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Synapsin peptide fused to *E. coli* heat-labile toxin B subunit induces regulatory T cells and modulates cytokine balance in experimental autoimmune encephalomyelitis

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ABSTRACT

We previously found that the preventive oral administration of a hybrid consisting of the C domain of synapsin and the B subunit of *E. coli* heat-labile enterotoxin (LTBSC) efficiently suppresses experimental autoimmune encephalomyelitis (EAE) development in rats. We investigated the effect of LTBSC on cytokine expression and on regulatory T (Treg) cells in rats with myelin induced EAE. LTBSC treatment increased the frequency of $CD4^+FoxP3^+$ Treg cells in lymph nodes prior to challenge and in the EAE acute stage. LTBSC also up-regulated the expression of anti-inflammatory Th2/Th3 cytokines and diminished myelin basic protein-specific Th1 and Th17 cell responses in lymph nodes. $CD4^+CD25^+$ Treg cells from LTBSC treated rats showed stronger suppressive properties than Treg cells from controls in vitro. Our observations indicate that LTBSC is a useful agent for modulating the autoimmune responses in EAE.

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is a neuroinflammatory and demyelinating T cell-mediated disease that is used as an animal model for multiple sclerosis (MS) in humans. Inflammation of the central nervous system (CNS) and demyelination in MS and EAE have an autoimmune basis. White matter disease is characterized by focal lesions with lymphocyte and macrophage infiltrates, demyelination and axonal injury (McFarland and Martin, 2007). In EAE and MS, it is well established that myelin antigens prime peripheral autoreactive T cell clones to become active effector $CD4^+$ T cells, which produce high quantities of T helper 1 (Th1) cytokines (INFγ, TNFα) that efficiently activate macrophages (Merrill et al., 1992). Moreover, Th17 cells were recently demonstrated to play a role in the immunopathogenesis of EAE (Aranami and Yamamura, 2008). Primed T cell clones infiltrate the CNS and generate a local immunologic attack orchestrated through a variety of pro-inflammatory mediators, chemokines and cytokines that activate both blood and resident macrophages leading to focal myelin disruption and axonal transection. These events correlate with the presence of characteristic histopathological lesions in the CNS that result in neurological deficiencies (Peterson and Fujinami, 2007). On the other hand, resistance or recovery from EAE has been classically associated with the up-regulation of $CD4^+$ Th2 responses (IL-4, IL-5 and IL-13) that are able to limit Th1/Th17-mediated inflammation (Harrington et al., 2005).

Autoreactive T cells, which are normally present in all individuals at very low levels, are finely controlled by mechanisms of peripheral tolerance to prevent the breakdown of self-tolerance and the subsequent development of autoimmunity. The main immunoregulatory mechanisms include regulatory T (Treg) cells and anti-inflammatory cytokines.

Several subsets of Treg cells have been described in humans and rodents. Most Treg cells are either natural thymus-derived $CD4^+CD25^+$ (nTreg) or are adaptive or induced Treg cells that differentiate from naïve T cells following antigen exposure in the periphery (iTreg) (Fontenot et al., 2003; Sakaguchi et al., 2006). Some Treg cells have been demonstrated to be phenotypically and functionally similar to $CD4^+CD25^+$ nTreg cells and can differentiate from naïve T cells in the periphery (Chen et al., 2003). The forkhead/winged helix transcription factor *FoxP3* expressed specifically by nTreg cells has been crucially related to their development and regulatory functions (Sakaguchi et al., 2006). Severe autoimmune disorders have been related to the depletion of FoxP3-expressing T cells, including $CD25^+$ and $CD25^-$ nTreg cells (Ono et al., 2006). Consequently, Treg cells appear as promising targets for the treatment of autoimmune and graft versus host diseases and new protocols for expanding these populations in vivo may have a great impact in the immunotherapy field. Treg cells exert their actions through cell to cell contact and/or cytokine release and may produce either TGF-β and IL-10, or both (von Boehmer, 2005). Additionally, $CD4^+CD25^+$ Treg cells are able to suppress the proliferation of responder T cells and exhibit an anergic phenotype in vitro (Baecher-Allan et al., 2004).

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Tolerance can be achieved by an oral administration of selected autoantigens that can be used to lower self-aggressive responses in EAE and other autoimmune diseases (Faria and Weiner, 2006). Additionally, the use of mucosal adjuvants can enhance oral tolerance. The B subunits of the *E. coli* heat-labile toxin (LTB) and cholera toxin (CTB) are nontoxic molecules that act as potent mucosal adjuvants and transmucosal carriers (Sun et al., 1994). CTB and LTB share several structural and functional features (Spangler, 1992). Both toxin B subunits are strong immunogens that have a great ability to break the intestinal barrier and to target linked antigens to the gut-associated lymphoid tissue (GALT) (Sun et al., 1994). Effective targeting of very small amounts of pathogen antigens or autoantigens has been shown to induce protective or tolerogenic responses, respectively, more effectively than that observed with the administration of the antigen alone. The strong immunotherapeutic effectiveness of the mucosal administration of autoantigens coupled to CTB has been demonstrated in various autoimmune disease models such as EAE, diabetes, uveitis, and arthritis as well as in a proof-of-concept pilot clinical trial in patients with Behcet's disease (Sun et al., 2010).

It has been shown that rats suffering from EAE raise antibodies and T cells against myelin basic protein (MBP) that are also able to recognize the neuronal protein synapsin (De Santis et al., 1992; De Santis and Roth, 1996). Recently, it has been demonstrated that T cell clones raised against MBP proliferated and secreted IL-2, IFN-γ and IL-10 upon in vitro stimulation with the N-terminal ABC-domains of synapsin (Degano and Roth, 2009). Intraperitoneal administration of a synaptosomal preparation prior to the induction of acute EAE was shown to suppress clinical signs and to reverse the expected CNS histological and biochemical alterations (Degano et al., 1998). The effect of synaptosomal antigens on EAE development was similar to the suppression induced by treatment with myelin antigens and could not be mimicked by antigens from unrelated tissue (Degano et al., 2004). Recently, we showed that rats orally fed four 10 μg doses of a hybrid between the C domain of synapsin and LTB (LTBSC) developed oral tolerance in rats (Scerbo et al., 2008). Additionally, when LTBSC was fed prior to the encephalitogenic challenge, the rats were protected from EAE, had diminished cellular infiltration in the spinal cord and reduced cellular reactivity against MBP. LTBSC-treated rats presented diminished Th1-mediated delayed type hypersensitivity and reduced lymph node cell proliferation by MBP stimulation in vitro (Scerbo et al., 2009).

In the present study, we investigated the effects of LTBSC oral administration on the cytokine production and Treg cell populations in rats with EAE that was induced with myelin antigens. Our data indicate that a preventive administration of LTBSC shifted the cytokine balance toward a Th2/Th3 profile and expanded the FoxP3$^+$ Treg cell population in peripheral lymph nodes; both factors may contribute to the immunomodulation of EAE by an oral administration of LTBSC.

