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# TREATMENT WITH A HYBRID BETWEEN THE SYNAPSIN ABC DOMAINS AND THE B SUBUNIT OF *E. COLI* HEAT-LABILE TOXIN REDUCES FREQUENCY OF PROINFLAMMATORY CELLS AND CYTOKINES IN THE CENTRAL NERVOUS SYSTEM OF RATS WITH EAE

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**Abstract—Multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE), are crucially dependent on the invasion of activated autoreactive lymphocytes and blood macrophages into the central nervous system (CNS). Proinflammatory mononuclear cells and activated local microglia mediate inflammation, demyelination and axonal damage at the target organ. Previously, we observed that the administration of a hybrid between the synapsin ABC domains and the B subunit of *Escherichia coli* heat labile-enterotoxin (LTBABC) to rats with EAE ameliorated disease by modulating the peripheral Th1 response to myelin basic protein (MBP). In the present study, we investigated the effect of LTBABC administration on proinflammatory cell frequency in the CNS of rats with EAE. Treatment with the hybrid in the inductive phase of EAE attenuated disease severity and diminished histological inflammatory infiltrates and demyelination in the spinal cord of rats with acute EAE. Lower frequencies of infiltrating and local macrophages as well as CD4+ T cells that produce the proinflammatory cytokines interferon-gamma (IFN-γ) and interleukin (IL)-17 were found at the target organ. Concomitantly, low levels of INF-γ and IL-17 and increased levels of IL-10 were measured in cultures of CNS infiltrating cells and spinal cord tissue. An increased frequency of CD4+CD25+Foxp3 cells was observed at the disease peak and at the beginning of the recovery stage. These results provide further evidence for the immunomodulatory properties of the fusion protein LTBABC in autoimmune demyelinating disease affecting the central nervous system.**



**Key words: experimental autoimmune encephalomyelitis, autoimmunity, synapsin, *E. coli* labile enterotoxin, immunomodulation.**

## INTRODUCTION

Multiple sclerosis (MS) is the most common inflammatory and demyelinating disease of the central nervous system (CNS). MS is a complex autoimmune disease that involves early dysfunction of the homeostatic mechanisms that maintain T cell tolerance to myelin antigens. Diverse inflammatory reactions mediated by extensive mononuclear leukocyte (phagocyte) infiltration and proinflammatory cytokine secretion initiate damage to myelin, oligodendrocytes and axons in the CNS of MS patients (McFarland and Martin, 2007; Comabella and Khoury, 2012).

Experimental autoimmune encephalomyelitis (EAE) is used as an animal model of MS. EAE development includes at least one acute episode of paralysis followed by spontaneous resolution or variable persistence of clinical neurological signs, which vary according to the animal species, strain and immunogens used (Mix et al., 2010). EAE in rodents is characterized histologically by CNS inflammation accompanied by a variable degree of demyelination and axonal damage (Zhu et al., 2003), which correlates with neurological deficiencies (Mix et al., 2010). Inflammatory lesion foci are populated by blood-derived infiltrating monocytes/macrophages, activated microglia, T cells and B cells (Sriram et al., 1982). Activated CD4+ T cells, blood macrophages and local microglia act as important effectors of inflammation and demyelination, orchestrating the secretion of elevated levels of several proinflammatory cytokines, chemokines and other inflammatory mediators (Hill et al., 2004; Murphy et al., 2010). In EAE, the peripheral activation of T cells promotes the differentiation of naïve CD4+ T cells into pathogenic T helper 1 (Th1) and T helper 17 (Th17) cells that produce the proinflammatory cytokines interferon-gamma (IFN-γ) (CD4+ interleukin (IL)–IFN-γ+) and IL-17 (CD4+ IL–IL-17+), respectively. It is accepted that IFN-γ is closely related to the pathogenesis of EAE (O'Connor et al., 2008). However, IL-17 has been

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*Abbreviations:* CFA, complete Freund's adjuvant; CNS, central nervous system; dpi, days post-immunization; EAE, experimental autoimmune encephalomyelitis; ELISA, enzyme-linked immunosorbent assay; HE, hematoxylin and eosin; LTB, *E. coli* heat-labile toxin B subunit; LTBABC, hybrid between the ABC domains of synapsin and the B subunit of the heat-labile toxin of *E. coli*; IFN-γ, interferon-gamma; IL, interleukin; MBP, myelin basic protein; MS, multiple sclerosis; PBS, phosphate-buffered saline; Treg cells, T regulatory cells.

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attributed to major pathogenic potential in the CNS autoimmunity because mice deficient in IL-17 or the IL-17 inducing IL-23 (Komiyama et al., 2006; Langrish et al., 2005) and due to the fact that mice treated with IL-17 neutralizing antibodies were resistant to induction or developed milder EAE (Park et al., 2005; Hofstetter et al., 2005; Uyttenhove and Van Snick, 2006).

Activated CD4+ T cells, specifically Th1 and Th17 cells, migrate across the disrupted blood–brain barrier, undergo activation by local antigens and infiltrate into the meninges and parenchyma of the CNS through complex mechanisms mediated by adhesins and chemokines (Reboldi et al., 2009; Bartholomäus et al., 2009).

Strategies to reduce the recruitment of blood-derived reactive T cells and macrophages and to decrease local microglia activation and modulate the cytokine microenvironment in the target tissue could result in amelioration of disease severity and damage to the CNS (Peron et al., 2010; Starossom et al., 2012). Oral tolerance to myelin antigens ameliorates EAE autoimmune pathology through proinflammatory cytokine modulation and the induction of CD4+CD25+Foxp3 T regulatory (Treg) cells (Chen et al., 1994; Scerbo et al., 2012). The forkhead/winged helix transcription factor Foxp3 expressed by natural Treg cells has been related to their development and regulatory functions (Sakaguchi et al., 2008). Treg cells exert their action via the secretion of immunosuppressive cytokines by interfering with the metabolic pathways of T cells or by contact inhibition (Sakaguchi et al., 2008; Josefowicz et al., 2012).

An attractive strategy to potentiate oral tolerance by the induction of Treg cells is to administer antigens genetically fused to a mucosal adjuvant, such as the atoxic B subunit of the cholera toxin or its close relative the *Escherichia coli* heat-labile toxin (Sun et al., 2010; Odumosu et al., 2010). Along this line, we have taken advantage of the functional cross-reactivity between myelin basic protein (MBP) and synapsin Ia (Degano and Roth, 2009) and used synapsin peptides for immunomodulating EAE (Scerbo et al., 2009, 2012). We have recently reported that oral administration of ABC synapsin peptide genetically fused to the B subunit of *E. coli* labile toxin (LTBABC) modulated the Th1 type of MBP reactivity in lymphoid nodes from rats with EAE (Bibolini et al., 2012). However, the effect of LTBABC administration on demyelination and inflammatory infiltration in the CNS of rats with EAE has not been sufficiently studied. In this study, we determined the frequency of local and infiltrating cells of myeloid lineage (CD11b+CD45hi) and the proinflammatory CD4+ T cells that secrete IFN-$\gamma$ and IL-17 or both cytokines (Suryani and Sutton, 2007). To explore a putative immunomodulatory mechanism of the hybrid at the target organ, the cytokine balance was determined based on the levels of the relevant proinflammatory cytokines IFN-$\gamma$ and IL-17, the modulatory cytokine IL-10 and the frequency of CD4+Foxp3+ Treg cells.

