).

Modification of expression of costimulatory molecules by in vivo *administration of IFN-β*

As IFN-β administration prior to MBP/CFA lessened the severity of EAE and suppressed the *in vitro* proliferative response, the ability of IFN-β to modify costimulatory molecule expression was examined. The *in vivo* administration of IFN-β inhibited the expression of CD80 in the treated group compared with immunized and naive groups (naive mice $6.5\% \pm 1.2\%$ vs $18\% \pm 0.8\%$ in the immunized group and $7.8\% \pm 0.6\%$ in the immunized and treated group, $p < 0.001$). There were no significant changes in the expression of CD28 ($p > 0.05$). Moreover, there was no significant change observed among the three groups in the expression of CD86 ($p > 0.05$) (Fig. 3).

The expression of CTLA4 was determined in the immunized group treated or not with IFN-β 24 h after the last dose of 10,000

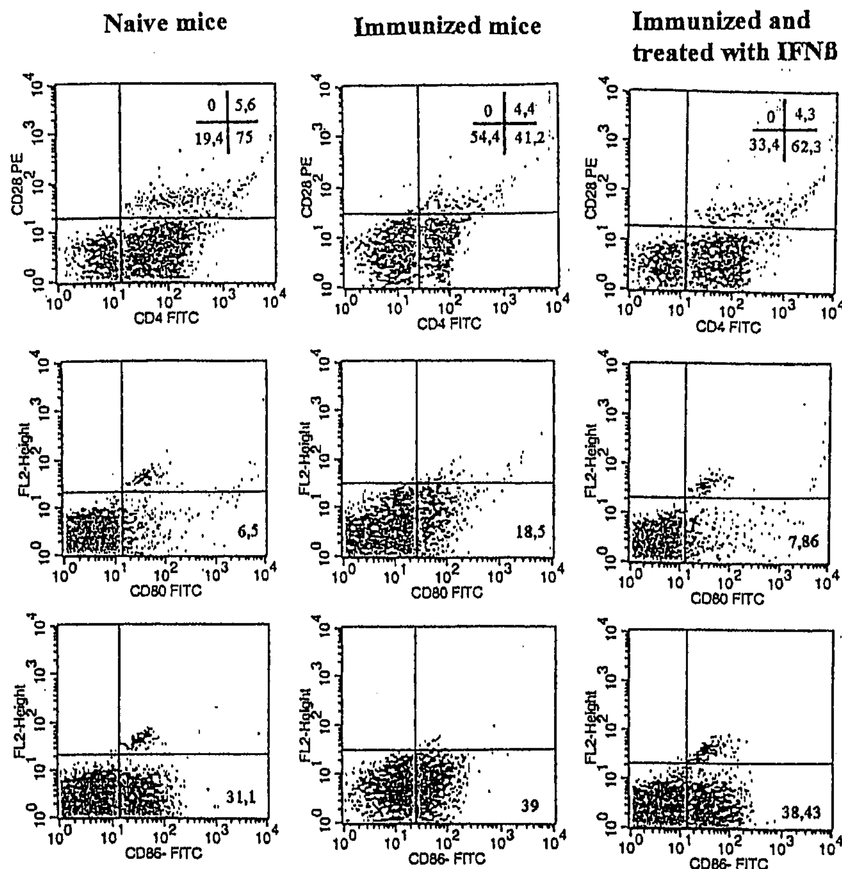

FIG. 3. Costimulatory molecule expression on leukocytes of SJL mice treated or not with IFN-β. Lymph node cells from naive mice and those treated or not with IFN-β were labeled with mAbs to CD28, CD80, and CD86, and the expression of these molecules was quantified by flow cytometry 24 h after the last treatment. The results are representative of eight experiments.

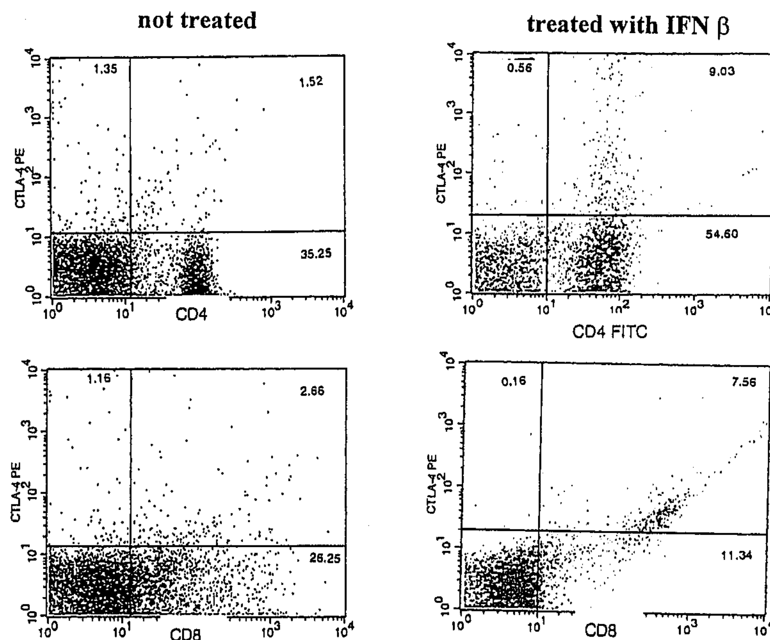

FIG. 4. CTLA4 (CD152) expression on lymphocytes from SJL mice treated or not with IFN-β. The expression of CTLA4 was quantified by flow cytometry on lymph node cells from SJL mice treated or not with IFN-β.