2. Materials and methods

2.1. Antigens

Myelin and MBP were purified from bovine spinal cords as previously described (Scerbo et al., 2009). Recombinant LTB, SC and the hybrid protein LTBSC were obtained from cultures of *E. coli* harboring the corresponding expression vectors. Construction of expression vectors, expression and purification of bioactive forms of LTB, LTBSC and SC were performed as previously described (Scerbo et al., 2008; Scerbo et al., 2009).

2.2. Animals

Forty-five-day-old male and female Wistar rats inbred at our institution and maintained under SPF conditions in the vivarium were used for the in vivo studies. The rats weighed 120–130 g at the beginning of the experiment. All animal experiments were conducted in accordance with the National Institutes of Health (Bethesda, MD, USA) regulations, and the Institutional Animal Care Committee approved all animal handling and experimental procedures (Exp. No. 15-99-40426).

2.3. Oral tolerance and EAE induction

The oral regimen with LTBSC and EAE induction were performed as previously reported (Scerbo et al., 2008). Briefly, rats were fed 10 μg of LTBSC (LTBSC group), an equimolar mixture of LTB plus SC (2.6 μg plus 7.4 μg of protein, respectively, LTB + SC group), or with vehicle alone (0.2 ml, 2 M urea in PBS, control group) on days −10, −8, −6 and −4 prior to either sacrifice or EAE induction. EAE was induced (day 0) in rats by intradermal injections in both hindfeet with 0.5 ml of an emulsion made with 0.2 ml of PBS and 0.3 ml of complete Freund's adjuvant containing 8 mg of bovine myelin. Animals were weighed and clinical signs of EAE were examined daily. The degree of clinical signs was scored as: 0, no clinical expression of the disease; 0.5, loss of tip tail tonus; 1, flaccid tail; 2, hind limb weakness; 3, complete hind leg paralysis accompanied by urinary incontinence; 4, quadriparesis, moribund state, or death. Animals were sacrificed 10 or 12 days post-induction (dpi) during the acute stage of the disease; otherwise, the animals were sacrificed at 5, 8, 12 and 15 dpi.

2.4. Isolation of mononuclear cells

Mesenteric lymph nodes (MLNs), inguinal lymph nodes (ILNs) and spleen were mechanically disrupted and single cell suspensions were filtered through a 70 μm cell strainer (Nylon membrane BD,

Table 1
Clinical features of EAE rats treated with LTBSC.

Treatment	*n*	Disease incidence (%)	M.M.C.S.[a]	Day of onset	Length of disease	Maximum weight loss (%)
Vehicle	15	13/15 (86.66)	2.71 +/− 0.21	11.96 +/− 0.31	4.25 +/− 0.33	13.58 +/− 1.34
10 μg LTBSC	15	5/15 (33.33)**,#	2.02 +/− 0.16	13.15 +/− 0.39	4.58 +/− 0.55	6.21 +/− 1.29*
10 μg LTB + SC	15	10/15 (66.66)	2.52 +/− 0.18	13.87 +/− 0.52	4.19 +/− 0.41	10.76 +/− 1.63*

Animals were fed with the indicated amount of LTBSC, an equimolar mixture of LTB and SC or vehicle alone at 10, 8, 6, and 4 days prior the active induction of EAE with bovine myelin in CFA. Rats were weighed and clinical signs were assessed daily. The results are expressed as mean ± SEM.
Maximum weight loss was observed at the acute period and was calculated from all individuals in each group.
[a] M.M.C.S., mean maximum clinical score is the sum of the highest clinical score (1–4) achieved by each sick rat during the acute phase of the disease divided by the number of rats that got sick in each group.
* $p<0.05$ treated vs. vehicle group.
** $p<0.0001$ treated vs. vehicle group.
# $p<0.001$ LTBSC vs. LTB + SC group.

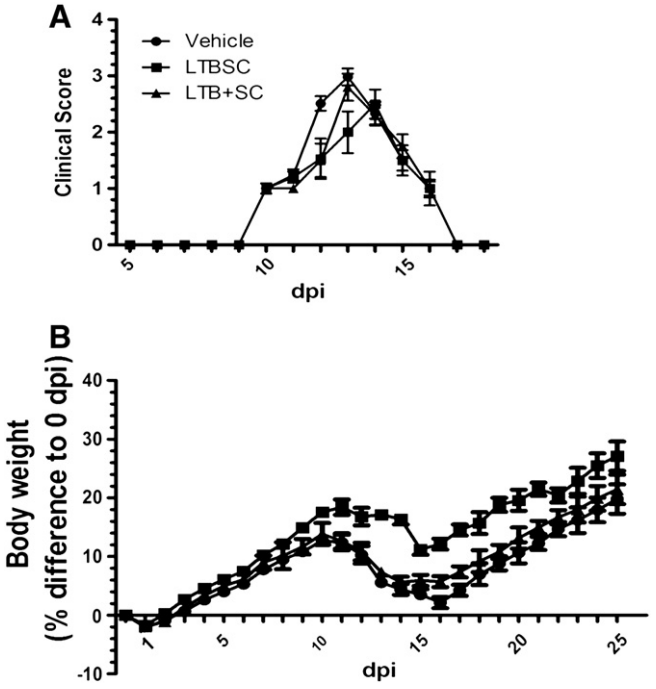


Fig. 1. Clinical evaluation of EAE. LTBSC, LTB + SC or vehicle were orally administered to rats prior to challenge for EAE. (A) Severity of clinical signs was scored as described in Materials and methods in animals that developed clinical signs in the three experimental groups. (B) Comparison of body weight variation of total number of animals (sick and non sick) in each group. All values are shown as mean ± SEM.

Becton Dickinson, Argentina) and washed. Mononuclear cells (MNCs) were resuspended in RPMI 1640 containing 10% fetal calf serum (FCS). Spleen MNCs were separated by Histopaque 1080 (Sigma-Aldrich USA) gradient centrifugation and then washed twice with RPMI 1640 containing 10% FCS. Cellular viability was assessed by Trypan blue exclusion.

2.5. Mononuclear cell culture and stimulation

Cytokine content was measured in MNC culture supernatants. MNCs were maintained in RPMI 1640 medium (Sigma-Aldrich, Buenos Aires, Argentina) supplemented with 30 μg/ml gentamicin, 10% FCS (Natocor, Córdoba, Argentina) and 50 μM 2-mercaptoethanol at 37 °C in a humidified CO_2 incubator.

MLNs, ILNs and spleen MNCs isolated from rats orally fed but not challenged for EAE (four days after the last feeding) were stimulated in culture (2.5×10^6 cells/ml) with 1 μg/ml of concanavalin A (ConA, Sigma Aldrich, Buenos Aires, Argentina), and after 48 h, supernatants were collected and stored at −70 °C until cytokine analysis.

Suspensions of ILN cells (1.25×10^6 cells/ml) from rats challenged for EAE were collected at 5, 8, 12 and 15 dpi. The $CD4^+CD25^+$ and $CD4^+CD25^-$ co-cultures were stimulated with 45 μg/ml of MBP for 48 h, and then, supernatants were collected and stored at −70 °C until cytokine detection.

