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The hybrid between the ABC domains of synapsin and the B subunit of *Escherichia coli* heat-labile toxin ameliorates experimental autoimmune encephalomyelitis

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ABSTRACT

The B subunit of *Escherichia coli* heat-labile enterotoxin (LTB) acts as efficient mucosal carrier for conjugated antigens. We expressed two heterologous proteins using *E. coli* as a host: a hybrid consisting of LTB and the A, B and C domain of synapsin (LTBABC) and the separated ABC peptide of this synaptic protein. Refolded LTBABC and LTB bound to the GM1 receptor and internalized into CHO-K1^{GM1+} cells. LTBABC showed enhanced solubility and cell binding ability respect to the former hybrid LTBSC. Several oral doses of LTBABC were administered to rats with experimental autoimmune encephalomyelitis (EAE) from induction to the acute stage of the disease. This treatment decreased disease severity, delayed type hypersensitivity reaction and lymph node cell proliferation stimulated by myelin basic protein. Amelioration of EAE was also associated with modulation of the Th1/Th2 cytokine ratio, increased TGF-β secretion in mesenteric lymph nodes as well as expansion of $CD4^+CD25^+Foxp3^+$ regulatory T cell population. These results indicate that the fusion protein LTBABC is suitable for further exploration of its therapeutic effect on EAE development.

1. Introduction

Type I *Escherichia coli* heat-labile enterotoxin (LT-I) and cholera toxin (CT) are the closest members of the AB5 toxin family constituted by five identical B chains and one A polypeptide. Both toxins exhibit more than 80% amino acid sequence homology in the A and B chains and also share functional and immunological properties [1]. The five B chains (~11 kDa) form a ring-like pentameric B subunit (CTB, LTB) [2,3]. Crystallographic data indicate that LTB is held by about twenty-six hydrogen bond and four salt bridges that confer great structural stability to the pentameric structure [4]. LTB and CTB are non-toxic subunits that recognize the oligosaccharide moiety of monosialoganglioside GM1 (II3NeuAc-Gg4Cer, Galb3Gal NAcb4-(NeuAca3) Galb4Glcb1Cer) with great affinity (Kd ~ 10^{-9} - M) [5]. Upon binding to GM1 containing-cells, the holotoxins or the B subunits undergo intracellular retrograde transport and they traffic through early and recycling endosomes, the Golgi and the endoplasmic reticulum (ER) [6]. During the course of the holotoxin natural action the A1-chain is released from the A-subunit in the ER and later translocated into the cytoplasm where it triggers signal transduction events leading to irreversible adenylate cyclase activation [7].

It was early shown that CTB and LTB may function as carriers for coupled antigens across the mucosal barrier and function as efficient mucosal adjuvants for antigens targeted to the gut associated lymphoid tissue (GALT) [8] and exhibit immunomodulatory properties [9,10]. In this direction, CTB and LTB have emerged as promising molecules for enhancing immunomodulation by tolerogenic antigens in several experimental autoimmune diseases [11]. Significant contributions have been made to the mechanisms involved in the suppression of autoimmunity by several self-antigens fused to CTB [11,12] and in type I diabetes model [13].

We have previously shown that the preventive oral administration of the well conserved- synapsin C-domain fused to LTB (LTBSC) [14] was very effective to induce oral tolerance in rats with experimental autoimmune encephalomyelitis (EAE). LTBSC diminished the incidence and histopathological features of this disease by decreasing cellular reactivity against myelin basic protein (MBP) and immunomodulation in EAE [15,16]. Although LTBSC was abundantly expressed in *E. coli* [14] it was unstable in solution. LTBSC exhibited greater tendency to aggregation than LTBABC and consequently, lost ability to bind to GM1 containing-cells.

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Since it was very relevant testing the therapeutic effect of the hybrid involving the synapsin C domain on EAE, in this study we aimed to evaluate the effect of a new hybrid on disease severity. We constructed LTBABC, a fusion protein between the synapsin C peptide preceded by the short N-terminal A and B domains and LTB. We found that biologically active LTBABC expressed in *E. coli* cells diminished clinical severity of EAE in rats and showed strong immunomodulatory effects.

2. Materials and methods

2.1. Antigens, animals

Myelin from bovine brains was isolated according to Norton and Poduslo [17]. MBP was purified from bovine spinal cords according to Deibler and Martenson [18].