## EXPERIMENTAL PROCEDURES

### Animals

Forty-five-day-old male and female Wistar rats weighing 120–130 g that were inbred at our institution and maintained under SPF conditions in the vivarium were used for the *in vivo* studies. Rats (four to five per cage) were kept in an environment with controlled temperature and humidity. They were housed under a 12-h-reversed light–dark cycle and allowed free access to food and water. All animal experiments were conducted in accordance with the National Institutes of Health regulations (NIH Publications, NIH No. 40-23, revised 1996), and the Institutional Animal Care Committee approved all animal handling and experimental procedures (Exp. No. 15-99-40426). Every effort was made to minimize both the number of animals used and their suffering.

### Antigens

Myelin from bovine brains was isolated according to Norton and Poduslo (1973). MBP was purified from bovine spinal cords according to Deibler and Martenson (1973). Recombinant LTB, ABC and the hybrid protein LTBABC were obtained from cultures of *E. coli* harboring the corresponding expression vectors. Construction of expression vectors and the expression and purification of bioactive forms of *E. coli* heat-labile toxin B subunit (LTB), LTBABC and ABC in *E. coli* cells were performed as previously described (Bibolini et al., 2012).

### Induction and assessment of EAE

EAE was induced (day 0) in rats by intradermal injections in both hind feet with 0.5 ml of an emulsion prepared as follows: per rat, 8 mg purified whole myelin from bovine spinal cord was suspended in 0.25 ml saline solution, mixed at 1:1 ratio with Complete Freund's adjuvant (CFA; Sigma–Aldrich, St Louis, MO, USA) and the mixture was aspirated and expelled through a syringe with a 21G-needle until a stable emulsion was formed. Animals were examined daily for clinical signs of disease and body weight variations. The degree of the clinical signs developed during the acute stage of the disease was scored as follows: 0, no clinical expression of the disease; 1, flaccid tail; 2, hind-limb paralysis; 3, hind-limb paralysis accompanied by urinary incontinence; 4, hind limb and fore limb paralysis; and 5, moribund state or death. Rats were sacrificed at 13 or 21 days post-immunization (dpi).

### Administration of recombinant proteins

EAE was induced, and the rats were randomized into different treatment groups following an oral regimen, as previously described (Bibolini et al., 2012). Briefly, rats were fed 0.3 ηmol of either LTBABC, an equimolar mixture of LTB plus ABC, LTB, ABC or vehicle alone (1 M ultra pure urea in phosphate-buffered saline (PBS), control group) on days 0, 2, 4, 6, 8 and 10 after EAE induction. Recombinant proteins in 0.2 ml of vehicle were administered with a feeding needle (Popers and Sons Inc., New York, NY, USA).

### Histological analysis

Rats were anesthetized and perfused intracardially with ice-cold PBS followed by the same volume of 4%

paraformaldehyde (w/v) in PBS, pH 7.4. Spinal cords were removed and fixed in the same solution for 24 h. The lumbar segments of the spinal cords (L2–L4) were isolated and embedded in paraffin for sectioning and staining. Paraffin-embedded spinal cords were sectioned at 5 μm, deparaffinized, rehydrated and processed with hematoxylin and eosin (HE) staining for inflammatory infiltrates or Luxol Fast Blue (LFB) for demyelination. Tissue sections from three rats per group (five serial sections per animal) were examined with an Axiovert 200 microscope, and images were captured using a camera and AxioVision Imaging software. The inflammation was evaluated based on the extent of cell infiltration and frequency of cell cuffing, according to the following scale: 0, no inflammatory cells; 1, inflammatory cells limited to the meninges and the subdural space; 2, inflammatory cells in the meninges, perivascular space and parenchymal infiltrates; 3, inflammatory cells in the perivascular space and scarce cell cuffing in the parenchyma; and 4, frequent cell cuffing in the perivascular space and abundant inflammatory cuffing in the parenchyma. Histologic white matter demyelination was scored as follows: 0 normal myelination; (1) mild demyelination, small focal areas of demyelination; (2) moderate demyelination, small and some wider areas of demyelination; and (3) severe demyelination, abundant areas of demyelination.

### Isolation of CNS-infiltrating mononuclear cells

For the analysis of CNS inflammatory cells, rats were deeply anesthetized and perfused through the left cardiac ventricle with cold PBS. The brain and spinal cord were aseptically dissected from each animal. Tissue was cut into small pieces and digested in PBS containing 1.5 mg/ml collagenase D (GIBCO Life Technologies, Carlsbad, CA, USA) and 50 μg/ml deoxyribonuclease (Sigma–Aldrich) at 37 °C for 45 min followed by mechanical disruption. The homogenate was passed through a cell strainer (70 μm, BD Biosciences, San Jose, CA, USA), adjusted to 30% (v/v) Percoll (Sigma–Aldrich) and overlaid over a 30%/70% discontinuous Percoll gradient followed by centrifugation at 400*g* for 20 min at 4 °C. Mononuclear cells were recovered from the 30%/70% Percoll interface and washed twice with RPMI 1640 (GIBCO Life Sciences, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (GIBCO Life Sciences, Carlsbad, CA, USA). Viable cell number was determined by Trypan Blue exclusion.

### Cell staining and flow cytometric analysis

Anti-CD45-biotin, anti-CD11b-PE (AbD Serotec, Raleigh, NC, USA), anti-CD4-FITC and anti-CD25 (eBioscience, San Diego, CA, USA) were used for the phenotype analysis of CNS-infiltrating cells. For intracellular Foxp3 staining, cells that were previously stained with anti-CD4-FITC antibodies (eBioscience) were fixed, permeabilized and stained with anti-Foxp3-PE according to the manufacturer's protocol (eBioscience). Biotin-coupled antibodies were used in conjunction with SAv–APC conjugate.