2.6. Cytokine detection

All cytokines were measured by sandwich ELISA following the manufacturer's instructions: IFN-γ and IL-10 with BD OptEIA Set (BD Argentina, Buenos Aires, Argentina) and TGF-β with TGF-β1 Multispecies Antibody Pair (Invitrogen Argentina Ltda, Buenos Aires Argentina). For IL-4 determination, capture and detection antibodies conjugated to biotin were from BD-Pharmingen (BD Argentina, Buenos Aires, Argentina). IL-17 was measured using the Ready-SET-Go kit from eBioscience (San Diego, CA, USA).

2.7. Flow cytometry analysis

Surface molecules were detected in freshly isolated MNCs (1×10^6 cells) by incubating with the appropriate concentration of the conjugated monoclonal antibodies anti-CD4-PECy5, anti-CD25-FITC, anti-CD4-FITC, anti-CD25-allophicocyanin and the corresponding isotype controls (Becton Dickinson, Argentina, Buenos Aires, Argentina). For intracellular FoxP3 staining, cells that were stained with anti-CD4-PECy5 and anti-CD25-FITC antibodies (eBioscience, San Diego, CA, USA) were fixed, permeabilized and stained with anti-FoxP3-PE according to the manufacturer's protocol (eBioscience, San Diego, CA, USA).

For intracellular IL-4, IL-10, TGF-β, IFN-γ and IL-17 staining, MNCs were treated in culture (1.25×10^6 cell/ml) with 100 ng/ml of phorbol myristate acetate and 10 ng/ml of ionomycin (Sigma Aldrich, Buenos Aires, Argentina) for 4 h, and then, 2 μM of monensin (eBioscience, San Diego, CA, USA) was added for 1 h. Cells were collected and stained for surface antigens with anti-CD4-FITC (eBioscience, San Diego, CA, USA). Following surface staining, the cells were fixed, permeabilized and stained with anti-IL-4-biotin, anti-IL-10-biotin, anti-IFN-γ-biotin (BD Argentina, Buenos Aires, Argentina), anti-TGF-β-biotin (Invitrogen Argentina Ltda, Buenos Aires, Argentina) or anti-IL-17-allophicocyanin (eBioscience, San Diego, CA, USA) antibodies. To detect the biotin-conjugated primary antibodies, cells were treated with Streptavidin-allophycocyanin (eBioscience, San Diego, CA, USA).

Samples were acquired on a FACSCanto Flow Cytometer (BD Argentina, Buenos Aires, Argentina) and data were analyzed with Wind MDi software. The analysis was restricted to the small lymphocyte gate as determined by their characteristic of forward and side scatter properties. At least 10,000 (for surface antigens) or 30,000 (for intracellular antigens) cells were assayed.

2.8. Purification of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells

ILNs were removed from animals treated with LTBSC or vehicle at 10 dpi, and single cell suspensions were obtained and stained with monoclonal antibodies against CD4 and CD25 as described in Section 2.7. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets were isolated using fluorescent activated cell sorting (FACS) on a FACSAria II cell sorter (BD Argentina, Buenos Aires, Argentina). The analysis and sorting gates were restricted to the small lymphocyte gate as determined by their characteristic forward and side scatter properties.

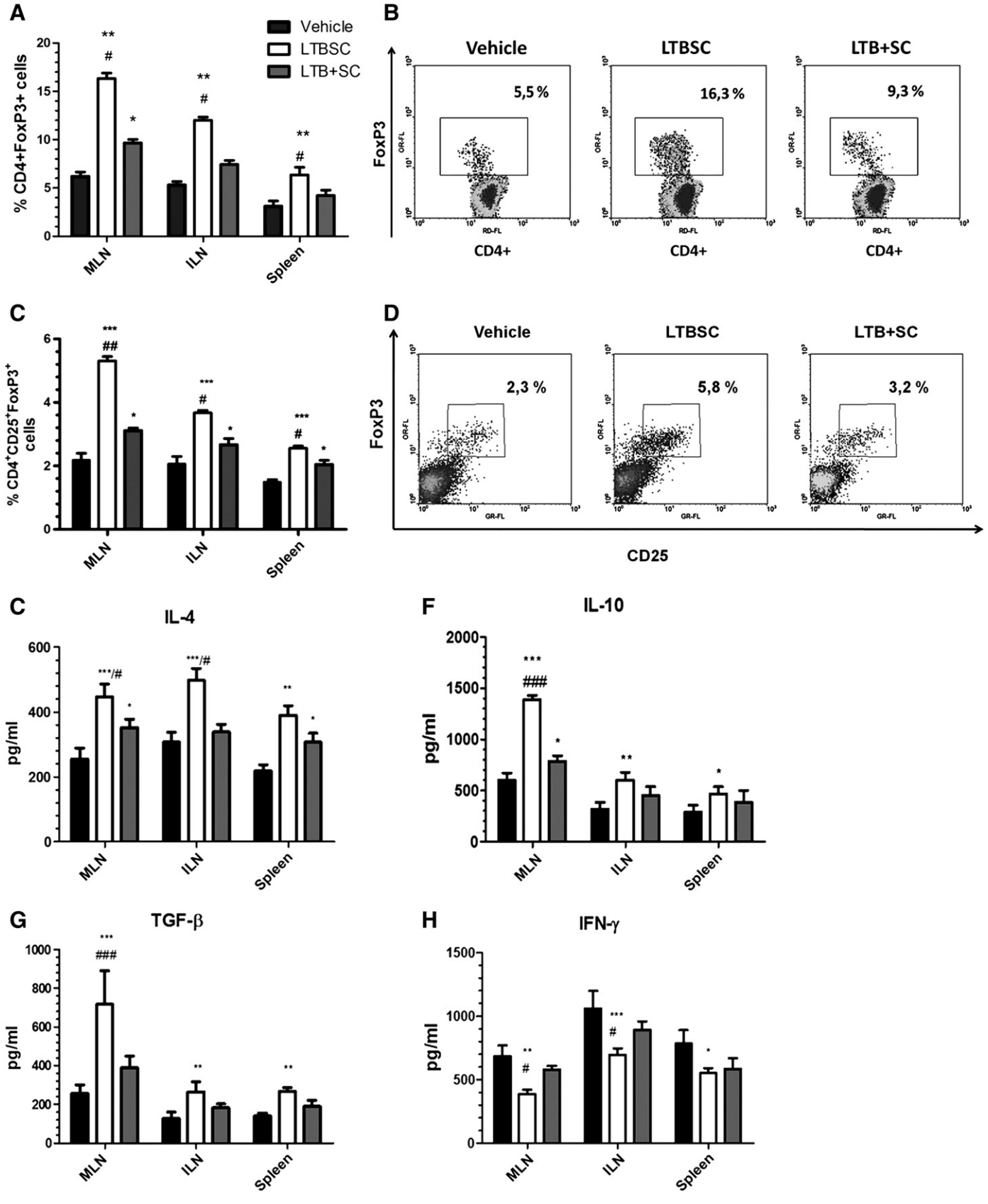
Sorted $CD4^+CD25^+$ cells were maintained in the presence of 2 pg/ml of recombinant IL-2 (BD Argentina, Buenos Aires, Argentina) for 3 days at