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). Every effort was made to minimize both the number of animals used and their suffering.

2.2. Plasmid construction, expression and purification of recombinant proteins

E. coli expression vectors pET-SABC and pET-LTBABC with the coding sequences of ABC synapsin peptide and LTBABC respectively, were constructed using the polymerase chain reaction (PCR) method and standard recombinant DNA techniques. Briefly, the DNA fragment containing the coding sequence of the A, B, and C domains of rat synapsin Ia (ABC, residues 1-308) (NCBI GeneBank Accession No. M27872) with the sequence of the *Sal*I restriction site at 5′-terminus and the *Xho*I site at 3′-terminus were amplified by PCR using as template vector p*GEX*6-P1 (kindly donated by Dr. P. Greengard, The Rockefeller University, NY, USA). This DNA fragment was digested and inserted into the corresponding restriction sites of plasmid pET-26b(+) (Novagen) to generate plasmid pET-SABC.

For construction of the fusion protein LTBABC, the coding sequence of LTB without leader sequence (NCBI Gen Bank Accession No. M17874, residues 1-114) including the sequences for the *Nco*I restriction site at 5′-terminus and at the 3′-terminus for Gly-Pro.Gly Pro Glu-Asp and the sequence of the *Sal*I restriction site was amplified by PCR. This *Nco*I-*Sal*I PCR restriction fragment carrying the coding sequence of LTB for fusion construct (LTBpf) was cloned in the corresponding restriction sites of plasmid pET-26b(+) to generate plasmid pET-LTBpf. Then, the *Sal*I-*Xho*I PCR restriction fragments carrying the coding sequence of the ABC fragment was digested and inserted in the corresponding restriction sites of plasmid pET-LTBpf resulting in plasmid pET-LTBABC. Both proteins, LTBABC and ABC, have a 6 His tag fused in frame at the C-terminus to facilitate purification. Sequences of LTBABC and ABC constructs were confirmed by automated sequencing. The LTBABC construct is illustrated in Fig. 1.

Construction of plasmid carrying the LTB and LTBSC genes were previously described [14]. LTBABC, LTBSC and LTB were expressed in *E. coli* Codon-plus (λDE3) (Novagen) transformed by electroporation (Gene Pulser X cell, Biorad) with pET-LTBABC, pET-LTBSC and pET-LTB plasmids, respectively. Cells were grown in terrific broth (TB) containing kanamycin 20 μg/ml and 2% glucose (culture medium) and incubated at 37 °C until culture OD_{600} reached OD_{600} 0.6–0.8, when protein expression was induced by the addition of 0.4 mM IPTG and cells were further incubated at 20 °C for 6 h. Then, bacteria were harvested and disrupted in ice-cold extraction buffer (PBS 50 mM phosphate, 0.5 M NaCl, pH 8.0) in an EmulsiFlex-C3 apparatus (Avestin, Ottawa, Canada). LTBABC and LTB cytoplasmic inclusion bodies were separated by centrifugation at 10000*g* for 20 min and washed with Triton X100 and high ionic strength solution as previously described [14].

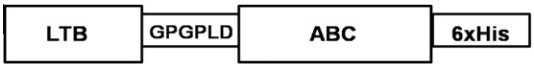

Fig. 1. Structural diagram of synapsin A, B and C domains (ABC) fused with *E. coli* heat-labile toxin B subunit (LTB).

LTBABC and LTB were purified from washed inclusion bodies solubilized for 12 h at 4 °C in binding buffer (20 mM sodium phosphate and 0.5 M NaCl pH 8.0) containing 10 mM imidazole and 8 M urea; insoluble material was removed by centrifugation at 100,000*g* for 20 min. Soluble recombinant protein was purified by affinity chromatography using a Ni–Sepharose Hi Trap chelating column (GE Healthcare, Argentina) equilibrated with binding buffer following manufacturer's guidelines. On the other hand, LTBSC was purified from washed inclusion bodies using a Ni–Sepharose Hi Trap chelating column [14].