The detection of IFN-γ and IL-17-producing cells was performed using intracellular staining after a short stimulation (4 h) with phorbol myristate acetate (100 ηg/ml) and ionomycin (400 ng/ml) in the presence of monensin (2 μM) (all from Sigma–Aldrich). First, cells were stained for CD4 by incubation with anti-CD4-FITC antibody for 30 min at 4 °C. Subsequently, the cells were fixed, permeabilized and stained with PE- and APC-conjugated anti-INF-γ and anti-IL-17, respectively, and the corresponding control isotypes (all from eBiosciences) according to the manufacturer's instructions. Samples were acquired on a FACSCanto Flow Cytometer (BD Biosciences and BD Argentina, Buenos Aires, Argentina), and data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

### Preparation of supernatants from spinal cord homogenates

Rats were deeply anesthetized and perfused through the left cardiac ventricle with cold PBS. The spinal cords were collected and dissected into cervical, thoracic and lumbar regions. Each spinal cord region was homogenized with PBS and centrifuged at 10,000*g*, and the supernatant was separated and stored at −20 °C for cytokine quantification. The protein content of the supernatants was determined by a Bradford–based Protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

### Cytokine detection

CNS-infiltrating cells in culture were seeded in 24-well plates ($5 \times 10^6$/ml) with RPMI 1640 medium supplemented with 30 μg/ml gentamycin, 10% fetal calf serum (GIBCO) and 50 μM 2-mercaptoethanol. Cells were stimulated for 48 h with 75 μg/ml MBP and then separated by centrifugation (500*g* for 5 min), and cell-free supernatants were frozen until cytokine determination. The concentrations of INF-γ, IL-17 and IL-10 were measured by enzyme-linked immunosorbent assay (ELISA) according to the protocol recommended by the manufacturers of the ELISA BD OptEIA Set (BD Biosciences).

### Statistical analysis

Statistical analyses were performed using the computer-based GraphPad Prism Program V5.0 (GraphPad Software Inc., San Diego, CA, USA). Data are presented as the mean ± SEM. The quantitative data between groups were analyzed by a one-way analysis of variance (ANOVA), and the Tukey multiple comparison test was used to examine statistical significance for the mean values. Differences in clinical score and percentage of body weight variations were analyzed by the Kruskal–Wallis test. *P* values less than 0.05 were considered significant.

## RESULTS

### Oral treatment with LTBABC alleviates clinical severity of EAE

EAE was induced in rats with an emulsion of myelin in CFA (0 dpi), and some rats were chosen at random for treatment with 0.3 ηmol LTBABC, an equivalent amount

of LTB + ABC, LTB or ABC separate proteins or vehicle (control animals) on alternating days from 0 to 10 dpi. When indicated, one group of rats received only an emulsion containing PBS and CFA (CFA group) administered in the same way. All animals were monitored daily for signs of clinical disease and body weight variation. Approximately 97% of rats challenged for EAE developed a monophasic acute disease with a peak of disease severity at approximately 13 dpi. Clinical signs of EAE included loss of tail tone, hindlimb paralysis and urinary incontinence. Development of neurological disabilities was associated with weight loss (Bibolini et al., 2012). However, rats treated with LTBABC lost less body weight compared with the control (vehicle) and LTB + ABC groups. As expected, animals challenged only with CFA did not develop EAE and showed no weight loss (Fig. 1A). In all sick rats, the peak of the disease was followed by a progressive decline of clinical signs to a minimum, which was observed at approximately 20 dpi. The group that received LTBABC showed a significant decrease in clinical severity of EAE between 11 and 14 dpi, but the administration of an equimolar amount of the separated molecules (LTB + ABC) failed to diminish the EAE clinical score at the symptomatic period (Fig. 1B). As previously observed (Bibolini et al., 2012), a similar treatment involving the administration of LTB, ABC or 0.15 ηmol LTBABC did not significantly affect the course or severity of EAE compared to vehicle-treated rats (results not shown). Hence, most of the shown experiments compared results for treatments with LTBABC or LTB + ABC.

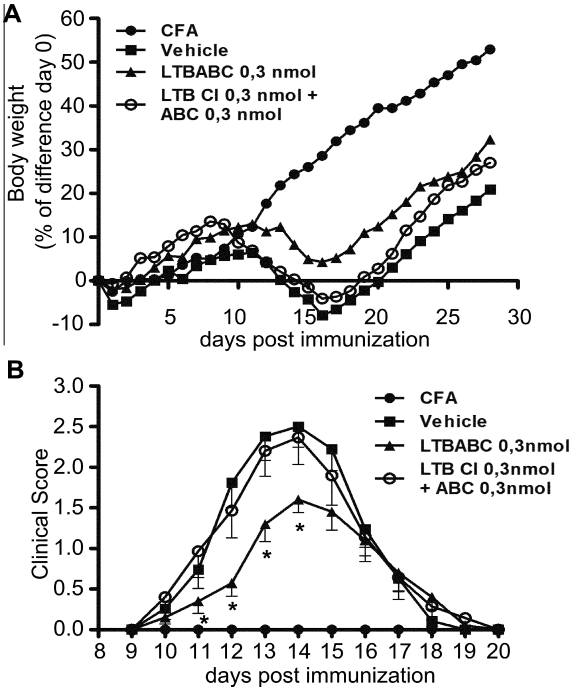


**Fig. 1.** LTBABC ameliorates EAE. Rats were challenged with myelin in CFA (0 dpi) and LTBABC, and an equivalent amount of LTB + ABC or vehicle was orally administered every other day from 0 to 10 dpi. Animals were monitored for clinical signs of disease and body weight variations. (A) Body weight variations measured as percentage of body weight at 0 dpi. Results from one experiment performed with six animals per group are shown. (B) Disease scores related to neurological impairment as indicated in the Experimental Procedures. Values are the mean ± S.E.M. from two experiments performed with six animals per group. $^{*}P < 0.05$.

### Attenuation of CNS histopathological inflammation and demyelination

HE staining of spinal cord sections from animals challenged for EAE and sacrificed at 13 dpi showed marked meningeal, perivascular and parenchymal mononuclear infiltration, mainly in white matter, which was similar to what was previously observed in our EAE model (Scerbo et al., 2009). Rats treated with LTBABC clearly showed lower inflammation scores compared with animals treated with vehicle (Fig. 2A). Demyelination was also investigated in spinal cord sections. Demyelinated areas in the spinal cords were often correlated with inflammatory lesions in the histological sections of all groups, but marked demyelination was indistinguishable between the control and the LTB + ABC groups. Contrarily, lower demyelination scores were observed in sections from rats treated with LTBABC compared with controls (Fig. 2A).