Fig. 2. Frequency of $FoxP3^+$ regulatory T cells and cytokine levels in peripheral lymphoid tissues after oral treatment with recombinant LTBSC, LTB and SC proteins. LTBSC, LTB + SC or vehicle was orally administered to rats, and mononuclear cells were isolated from mesenteric lymph nodes (MLNs), inguinal lymph nodes (ILNs), and spleen four days after the last feeding. Cells were stained with anti-CD4, anti-CD25 and anti-FoxP3 antibodies and analyzed by flow cytometry. (A) Relative percentages of $CD4^+FoxP3^+$ cells within the $CD4^+$ population from the MLNs, ILNs and spleens of rats that received the different oral treatments. (B) Dot plots showing FoxP3 expression on gated $CD4^+$ T cells from MLN corresponding to an animal of each experimental group. Numbers adjacent to the outlined areas indicate the proportion of cells that were positive for both antibodies. (C) Relative percentages of $CD4^+CD25^+FoxP3^+$ cells from the MLNs, ILNs, and spleen of animals that received the different treatments determined in the $CD4^+$ population. (D) Dot plots of $CD4^+$ MLN T cells from individual animals of each experimental group stained with anti-CD25 (*x*-axis) and anti-FoxP3 (*y*-axis) antibodies. Numbers adjacent to the outlined areas indicate the proportion of $CD25^+FoxP3^+$ T cell subset on gated $CD4^+$ T cells. Cytokine concentrations of IL-4 (E), IL-10 (F), TGF-β (G) and IFN-γ (H) in culture supernatants of MLN, ILN and splenic cells from rats treated with LTBSC, LTB + SC or vehicle and stimulated with ConA (1 μg/ml) for 48 h. Data in (A), (C), and (E–H) correspond to two experiments performed with 3 animals per group. Each value represents the mean ± SEM. Significant differences are indicated for treated group vs. vehicle: $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; and LTBSC vs. LTB + SC group: $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$.

37 °C before use. Because of the low yield of highly pure $CD4^+CD25^+$ FACS-isolated cells, the wells were seeded at a very low density with fresh medium. On the other hand, the ILN $CD4^+CD25^-$ T cell subset was used shortly after isolation.

2.9. In vitro suppression assay

Sorted $CD4^+CD25^+$ cells were used as the Treg cell subset, and freshly isolated $CD4^+CD25^-$ cells were used as the responder/



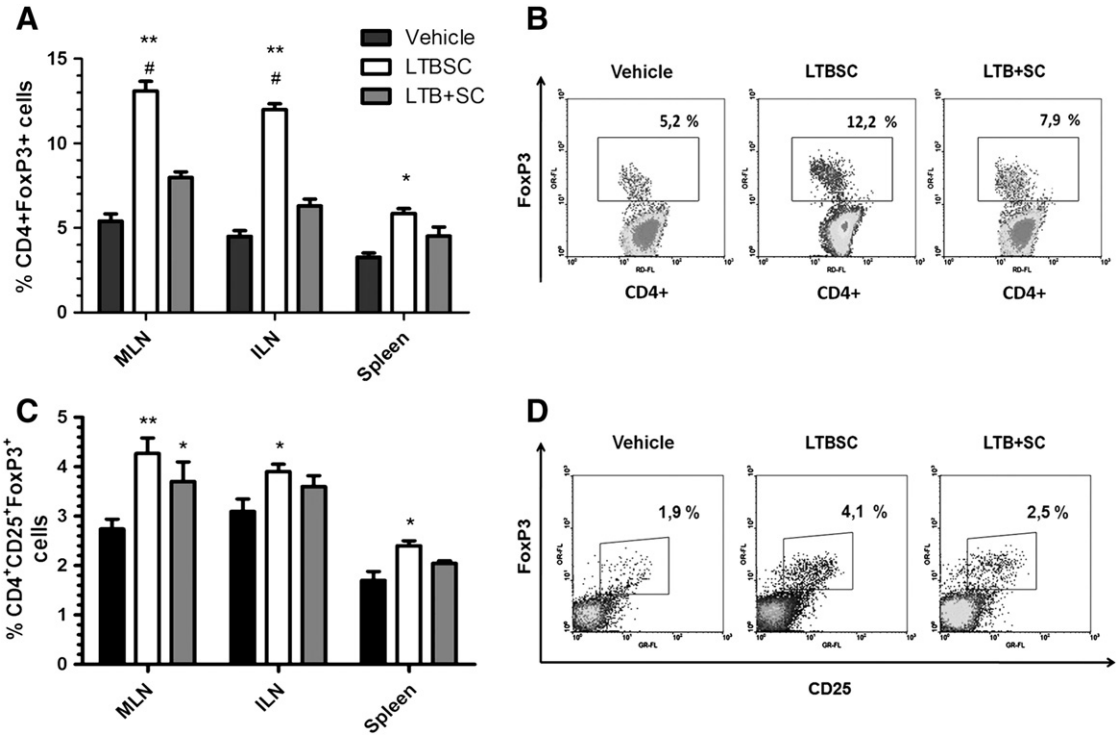


Fig. 3. Frequency of FoxP3$^+$ regulatory T cells in rats that were orally treated with recombinant proteins LTBSC, LTB and SC prior to EAE challenge. LTBSC, LTB + SC or vehicle was orally administered to rats and four days later EAE was induced with myelin. Mesenteric lymph nodes (MLNs), inguinal lymph nodes (ILNs), and splenic cells were obtained at 12 days post EAE induction and stained with anti-CD4, anti-CD25 and anti-FoxP3 and then analyzed by flow cytometry. (A) Relative percentages of CD4$^+$FoxP3$^+$ cells within the CD4$^+$ cell population from MLNs, ILNs, and spleens of animals that received the different treatments. (B) Representative dot plots showing FoxP3 expression on gated CD4$^+$ T cells from ILN of an animal of each experimental group. Numbers adjacent to the outlined areas indicate the percentage of cells that were positive for both antibodies. (C) Percentages of CD4$^+$CD25$^+$FoxP3$^+$ cells from the MLNs, ILNs, and spleens of animals that received the different treatments. (D) Representative dot plots of CD4$^+$ MLN cells from one animal of each experimental group that were stained with anti-CD25 (*x*-axis) and anti-FoxP3 (*y*-axis) antibodies. Numbers adjacent to the outlined areas indicate the percentage of CD25$^+$ and FoxP3$^+$ cells on gated CD4$^+$ T cells. Results in (A) and (C) correspond to two experiments performed with 3 animals per group. Each value represents the mean ± SEM. Significant differences are indicated for treated group vs. vehicle: $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; and LTBSC vs. LTB + SC group: $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$.

proliferative subset (Tresp) to assess the regulatory function in vitro. Treg cells from either LTBSC or vehicle-treated rats were co-cultured with Tresp cells from vehicle-treated rats at a final cell density of 1×10^6 cell/ml. Cell cultures were stimulated with plate-bound anti-CD3 and anti-CD28 at 2.5 μg/ml and 50 ng/ml, respectively, for 72 h. In some cases, cell cultures were stimulated with 45 μg/ml of MBP plus 1.5×10^5 cells/ml syngeneic APC (3000 rads, ^{60}Co-irradiated spleen MNCs). To measure cell proliferation, cultured cells were pulsed with 1 μCi [^{3}H] thymidine (NEN Biolabs, Migliore Laclaustra SRL, Buenos Aires, Argentina) during the last 18 h of culture. To measure the incorporation of [^{3}H] thymidine cells were harvested onto fiberglass filters, and the radioactivity (as counts per minute, CPM) was determined using a standard liquid scintillation technique. The inhibition of proliferation was calculated from the sample CPM with respect to the Tresp cells CPM alone (100% proliferation) (Scerbo et al., 2008). Treg cell proliferation was assumed as a background. Cultures were stimulated in replicate with plate-bound anti-CD3 or anti-CD28 for 72 h before collecting culture supernatants for cytokine measurement.