Synapsin ABC peptide was expressed in *E. coli* Codon plus-RIL (Novagen) transformed with the expression vector pET-SABC. Cells were grown at 37 °C in TB containing kanamycin and glucose. When OD_{600} of culture reached 0.8, protein synthesis was induced with 1 mM IPTG for 5 h at 30 °C. After culture, bacteria were harvested and disrupted in ice-cold extraction buffer (PBS 50 mM phosphate, 0.5 M NaCl, pH 8.0) supplemented with 4.5 M urea in an EmulsiFlex-C3 apparatus. The supernatant containing ABC was first obtained at 10,000*g* and further centrifuged at 100,000*g*. The synapsin peptide ABC expressed in the cell soluble fraction was purified using a Ni-Sepharose Hi Trap chelating column equilibrated with binding buffer containing 4.5 M urea following the manufacturer's guidelines. To eliminate imidazol, the fractions containing the ABC peptide were pooled and dialyzed against PBS containing 1 M urea. The level of recombinant protein expression and protein homogeneity after purification steps was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Protein bands were detected with Coomassie Brillant Blue R-250.

2.3. Protein determination

Protein concentration was determined using the Bio-Rad Protein Assay with bovine serum albumin (BSA) as reference protein. The relative abundance of protein bands in SDS–PAGE or Western blots was evaluated by densitometry analysis using UVP Biolmaging System (Upland CA, USA) and the VisionWorksLS software.

2.4. In vitro refolding and storage of assembled proteins

To allow refolding, the denaturant concentration was lowered by step dialysis [19]. For this, 1–5 ml of 500 μg/ml affinity purified LTBABC, LTB or LTBSC were dialyzed successively against 100-volumes of refolding buffer (PBS, 0.05 M sodium phosphate pH 8.0 with 0.15 M NaCl) containing 3, 2, 1 and 0.5 M urea for 3 h at 4 °C. In some experiments additives were added to the refolding buffer containing 1 M urea (0.4 M L-arginine, GSH-GSSG). At the end of each refolding step the OD_{450} nm of protein solution was measured. The soluble protein after refolding in selected conditions was measured after centrifugation at 20,000*g* for 20 min

and compared as a percentage to the total protein before refolding (refolding yield).

Ability of refolded LTBABC, LTB and LTBSC to remain in solution was tested upon storage. Sterile-filtered solutions of refolded LTBABC, LTB and LTBSC (500 μg/ml) in the selected refolding buffer were stored at 4 °C for 2, 24, 168 and 240 h. Particles were eliminated by centrifugation at 20,000*g* for 20 min and concentration of protein was measured as indicated in Section 2.3.

2.5. Western blot analysis

For Western blot 15% SDS–PAGE gels were currently used to analyze samples of recombinant proteins except that 6–17% gradient gels were used in the case of refolded LTBABC. Proteins separated in SDS–PAGE were electrotransferred to nitrocellulose sheets with a Bio-Rad apparatus. Presence of LTB epitopes were analyzed with a rabbit polyclonal anti-LT-I antibody prepared at our laboratory [14,20]. Western blot with anti-LT-I antibody was performed essentially as previously described [14] except for the binding of the LT-I primary antibody which was detected with IR-800 coupled-mouse anti-rabbit IgG antibody (Li-COR, diluted 1/25,000) incubated for 30 min. IR800 probe was detected on an Odyssey infrared imaging system.

To detect the synapsin ABC epitopes in the recombinant protein blotted nitrocellulose, membranes were blocked with 5% skim powder milk in PBS-for 1 h at room temperature and then incubated with polyclonal anti-synapsin antibody (diluted 1/1,000, Invitrogen Argentina) for 16 h at 4 °C. Primary rabbit antibody was detected using protein A coupled to horseradish peroxidase combined with a chemiluminescence detection kit (SuperSignal West Pico Chemiluminescent Substrate, Pierce, IL, USA) and Hyperfilm MP film (GE Healthcare, Argentina).