### LTBABC reduces macrophage frequency in the CNS of treated rats

To ascertain whether oral treatment with LTBABC affects the frequency of macrophages in the CNS of rats challenged for EAE, infiltrating blood-derived macrophages (monocytes and polymorphonuclear (PMN) cells) and local activated microglia (CD11b + CD45hi) were analyzed by flow cytometry. Activated microglia up-regulate their CD45 expression to high levels, which has been related to antigen-presenting ability (Sedgwick et al., 1991; Carson et al., 1998), while resting microglia show low expression of CD45. Mononuclear cells isolated from the whole CNS of animals from the three experimental groups were isolated and treated with CD11b antibody (myeloid cell lineage) and CD45 antibody. Treatment with LTBABC diminished the proportion of CD11b + CD45hi cells by approximately 50% compared with the control and LTB + ABC groups. The number of CD11b + CD45hi cells was also diminished in the LTBABC-treated rats compared with the controls. The separated LTB + ABC molecules induced much less marked diminution of the percentage of pathogenic CD11b + CD45hi cells compared with the controls (Fig. 3A). On the other hand, the administration of the hybrid also reduced the percentage and number of CD11b + CD45lo cells in the rat CNS (Fig. 3B). At 21 dpi, when the clinical signs declined, the proportions and number of CD11b + CD45hi cells were generally diminished compared with the values observed at 13 dpi and the percentage of CD11b + CD45lo cells increased compared with 13 dpi. However, no difference between experimental groups was observed for CD11b + CD45hi and CD11b + CD45lo cell percentage or number at 21 dpi (results not shown).

### LTBABC reduces frequency of proinflammatory CD4+ T helper cells

In EAE, pathogenic CD4+ T cells are mainly Th1 or Th17- type, which secrete the proinflammatory cytokines

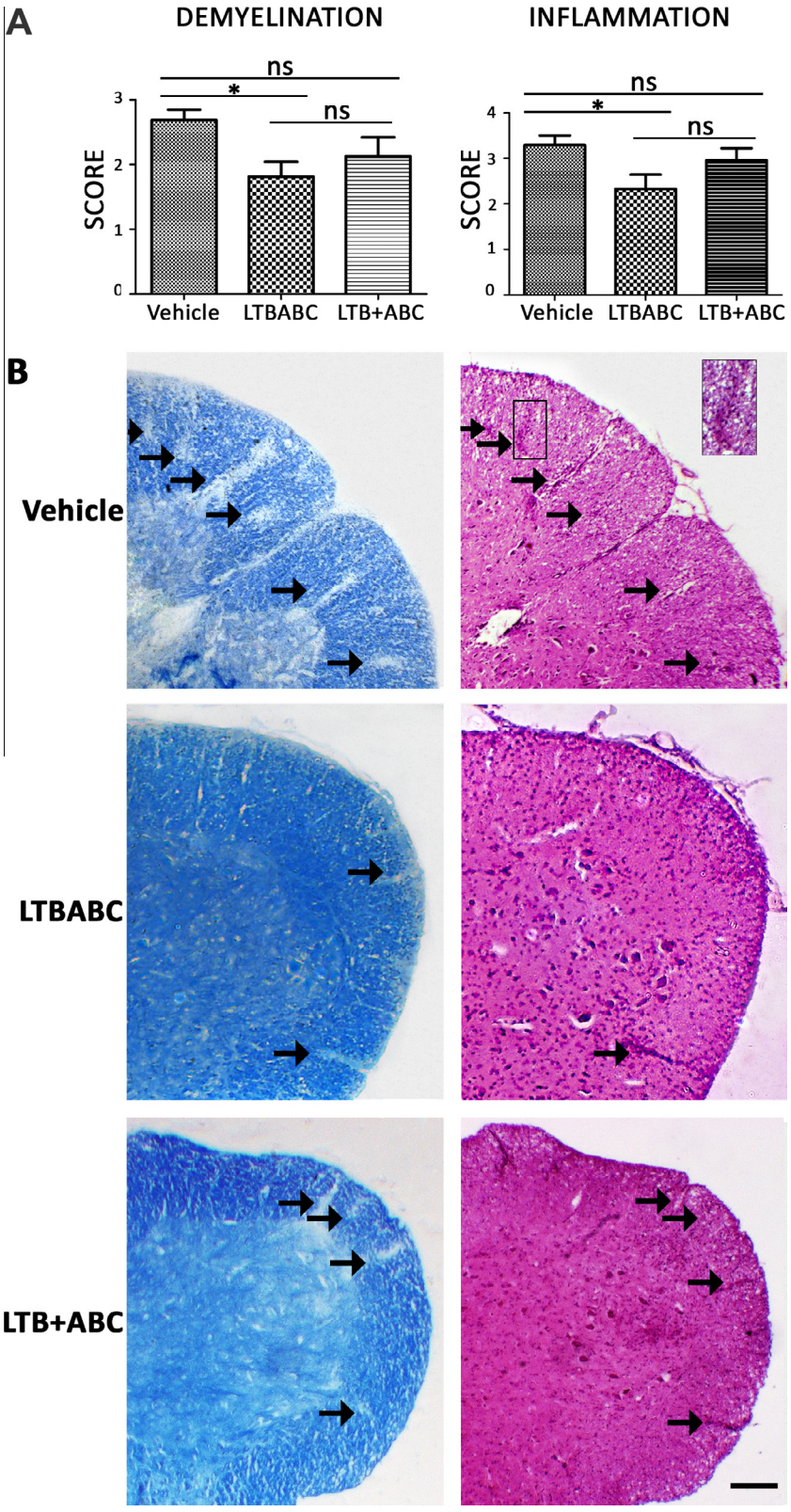


**Fig. 2.** LTBABC treatment protects CNS from histological inflammation and demyelination. Lumbar spinal cords were excised at 13 dpi from rats treated with LTBABC, LTB + ABC or vehicle, embedded in paraffin and 5 μm-sections were stained with HE or LFB. (A) Inflammatory cell infiltrates and demyelination were scored as described in the Experimental procedures; data are representative sections from three rats examined per group. $^{*}P < 0.05$, significant difference between treated group and vehicle. (B) Representative histological sections stained with HE and LFB from spinal cords corresponding to the control, LTBABC, and LTB + ABC groups. Arrows point to coincident inflammatory foci and demyelinated areas. Amplification of inflammatory foci is shown in the insert. Scale bar = 30 μm.