2.10. Statistical analysis

Data are presented as mean ± SEM. An analysis of the quantitative data between groups was tested by a one-way ANOVA, and the Tukey multiple comparison test was used to examine statistical significance in mean values. Differences in percentage values were analyzed by the χ^2 test. The significant differences were informed as an error probability of ≤0.05. Prior to the analysis, residuals of data were tested for deviation from the normal distribution by the Kolmogorov–Smirnov test.

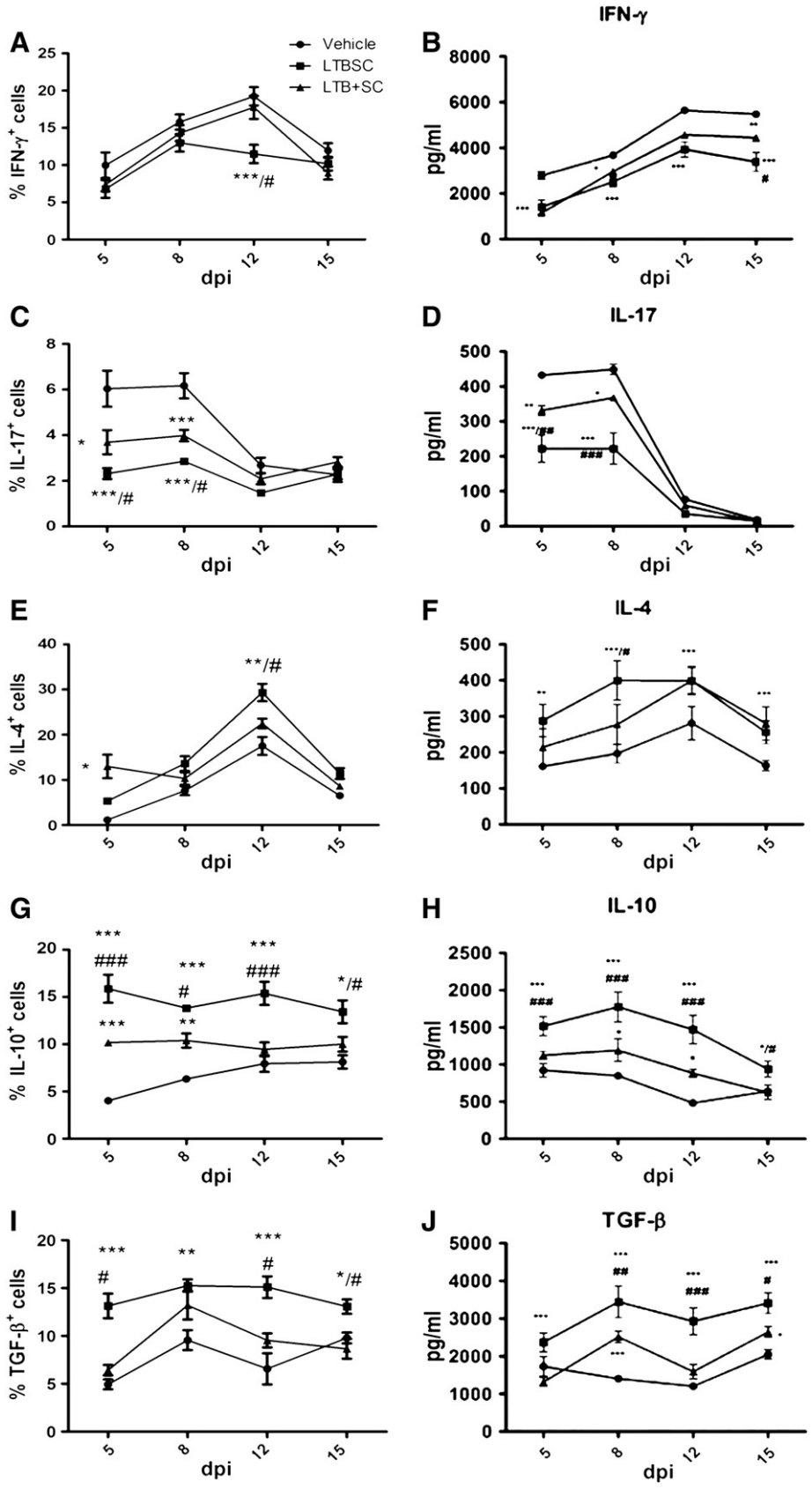
3. Results

3.1. Pretreatment with oral LTBSC protect rats from EAE

Groups of rats were fed with four doses of 10 μg of LTBSC, an equimolar mixture of LTB plus SC or vehicle alone at days 10, 8, 6, and 4 prior to the active induction of EAE with myelin. Clinical signs of EAE which developed at the acute stage of the disease EAE (10–14 dpi) were scored and body weight was determined daily. Experimental data is summarized in Table 1. As previously observed (Scerbo et al., 2009) about 87% (13/15) of the animals that received vehicle alone developed the characteristic signs of the disease but animals that were fed with LTBSC showed a diminished disease incidence (5/15). In contrast, the group that was orally treated with an equimolar amount of the separated molecules LTB + SC showed similar disease incidence with respect to the control group. However, animals of all groups that developed clinical signs did not show any

Fig. 4. Prior oral treatment with LTBSC shifted immune responses from Th1 and Th17 to Th2/Th3 in EAE. Vehicle, LTBSC or LTB + SC was orally administered prior to the encephalitogenic challenge and single cell suspensions from inguinal lymph nodes were obtained at 5, 8, 12 and 15 days post EAE induction (dpi). Cell suspensions were stained with anti-CD4 antibody on the surface and intracellularly with anti-IFN-γ, anti-IL-17, anti-IL-4, anti-IL-10 or anti-TGF-β antibodies for flow cytometry or were stimulated in culture with myelin basic protein for cytokine detection. Percentages of CD4$^+$IFN-γ$^+$ (A), CD4$^+$IL-17$^+$ (C), CD4$^+$IL-4$^+$ (E), CD4$^+$IL-10$^+$ (G), and CD4$^+$TGF-β$^+$ (I) double positive T cell populations determined within the CD4$^+$ T cell population. Concentration of IFN-γ (B), IL-17 (D), IL-4 (F), IL-10 (H) and TGF-β (J) in culture supernatant of ILN cells stimulated with myelin basic protein as described in Materials and methods. Data are representative of two independent experiments done with 2 animals per group. Each value represents the mean ± SEM. Significant differences are indicated for treated group vs. vehicle: $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; and LTBSC vs. LTB + SC group: $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$.

difference in day of onset, severity or length of the disease. EAE neurological disabilities were also associated with weight loss, which reached about 13% in the control group and 11% in the group that received LTB + SC, whereas this parameter was reduced to about 6% in the LTBSC group. The time course of the clinical severity among animals that showed clinical disease and body weight variation in the three experimental groups (sick and non sick animals) are shown in Fig. 1A and B, respectively.