IU (total of 30,000 IU mouse in three doses). The results presented in Figure 4 are representative of eight experiments. The data show that the expression of CTLA4 on the surface of leukocytes increased significantly after administration of IFN-β compared with that of those of the nontreated group. This increase was observed for the expression of CTLA4 in $CD4^+$ cells (1.52% $\pm$ 0.2% for nontreated group vs. 9.03% $\pm$ 0.9% for the treated group, $p < 0.001$) and $CD8^+$ cells (2.6% $\pm$ 1.2% in the nontreated group vs 7.56% $\pm$ 0.7% in the treated group, $p < 0.001$) (Fig. 4).

DISCUSSION

This study showed that the *in vivo* administration of IFN-β to SJL mice reduces the severity of EAE by altering the expression of costimulatory molecules on leukocytes. As demonstrated in Figure 1, administration of IFN-β markedly reduces the clinical signs of EAE when given before the onset of the disease. Treatment with IFN-β caused a stable, less severe form of the disease, whereas the control animals continued to have relapses of the acute form of the disease. These results are in agreement with previous data demonstrating the beneficial effects of IFN-β in murine EAE.[3,4] The *in vivo* administration of IFN-β also alters the *in vitro* immune response. As demonstrated in Figure 2, administration of IFN-β inhibited the proliferative response of lymph node T cells from MBP/CFA-immunized mice and of spleen cells from nonimmunized animals stimulated with ConA. These results are also in agreement with previous observations[3,19,20] indicating that IFN-β can provide effective immunosuppression, resulting in inhibition of the proliferative response of autoreactive T cells.

The role of costimulatory molecules in the maintenance and loss of tolerance has been described.[21] T cell clones activated solely by T cell receptor (TCR) binding to peptides and MHC molecules without efficient costimulatory stimulation become anergic.[19] Moreover, CTLA4/CD80/86 interactions may play a role in the induction of anergy in the presence of low costimulatory molecule expression.[20]

In the present study, a significant increase in the expression of CD80 molecules in mice immunized with MBP was found in relation to the normal expression of these molecules in naive animals, but with IFN-β treatment, this was reduced to normal levels. No significant changes in the expression of CD28 and CD86 were observed. The role of CD80 in the development of organ-specific autoimmune diseases, such as EAE and MS, involves expression on activated microglia,[22,23] infiltrating macrophages, and perivenular lymphocytes in active MS brain lesions, but not in normal brains, as previously described.[24] Moreover, actively induced EAE in mice is prevented by treatment with anti-CD80, whereas treatment with anti-CD86 antibody significantly worsened both clinical and histologic disease. The ability of anti-CD80/CD86 antibodies to inhibit or enhance EAE relates to the capacities of these antibodies to activate Th1 or Th2 cell cytokine.[13] The increase in anti-inflammatory cytokine *in vivo* could be responsible for the decrease in CD80 expression, as it has been shown that IFN-β enhances IL-10 and TGF-β production,[3,9] and these cytokines decrease CD80/CD86 expression.[23,24] IFN-β also diminishes the production of IFN-γ, which in turn induces the expression of CD80 and CD86 molecules.[25] MS patients treated with IFN-β have shown decreased CD80 expression on circulating B lymphocytes[26,27] and increased CD86 expression on monocytes.[27] The *in vitro* addition of IFN-β upregulated the expression of CD80 and CD86 molecules in both dendritic and monocyte cells.[28,29]

Concomitant with the reduction in CD80 molecule expression, IFN-β therapy was found to cause a moderate increase in the early expression of CTLA4 molecules. Despite various research efforts, the mechanisms by which CD28 and CTLA4 exert their effects remain poorly understood. There is some evidence that CTLA4 may function, at least in part, by competing with CD28 for CD80/86 ligands, thereby acting as an indirect attenuator of costimulatory signals, and that the competition of CTLA4 may be most effective when CD80/86 molecule levels are low.[30] The CD28 molecule is constitutively expressed on T cells, whereas CTLA4 is not readily detectable until 24–48 h after activation. The present data show that treatment with IFN-β activated the early expression of CTLA4 *in vivo*, as the cells analyzed here were not activated *in vitro*.

In recent years, various models have been proposed to explain whether CTLA4 might preexist, although expressed at low levels, or can be induced rapidly even in naive cells on engagement of the TCR and CD28.[30] This would be a possible mechanism for ensuring peripheral tolerance by preventing activation when a T cell encounters a self-antigen. The early expression of CTLA4 in T cells may also explain observations that a CTLA4 blockade accelerates the onset of experimental autoimmune diseases, including EAE.[14–16] We have demonstrated that immunotherapy with IFN-β induces a moderate expression of CTLA4, but it is possible that even very low levels of this molecule may be sufficient to bind to the CD80 molecule, which is reduced by the treatment. MBP-specific T cell activation might then be reduced, resulting in less severe EAE.

We have demonstrated here that therapy with IFN-β markedly reduces the expression of costimulatory molecules (CD80) and increases CTLA4 expression. These observations could explain, at least in part, the reduction of T lymphocyte activation and, consequently, the beneficial effect of this treatment in EAE.

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Address reprint requests or correspondence to:
Dr. Leonilda M.B. Santos
Departamento de Microbiologia e Imunologia
Instituto de Biologia
UNICAMP
Campinas-SP
Brazil CEP 13083-970

Tel: *55 19 3788-6262*
Fax: *55 19 3788-8190*
E-mail: leonilda@unicamp.br

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