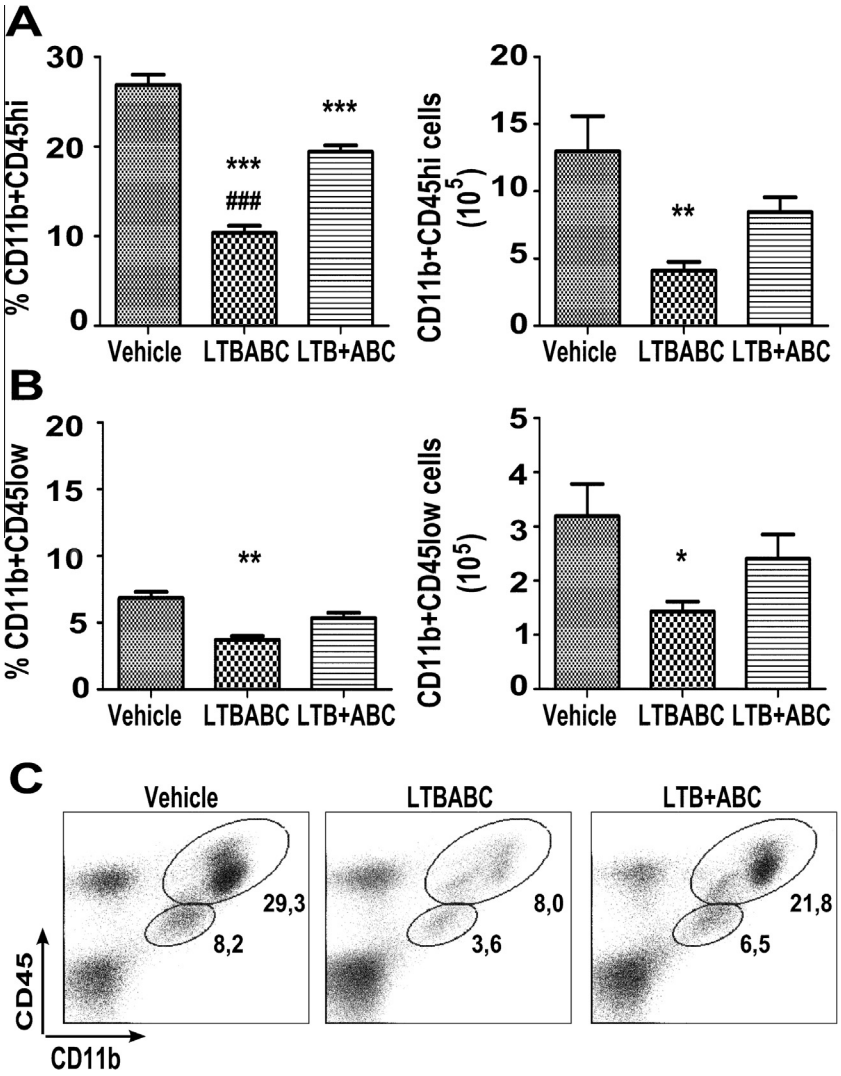


**Fig. 3.** LTBABC treatment diminished infiltration of CD11b + CD45hi cells in the CNS at the acute stage of EAE. Mononuclear cells from the whole CNS were isolated from rats challenged for EAE and treated with LTBABC, LTB + ABC or vehicle at 13 dpi and stained for CD45 and CD11b molecules and analyzed by flow cytometry. (A) CD11b + CD45hi (PMN, monocytes from blood and local activated microglia) and resting microglia (CD11b + CD45lo) (B) were distinguished on the basis of differential CD45 antibody staining. Results from one of three experiments with similar results performed with three rats per group are shown. Significant differences between the LTBABC group and vehicle ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) and LTBABC and LTB + ABC ($^{\#\#\#}P < 0.001$) are indicated. (C) Representative dot plots of CD11b + CD45hi and CD11b + CD45lo cells from one animal from each experimental group are shown.

IFN-γ and IL-17, respectively, or secrete both cytokines. To explore the ability of LTBABC treatment to limit these proinflammatory populations in the target organ, we used flow cytometry to estimate the abundance of pathogenic CD4 + T cells in the CNS by intracellular staining of IFN-γ and IL-17. In general, greater percentages and numbers of CD4 + IFN-γ + and CD4 + IL-17 + cells were found compared with CD4 + cells secreting both cytokines (CD4 + IFN-γ + IL-17 + cells). At 13 dpi, treatment with LTBABC greatly diminished the percentage and number of CD4 + IL-17 + (Fig. 4A) and CD4 + IFN-γ + IL-17 + (Fig. 4C), and less markedly decreased CD4 + IFN-γ + (Fig. 4B) cells compared with the control (vehicle). Comparatively, treatment with LTB + ABC showed much less ability to diminish the proportion of the three proinflammatory populations in the CNS.

At 21 dpi concomitantly with the initiation of the recovery period, lower number of the three cell populations was found in all groups compared to 13 dpi. The frequency of CD4 + IL-17 + and CD4 + IFN-γ + IL-17 + cells were similar in all experimental groups, meanwhile the proportion of CD4 + IFN-γ cells were lower in the group treated with LTBABC compared to the control group (results not shown).

### LTBABC modulates proinflammatory cytokine levels

We evaluated whether the lower frequency of Th17 and Th1 proinflammatory cells in the CNS of rats treated with LTBABC at the acute phase was associated with a down-regulation of proinflammatory cytokines (IL-17 and IFN-γ). Additionally, we determined the levels of IL-10 which is a major modulatory cytokine. Cells infiltrating