3.2. Oral regimen with LTBSC induced regulatory T-cell expansion and shifted cytokine milieu in local and peripheral immune sites in naïve rats

To study the putative induction of Treg cells and the immune response after treatment of naïve rats with the recombinant proteins or vehicle, we determined the frequency of $CD4^+Foxp3^+$ cells in lymphoid tissues from animals of the three experimental groups. Four days after the last feeding, MNCs from MLNs, ILNs and spleens were isolated and analyzed by flow cytometry. Animals treated with LTBSC showed almost threefold expansion of the $CD4^+FoxP3^+$ T cell compartment in mucosa inductive sites (MLNs), but in the ILNs and spleens, the increment was approximately twofold with respect to vehicle-treated animals. Expansion of $CD4^+FoxP3^+$ T cells by LTBSC administration was significantly higher than that observed with LTB + SC separated molecules. Treatment with LTB + SC only induced a modest increase of $CD4^+FoxP3^+$ T cell subset with respect to the control in MLNs (Fig. 2A). When we measured the percentage of $CD4^+CD25^+FoxP3^+$ cells, we also found significant increase in this T cell subpopulation in LTBSC-treated animals with respect to controls and the LTB + SC-treated group (Fig. 2C).

Additionally, we analyzed different cytokines in MNC culture supernatants after a polyclonal stimulus (ConA). Higher concentrations of IL-4 were produced by the MNCs from animals treated with LTBSC than the MNCs from control animals. LTBSC was also clearly more effective than LTB + SC at increasing IL-4 levels in MLN and ILN MNCs. Modest production of IL-4 was induced by LTB + SC administration in MLN and splenic cells (Fig. 2E). Moreover, marked up-regulation of IL-10 and TGF-β secretion was induced by LTBSC in MLN cells and, to a lesser extent, in ILN and splenic cells. Additionally, LTBSC treatment was more effective at increasing both of IL-10 and TGF-β anti-inflammatory cytokines than the treatment with LTB + SC in MLN cells. Conversely, the administration of the separate LTB and SC molecules only induced modest production of IL-10 levels in MLN cells (Fig. 2F, G). On the other hand, the administration of LTBSC diminished the levels of the Th1 cytokine IFN-γ in MNCs from all lymphoid tissues analyzed. On the contrary, the administration of LTB + SC did not affect IFN-γ levels in any of the three lymphoid tissues (Fig. 2H). These combined results show that treatment with LTBSC fusion protein increased Treg cell populations and shifted immune balance toward a Th2 phenotype in naïve animals.

3.3. $CD4^+FoxP3^+$ Treg cell expansion induced by oral administration of LTBSC persisted after induction of EAE

Randomly assigned animals were fed with LTBSC, LTB + SC or vehicle, and four days after the last feeding all rats received an encephalitogenic challenge (0 dpi). At 12 dpi, animals were sacrificed, and $CD4^+FoxP3^+$ Treg cells in MLN, ILN and spleen cell suspensions were analyzed. The results showed that at the acute stage of disease the expansion of the $CD4^+FoxP3^+$ T cell compartment observed in LTBSC-treated animals prior to encephalitogenic challenge persisted not only in the local inductive site MLNs but also in other peripheral sites (ILNs and spleens) after EAE induction. By comparison, oral treatment with LTB + SC had no effect on this parameter (Fig. 3A). Similarly, we also found an expansion of $CD4^+CD25^+FoxP3^+$ Treg cell population in those animals fed with LTBSC with respect to the control group in all lymphoid compartments analyzed (Fig. 3C).

3.4. Prior oral treatment with LTBSC shifted immune responses from Th1 and Th17 to Th2/Th3 in EAE

We fed groups of rats with LTBSC, LTB + SC or vehicle, and four days after the last feeding, they were all challenged for EAE in order to investigate Th1 and Th17 inflammatory responses during induction and acute stages of EAE and how these responses are affected by oral treatments. Intracellular IFN-γ or IL-17 concentrations were assayed in MNCs from ILNs separated at 5, 8, 12 and 15 dpi and stained on the surface with anti-CD4 antibody.

Additionally, replicates of MNC suspensions were stimulated in vitro with MBP, and levels of IFN-γ and IL-17 were analyzed in culture supernatants. The frequency of IFN-γ^+ T cells from control and LTB + SC groups increased from 5 dpi to a maximum at 12 dpi whereas the percentage of IFN-γ-secreting T cells remained low and constant between 5 and 15 dpi in LTBSC-treated animals (Fig. 4A). Accordingly, similar results were observed when MBP-specific IFN-γ secretion was measured in culture supernatants (Fig. 4B). The frequency of IL-17^+ T cells in the control group peaked during the inductive phase of the disease (8 dpi) and decreased in the symptomatic acute period. Although frequencies of IL-17^+ T cells were diminished in LTBSC and LTB + SC groups at 5 and 8 dpi, the LTBSC group exhibited the lowest levels, and they remained unaltered throughout the course of the disease (Fig. 4C). Accordingly, MBP-specific IL-17 secretion was up-regulated in MNC culture supernatants from controls during inductive EAE stage and decreased abruptly in the acute phase of the disease. However, IL-17 in cell supernatants from LTBSC group was approximately half the level found in the control group and lower than LTB + SC levels. Treatment with LTB + SC was clearly less effective than LTBSC at down-regulating the expression of IL-17 (Fig. 4D).

IL-4^+ T cells reached a maximum at 12 dpi in all groups, but in the LTBSC group, this T cell subset showed the highest frequency (Fig. 4E). Concomitantly, levels of secreted MBP-specific IL-4 were significantly elevated in animals that received the fusion protein during the whole EAE period analyzed (Fig. 4F). $CD4^+$IL-10^+ T cells were consistently expanded at all times in animals treated with LTBSC compared with control and LTB + SC-treated animals (Fig. 4G). Elevated concentrations of MBP-specific IL-10 were accordingly secreted by MNCs from animals treated with LTBSC at all times analyzed, and only modest increases in this cytokine were observed in the MNC cultures from rats treated with LTB + SC at 8 and 12 dpi (Fig. 4H). Similarly to that observed for $CD4^+$IL-10^+ T cells, $CD4^+$TGF-β^+ cells were also increased in rats treated with LTBSC at all times (Fig. 4I). This effect correlated with high MBP-specific TGF-β levels in the respective cell culture supernatants. MNCs from LTB + SC group only exhibited modest increases in TGF-β secretion at 8 and 15 dpi. By comparison, cells from control rats secreted very low levels of this anti-inflammatory cytokine (Fig. 4J).

3.5. Functional activity of regulatory T cells

Functional activity of Treg cells from LTBSC-treated and control rats was measured in vitro as inhibition on proliferation of syngeneic $CD4^+$ T responder (Tresp) cells. ILN $CD4^+CD25^+$ (Treg) cells from LTBSC or vehicle-treated animals were co-cultured with ILN $CD4^+CD25^-$ (Tresp) cells from vehicle-treated rats at different cell number ratios (Treg to Tresp at 2:1, 1:1 and 1:2). The results showed that stronger Tresp proliferation inhibition was observed in co-cultures containing Treg cells from LTBSC-treated animals than in the respective co-cultures with Treg cells from control rats either when anti-CD3/anti-CD28 (Fig. 5A) or MBP (Fig. 5B) was used as stimulant. Proliferation data for Fig. 5A and B is shown as Complementary Fig. 1.

We also analyzed cytokines in culture supernatants of cell co-culture replicates stimulated with anti-CD3/anti-CD28. Higher concentrations of IL-10 (Fig. 5C) and TGF-β (Fig. 5D) were found in co-cultures including LTBSC Treg cells at all Treg to Tresp cell number ratios assayed. On the contrary, higher levels of pro-inflammatory IFN-γ (Fig. 5E) and IL-17 (Fig. 5F) were found in supernatants of co-cultures that included Treg cells from control rats with respect to those co-cultures including Treg cells from LTBSC-treated rats.

4. Discussion

In this work, we provide data for the immunological factors that may mediate the suppression of EAE by LTBSC. We have previously

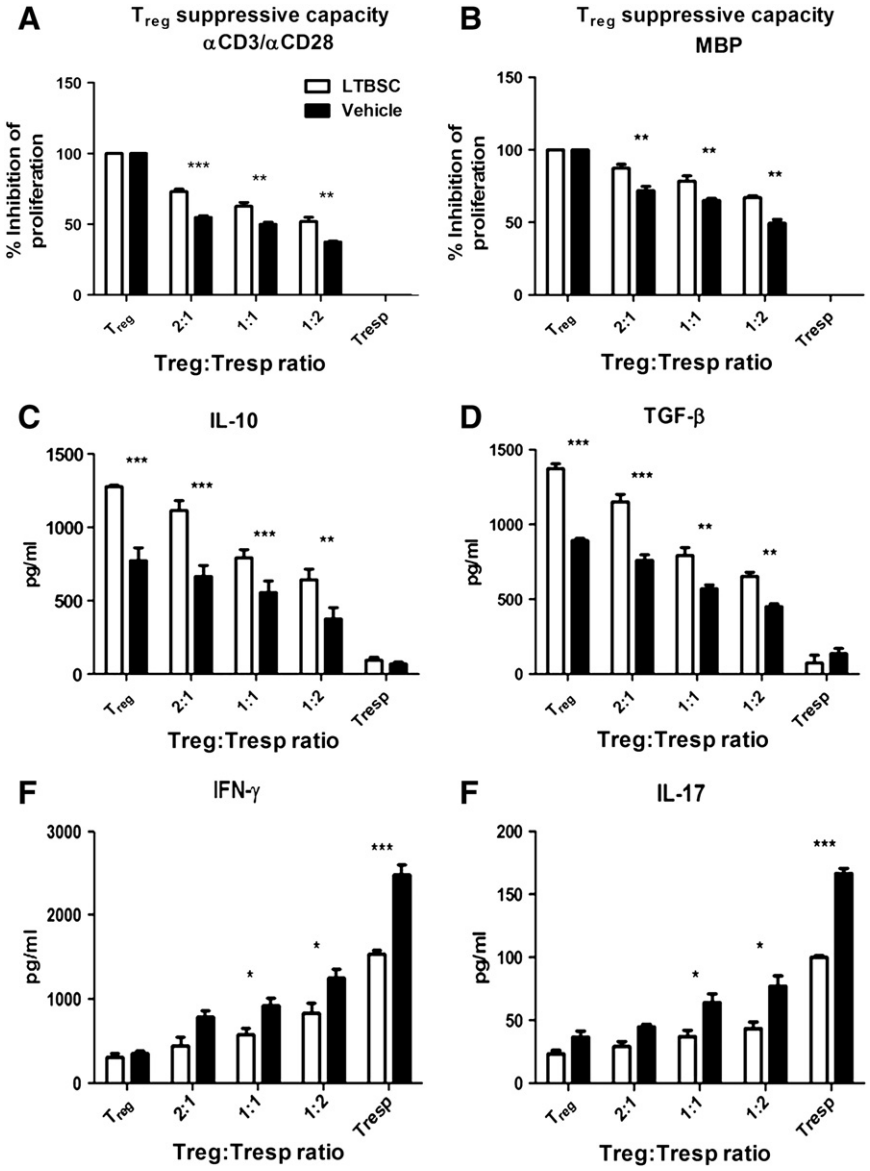


Fig. 5. Functional activity of $CD4^+CD25^+$ Treg cells in vitro. Rats were treated with vehicle or LTBSC prior to EAE induction and inguinal lymph node cells were obtained at 10 days post EAE induction. Cells were stained with anti-CD4 and anti-CD25 antibodies and $CD4^+CD25^+$ (Treg) cells and $CD4^+CD25^-$ (Tresp) cells were separated using fluorescent activated cell sorting (FACS). Treg and Tresp cells from vehicle treated-rats were co-cultured at different cell number ratios and were stimulated with plate-bound anti-CD3/anti-CD28 (A, C, D, E, F) or myelin basic protein plus irradiated syngeneic antigen presenting cells (B). Proliferation inhibition was calculated as a percentage respective to proliferation of Tresp alone (100% proliferation). ELISA concentrations of IL-10 (C), TGF-β (D), IFN-γ (E) and IL-17 (F) were measured in replicated co-culture supernatants. Data are representative of three independent experiments performed with 2 animals per group. Significant differences are indicated for treated group vs. vehicle: $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

shown that the oral administration of very small amounts of the synapsin C domain genetically fused to LTB prevented the development of EAE in Wistar rats. Since myelin induced-EAE is mediated by a cellular autoimmune response against MBP and other myelin components, we previously demonstrated that in vivo and ex vivo cellular reactivity against MBP was diminished in rats in which LTBSC was preventively administered before active EAE challenge (Scerbo et al., 2009). In the present study, we showed that the oral administration of LTBSC induced significant peripheral expansion of the $CD4^+FoxP3^+$ regulatory T cell compartment and shifted the Th1 and Th17 pathogenic cytokine balance in EAE toward a beneficial Th2 and Th3 profile. We expect that peripheral inactivation of MBP-reactive T cells by expanded $FoxP3^+$ Treg cells should be reflected at the target organ the CNS. Differential Treg cell recruitment and an anti-inflammatory cytokine milieu into the CNS from animals treated with LTBSC may account for the lower spinal cord inflammation previously observed (Scerbo et al., 2009). In this direction, we found that therapeutic administration of a hybrid involving a peptide consisting of an extended C domain of synapsin and LTB in rats with EAE induced higher Treg cell recruitment into CNS compared to non-treated animals (unpublished results). Another mechanism that is worth to be investigated which may account for lower CNS infiltration would be that peripheral impairment of MBP effector T cells by expanded Treg cell populations disrupted migration of the pathogenic T cells into the CNS. Interestingly, Tischner et al. (2006) demonstrated that peripheral expansion of $CD4^+CD25^+$ naturally occurring Treg cells induced by a monoclonal anti-CD28 antibody interferes with effector T cells migration into the target organ in EAE.