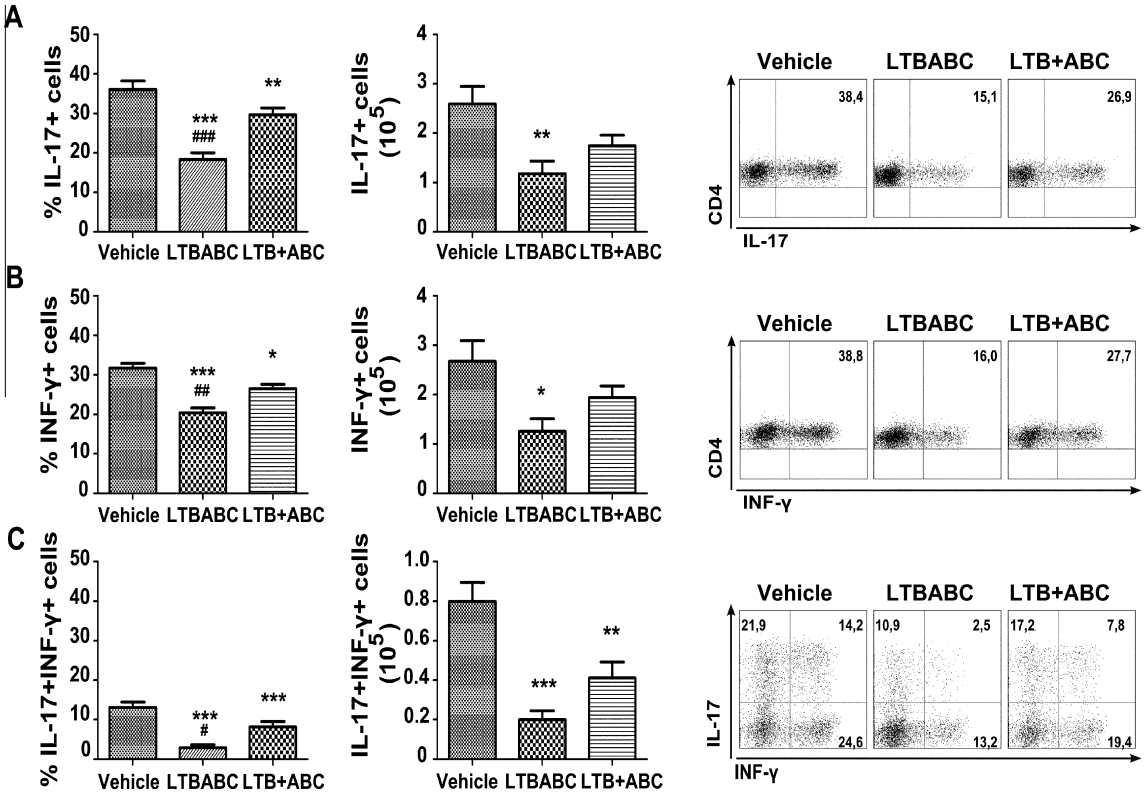


**Fig. 4.** LTBABC treatment in EAE modulated proinflammatory CD4 T cells secreting IL-17, IFN-γ and both cytokines in the CNS of rats with EAE. Mononuclear cells from the whole CNS of rats challenged for EAE and treated with equivalent amounts of LTBABC and LTB + ABC or vehicle were isolated at 13 dpi and stained for CD4 and intracellularly for IFN-γ and IL-17 and analyzed by flow cytometry. Results correspond to CD4+ cells positive for IL-17+ (A), IFN-γ+ (B), and IFN-γ-IL-17+ (C) gated in the CD4+ T cell population. Results (mean ± S.E.M.) correspond to one of three experiments with similar results performed with three rats per group. Significant differences between LTBABC and control group are $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$. Representative dot plots of the three cell populations analyzed from one individual of each experimental group are shown in the corresponding right panels.

the CNS were collected at 13 dpi and stimulated in culture with MBP. Cytokine concentrations were determined in culture supernatants by ELISA. Infiltrating cells from rats treated with LTBABC produced less MBP-specific IL-17 (Fig. 5A) and IFN-γ (Fig. 5B) than the controls. In contrast, higher amounts of IL-10 were found in rats treated with LTBABC compared to the controls (Fig. 5C). To further assess the target organ cytokine microenvironment, IFN-γ, IL-17 and IL-10 levels were determined in supernatant from homogenates obtained from the cervical, thoracic and lumbar regions of spinal cords. The results observed were consistent with those obtained from the supernatant of infiltrating cell cultures stimulated with MBP. Supernatant of spinal cord homogenates from rats treated with LTBABC contained lower levels of IL-17 (Fig. 6A) and IFN-γ (Fig. 6B) but contained increased levels of IL-10 (Fig. 6C) in all regions (except cervical IL-17). LTB + ABC only slightly diminished IL-17 levels in the lumbar spinal region (Fig. 6A).

### Expansion of CD4+Foxp3+ Treg cells in the CNS

The expansion of Treg cells has been associated with suppression or spontaneous recovery from EAE (Chen et al., 1994; Scerbo et al., 2012). We determined the abundance of CD4+FoxP3+ Treg cell population in the CNS of rats from the three experimental groups at 13 and 21 dpi. There was an abundance of CD4+Foxp3+ Treg cell population in the CNS of rats treated with LTBABC at 13 dpi that persisted or even increased at 21 dpi (Fig. 7A). However at 21 dpi, the CNS of rats treated with LTB + ABC also showed CD4+Foxp3+ Treg cell expansion compared with controls (Fig. 7B).

## DISCUSSION

The present study demonstrates the beneficial effect of the fusion protein LTBABC on CNS inflammation and demyelination in an acute EAE rat model. Oral administration of recombinant hybrid LTBABC throughout the inductive phase of disease diminished the clinical score and weight loss at the acute stage of disease. Myelin antigens are common early targets of the immune attack in MS and EAE, and demyelination appears as a consequence of the inflammatory process mediated by cells of the adaptive and innate immune system (Bhat and Steinman, 2009). In this study, spinal cord sections from rats with EAE treated with LTBABC showed reduced mononuclear cell infiltration and myelin loss score. Additionally, the attenuation of EAE by LTBABC concurred with the modulation of the major pathogenic proinflammatory cell populations in the CNS from rats with EAE. The results of this study and other

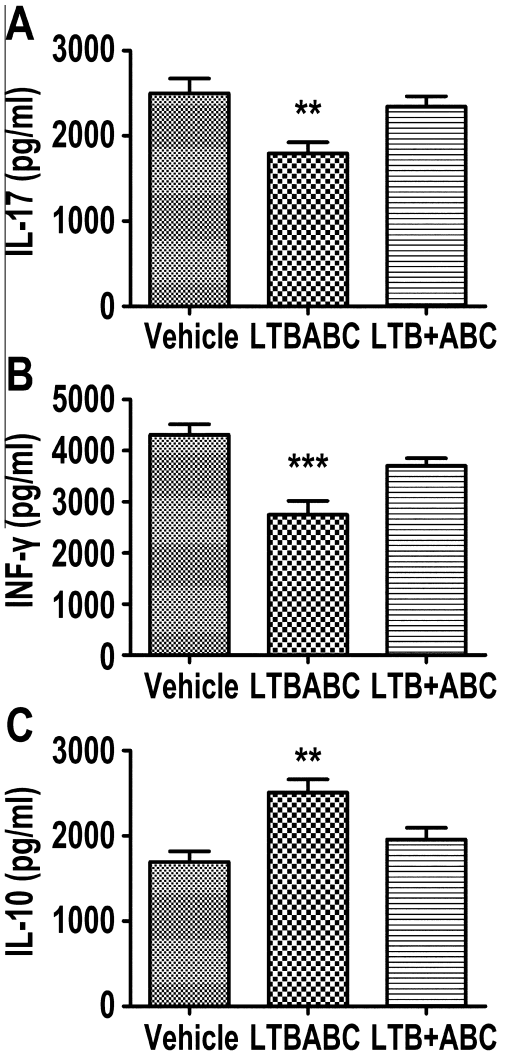


**Fig. 5.** LTBABC treatment decreased myelin basic protein-specific IL-17 and IFN-γ and increased secretion of IL-10 in rats with EAE. Infiltrating cells were isolated from the whole central nervous system from rats treated with LTBABC, LTB + ABC or vehicle at 13 dpi. Cells were stimulated in culture with myelin basic protein, and IFN-γ (A), IL-17 (B) and IL-10 (C) were measured in culture supernatants as described in the Experimental procedures. Data (mean ± S.E.M.) correspond to one of three experiments with similar results performed with three animals per group. Differences between LTBABC and vehicle were: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

(Bibolini et al., 2012) support administrating ABC synapsin peptide fused to the atoxic LTB adjuvant instead of the separated peptides.