Our data suggest that LTB could deliver the genetically fused SC synapsin peptide to the GALT inducing oral tolerance. The administration of LTBSC expanded $CD4^+FoxP3^+$ Treg cells and promoted an immunomodulatory milieu as indicated by prevailing levels of IL-4, IL-10 and TGF-β protective cytokines not only in a major mucosal inductive site (MLNs) but also in other peripheral sites (ILN and spleen). Antigen

presentation by immature mucosal DCs in the context of a non-inflammatory milieu is known to preferentially stimulate differentiation of naïve T lymphocytes to Th2 cells or to Th3 Treg cells producing IL-4, IL-10 or TGF-β respectively (Coombes and Powrie, 2008). This phenomenon is relevant for the intestine since it has been shown that the GALT preferentially promotes development of FoxP3$^+$ Treg cells (Sun et al., 2007). In this respect, it has been demonstrated that the close related CTB may maintain immature human myeloid DCs that produce IL-10 that drives differentiation to Th2 lymphocytes or to Treg cells (D'Ambrosio et al., 2008). The potential ability of CTB-antigen conjugates to target mucosal DCs and to set these cells in such status inducing immunosuppression was recently demonstrated for a CTB-pro-insulin fusion protein in type I diabetes (Odumosu et al., 2010). This result supports the putative mucosal adjuvant properties of CTB in the immunotherapy field.

In the present work, when the oral administration of LTBSC or LTB + SC was followed by an encephalitogenic challenge, a marked expansion of CD4$^+$FoxP3$^+$ regulatory T cells persisted only in lymphoid compartments of LTBSC-treated animals during EAE acute stage, but not in those rats that received LTB + SC. Therefore, the expanded Treg cell population induced by LTBSC treatment could effectively disrupt the priming of autoreactive T cell in mucosal inductive sites and peripheral lymph nodes and induce oral tolerance. LTBSC and LTB were assayed on an equimolar basis, a condition in which LTB is approximately ten times more active than LTBSC in recognizing GM1 adsorbed to a polystyrene surface (Scerbo et al., 2008). If this activity ratio equally occurs in vivo, it can be expected that LTB has preferential targeting and contact with GALT compared with the LTBSC fusion protein. This apparent drawback supports the hypothesis tested in this work that EAE suppression by the hybrid LTBSC may be attributed to the synapsin moiety potentiated by genetically fused-LTB since the administration of equimolar mixture of the separate peptides (LTB + SC) did not replicate most of the hybrid effects. Our results also support previous observations of the ability of synaptosomal antigens to suppress EAE by modulating MBP reactivity (Degano et al., 1998; Degano et al., 2004; Scerbo et al., 2009).

Th1 and Th17 responses were elicited in our EAE model, which is in agreement with data showing that both pathogenic cells participate in EAE and MS (Aranami and Yamamura, 2008). We found that the frequency of CD4$^+$IL17$^+$ cells was increased during the EAE inductive stage, whereas CD4$^+$IFN-γ$^+$ cells were enhanced during the acute stage. Similar observations were reported in another rat EAE model (Momcilović et al., 2008).

Immunomodulation of EAE by LTBSC treatment is further supported by the fact that an antigen-specific up-regulation of anti-inflammatory IL-4, IL-10 and TGF-β cytokines was detected during the inductive and acute stages of EAE. These observations were consistent with expansion of the respective IL-4, IL-10 and TGF-β cytokine secreting CD4$^+$ T cells. LTBSC treatment strongly induced the secretion of Th2 and Th3 cytokines that have been shown to be involved in the suppression and remission of clinical signs in EAE (Faria et al., 2003; McGeachy and Anderton, 2005). The well-known anti-inflammatory properties of IL-10 and TGF-β produced by Treg cells may constitute the major immune modulators that control autoreactive T cells in the suppressive effect of LTBSC. However, it should be considered that IL-10 can be produced in vivo by several immune cell types (Hedrich and Bream, 2010) and also TGF-β may be produced by many immune and non immune cells (Li et al., 2006). Further identification of the specific IL-10 and TGF-β producing-cells in the ex vivo experiments of this study is necessary to characterize other relevant cells as IL-10 producing-dendritic cells.

Our results also agree with studies showing that oral tolerance induced by low doses of CTB antigen conjugates expands Treg cells, up-regulates IL-10 and TGF-β and reduces levels of IFNγ and other Th1-associated cytokines (Sun et al., 2000; Aspord and Thivolet, 2002; Sun et al., 2006). Furthermore, we showed that oral tolerance induced by LTBSC treatment also caused a drastic reduction in Th17 response. The relationship between oral tolerance and Th17 cells has not been completely elucidated, but recent studies have shown the relevance of CD11b expression on APCs being detrimental to the peripheral Th17 cell differentiation (Ehirchiou et al., 2007). It has been demonstrated the suppression of IL-17 responses, both in the periphery and in the CNS, after oral MOG administration in mice (Peron et al., 2010). Recently, a putative role for IL-10 was suggested in limiting Th17 development in rheumatoid arthritis patients (Yu-Jung et al., 2010).

In the present study, we have also demonstrated that LTBSC induced-CD4$^+$CD25$^+$ Treg cells exhibit the functional ability to suppress effector T cells ex vivo more efficiently than Treg cells from control animals. This effect was observed upon the stimulation of cells either with anti-CD3/anti-CD28 or, more important, with MBP. Concomitantly, enhanced release of IL-10 and TGF-β and diminished production of IL-17 and IFN-γ was observed in Treg and Tresp cell co-cultures containing Treg cells from animals treated with LTBSC. Reduced number or functional impairment of Treg cells was found to cause autoimmunity in several animal models (Sakaguchi, 2000) indicating that CD4$^+$CD25$^+$ Treg cells are crucial for controlling other autoreactive T cells. Importantly, correlative deficiencies in either Treg cell quality or quantity also characterize several human autoimmune diseases such as MS, rheumatoid arthritis and type I diabetes (Ehrenstein et al., 2004; Viglietta et al., 2004; Lindley et al., 2005).

Since autoimmunity is associated with disturbances in the functional balance between immunoregulatory and inflammatory cytokines, the ability to induce, expand or improve Treg cell suppressive function may have great impact in treating autoimmunity. The present work adds to the current knowledge of molecular pathways underlying the induction of tolerance in EAE by targeting the GALT with a synapsin peptide fused to LTB; therefore, this approach may be useful to modulate CNS inflammation in EAE and MS.

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