We found that infiltrating and local macrophages (CD11b + CD45hi cells) were less frequent in the CNS of rats treated with LTBABC compared with the control and LTB + ABC groups. Monocyte-derived macrophages and local activated microglia interact with T cells that are recruited to the inflamed CNS and may secrete cytokines and other mediators of inflammation. However, macrophage–microglia cells are regarded as a population involved in several functions like antigen presentation, damage and repairing processes in the CNS (Chastain et al., 2011; Gao and Tsirka, 2011). Severity of EAE was associated with abundance of macrophages and up regulation of TNF-α and inducible nitric oxide synthase expression in these cells (Ahn et al., 2001). The pathogenic role of macrophages was proved in a previous study which showed that blocking macrophages ameliorated EAE in rats (Huitinga et al., 1993).

Treatment with LTBABC also decreased the frequency of CNS CD4 + IFN-γ +, CD4 + IL-17 + and CD4 + IFN-γ + IL-17 + cells at the peak of the disease, all proinflammatory populations involved in EAE and MS pathology (Kebir et al., 2009; El-behi et al., 2010; Murphy et al., 2010). CD4 + T helper cells are found at the peak of disease in the CNS of rats and mice with EAE (Rigolio et al., 2008; O'Connor et al., 2008) and they can transfer EAE to susceptible animals (Ben-Nun et al., 1981). Frequency of CD4 + T cells also correlated with attenuation of clinical signs in several treatments to ameliorate EAE (van der Laan et al., 2002; Peron et al., 2010; Scerbo et al., 2012). Coincidently, we observed that LTBABC treatment exerted marked diminution of the frequency of proinflammatory CD4 + T cells and CD11b + CD45hi cells in the CNS and concomitantly ameliorated neurological signs at the acute stage in our monophasic EAE model.

Consistent with the reduced frequency of CD4 + proinflammatory T cells at the clinical peak of the disease, the corresponding levels of IFN-γ and IL-17 secreted in response to MBP as well as the cytokine levels in the tissue were significantly reduced in animals treated with LTBABC. Most importantly, under the same conditions, mononuclear cells from LTBABC-treated rats secreted increased levels of the modulatory cytokine IL-10. This cytokine is involved in natural recovery as well as in the suppression of EAE (Rott et al., 1994; Zhang et al., 2004; McGeachy and Anderton, 2005), and it is most likely responsible for the balance of the EAE proinflammatory microenvironment assessed in the CNS of LTBABC-treated rats.

Administration of LTBABC from 0 to 10 dpi influenced the peripheral immune system probably affecting disease induction through the modulation of proinflammatory cytokine milieu and inducing expansion of Treg cells and this effect later attenuated the inflammatory status of the CNS. We previously observed that LTBABC treatment attenuated the Th1 response to MBP in lymph nodes by modulating the proinflammatory cytokine microenvironment based on a reduction of the INF-γ/IL10 ratio, although no changes in IL-17 secretion were observed (Bibolini et al., 2012). In consequence LTBABC peripheral immuno modulation controlled the cytokine pro inflammatory environment in the CNS even tighter than in the lymph nodes. Significant differences and higher levels of MBP-specific cytokines were clearly observed among experimental groups in the target organ compared to lymph nodes. CNS mononuclear cells from rats with EAE produced increased amounts of IL-17 (more than 10-fold), IFN-γ and IL-10 (2–5-fold) than inguinal lymph nodes.

Reduced proportions of the proinflammatory cells CD4 + IFN-γ +, CD4 + IL-17 + and CD4 + IFN-γ + IL-17 + and their respective cytokines in the CNS of rats treated

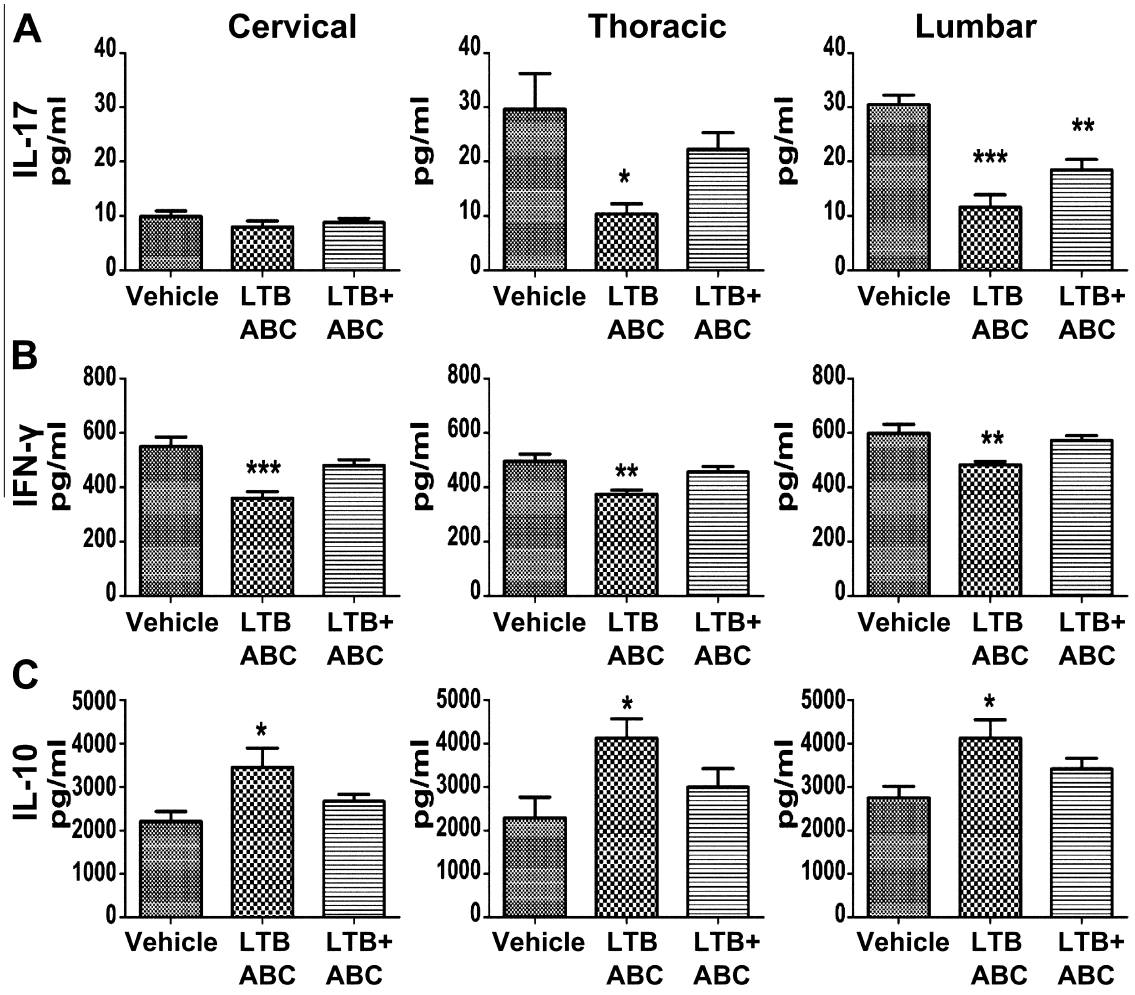


**Fig. 6.** LTBABC treatment in EAE modulated cytokine IL-17 and INF-γ levels and increased level of IL-10 in the CNS of rats with EAE. Rats were challenged for EAE and treated with LTBABC, LTB + ABC or vehicle as described in the Experimental Procedures. At 13 dpi, spinal cords were excised and the different regions were homogenized and analyzed for IL-17 (A), IFN-γ (B) and IL-10 (C) levels, as indicated in the Experimental procedures. Data (mean ± S.E.M.) correspond to one of three experiments with similar results performed with three animals per group. Differences between LTBABC and vehicle were: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

with LTBABC may account for the amelioration of clinical severity of EAE. Along these lines, oral tolerance in EAE has been associated with a reduction of Th17 cells and CNS inflammation in mice (Peron et al., 2010).

Additionally, the frequency of CD4 + Foxp3 + regulatory cells was increased in the CNS of rats treated with LTBABC not only at the EAE peak but also at 21 dpi, when clinical signs almost completely disappeared. Coincidently, several studies have shown that Treg cells play an important role in protection, recovery and regulation of disease severity (McGeachy et al., 2005; Tischner et al., 2006; O'Connor et al., 2007) The persistence of CD4 + Foxp3 Treg cells after the acute disease stage observed in LTBABC-treated rats suggested an enhancement in the homeostatic restoration process of tissue inflammation. Treg cells induced/expanded by LTBABC treatment may also regulate antigen presentation during the EAE inductive stage, but no specific studies were conducted to better define the regulatory mechanisms involving Treg cells in this work. We previously observed peripheral induction/expansion of Treg cells by LTBABC (Bibolini et al., 2012) and also by the related hybrid involving the C-synapsin domain and LTB (Scerbo et al., 2012), which resulted in functional Treg cells in *in vitro* assays. These results, as well as results from other authors (Sun et al., 2006 Sun et al., 2010; Odumosu et al., 2010), strongly indicate that the induction/expansion of Foxp3 Treg cells is a major immune regulatory mechanism associated with mucosal tolerance caused by hybrids involving cholera toxin B subunit (CTB) and the closely related LTB.

## CONCLUSION

Lower percentages of pathogenic CD11b + CD45hi macrophages and CD4 + T cells that are reactive against MBP and secrete proinflammatory IFN-γ and IL-17 were concomitant with modulation of the CNS cytokine microenvironment by IL-10 in rats treated with the hybrid LTBABC. The immunomodulatory effect of LTBABC at the target organ was correlated with an attenuation of clinical disease and lower histological inflammation and

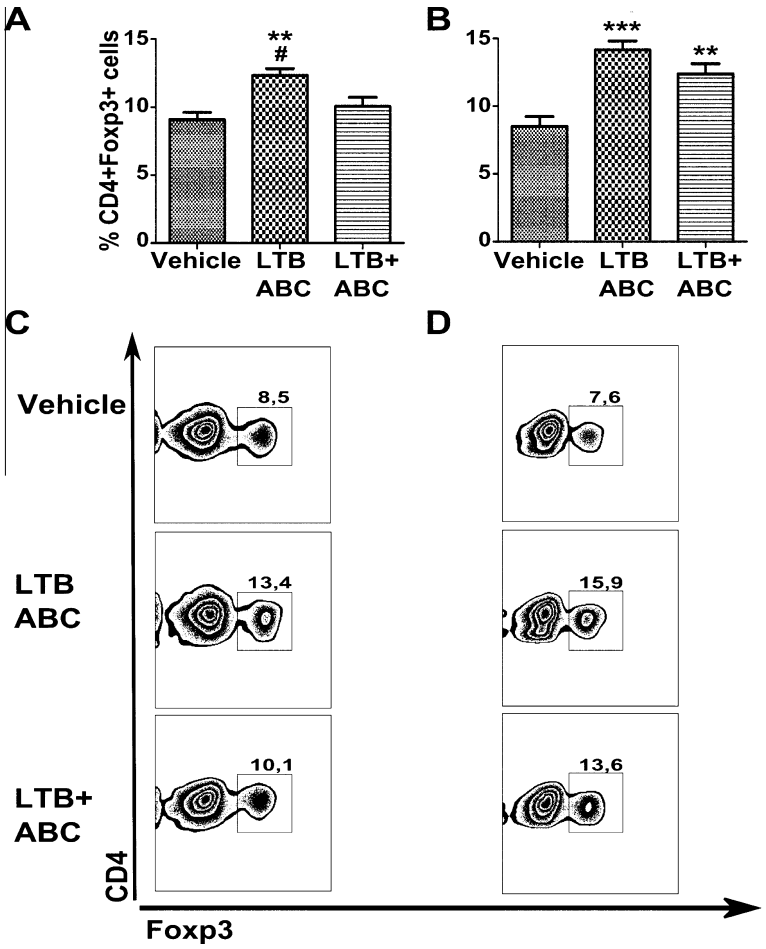


**Fig. 7.** LTBABC treatment expands CD4 + Foxp3 + T regulatory cells in the CNS from rats with EAE. Infiltrating cells from the CNS of rats challenged for EAE and treated with LTBABC, LTB + ABC or vehicle were isolated at 13 dpi (A) and 21 dpi (B), stained for CD4 and intracellular Foxp3 transcription factor and analyzed by flow cytometry. Results (mean ± S.E.M.) correspond to one of three (A) and one of two (B) experiments with similar results performed with three rats per group. The statistical difference between the experimental group and the control was: $^{**}P < 0.01$, $^{***}P < 0.001$, and the significant difference between LTBABC and LTB + ABC was $^{\#}P < 0.05$. Representative dot plots of CD4 + Foxp3 + gated on CD4 + T cells at 13 dpi (C) and 21 dpi (D) from one individual of each experimental group are shown.

demyelination in the spinal cords of LTBABC-treated rats compared with controls. This work supports the concept that oral tolerance induced by neuronal synaptic peptides (synapsin ABC domains) genetically coupled to the atoxic B subunits of the cholera toxin family is an effective tool for modulating the immunological milieu in the CNS and ameliorating inflammation caused by autoimmune responses to myelin antigens.

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