2.6. GM1-ELISA

The ability of refolded fusion protein LTBABC and LTB to bind the GM1 and GD1a gangliosides were assayed by an enzyme linked immunosorbent assay (ELISA) essentially as previously described [14]. For this, 60 pmol/well GM1 (Galβ3GalNAcβ4-(NeuAc3) Galβ4 Glcβ1Cer, Fidia Res., Italy) or GD1a (NeuAcα3Galβ3GalNAcβ4(NeuAcα3)Galβ4GlcβCer, kindly donated by Dr. G.A. Nores, CIQUIBIC, Universidad Nacional de Córdoba, Argentina) were adsorbed to 96-well-polystirene plates (3590, Costar) and incubated with serially diluted samples of LTBABC and LTB or CTB. To detect LTB and CTB epitopes from bound proteins anti-LT-I or anti-CT antibodies [14,20], respectively were used in similar dilution (1/1,000). Bound rabbit IgG was detected with horse radish peroxidase-conjugated-Protein A and with peroxidase substrate (o-phenylenediamine/H_2O_2 in citrate–phosphate buffer pH 4.5). The OD at 490 nm was measured with a Microplate Reader 680 (Bio-Rad).

2.7. Binding to cells and internalization assays

CHO-K1^{GM1+} cells, kindly donated by Dr. J.L. Daniotti (Universidad Nacional de Córdoba, Argentina) is a double stable transfectant clone of wild type Chinese hamster ovary (CHO)-K1 cells that express UDP-GalNAc:LacCer/GM3/GD3 N-acetylgalactosaminyltransferase (GalNAc-T) and UDP-Gal:GA2/GM2/GD2 galactosyltransferase (Gal-T2), which synthesize GM1 and GD1a [21]. CHO-K1^{GM1+} cells were grown and maintained at 37 °C in 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (complete medium). To measure binding of proteins to CHO-K1^{GM1+} cells the 'In cell-Western' assay was performed (Odyssey Li-COR Protocols). Briefly, CHO-K1^{GM1+} cells were grown to confluence in 96 well- culture plates (Cell Star, Greiner Bio-One) and placed in ice during the whole assay. Different concentrations of LTBABC, LTB and LTBSC in complete culture medium were incubated for 90 min. Cells were washed with ice-cold PBS and incubated with anti-LT-I or anti-CT antibodies (1/1,000 dilution in complete medium, [20,14] for 1 h. Rabbit anti-LT-I or anti-CT antibodies bound to recombinant proteins were detected with secondary IR-800 coupled-mouse anti-rabbit IgG antibody (Li-COR, diluted 1/25,000 in complete medium) for 30 min. The infrared signal was measured in the Odyssey imaging system, Li-COR.

For the endocytosis assays CHO-K1^{GM1+} cells grown on cover slips were maintained in ice for 20 min to stop intracellular transport. LTB, CTB-Alexa555 or LTBABC (3–20 nM) in D-MEM supplemented with 10% FCS were added incubated in ice for 30 min and cells were washed with cold PBS. Fresh pre-warmed medium containing FBS was added and the cells were transferred to 37 °C for 30–120 min and then the plates were returned to ice. When indicated, 6 μg/ml Alexa 647 coupled-transferrin (Alexa 647-Tf, Molecular Probes) was present in the culture medium during the incubation at 37 °C.

2.8. Confocal immunofluorescence microscopy

After the endocytosis assay, cells were washed with PBS and fixed with 4% buffered paraformaldehyde for 30 min at room temperature. Cells were permeabilized with 0.1% saponin/2% BSA in PBS at room temperature for 20 min, washed with PBS and incubated with primary polyclonal rabbit anti-LT-I antibodies [14,20] diluted 1/1,000 for 60 min at room temperature. Cells were washed with PBS and incubated with secondary antibodies (Cy3 coupled-goat anti rabbit IgG, Jackson ImmunoResearch, Inc. diluted 1/1,000 for 60 min at room temperature. Cells were mounted in FluorSave reagent (Calbiochem, EMD). Confocal images were collected using a Carl Zeiss LSM5 Pascal laser-scanning confocal microscope (Carl Zeiss, Jena, Germany) equipped with an argon/helium/neon laser and a 60 × 1.4 numerical aperture, oil immersion objective (Zeiss Plan-Apochromat). Single confocal sections of 0.7 μm were taken parallel to the coverslip (xy sections). The images were deconvoluted using the 'advanced maximum likelihood estimation algorithm' (AMLE) for Cell R software (Olympus Soft Imaging Solutions, Munchen, Germany), version 3.3, set with 20 iterations and an overlay sub-volume of 10 pixels. A theoretical point spread function (PSF) was used. Final images were compiled with Adobe Photoshop 9.0. The fluorescent micrographs shown in this manuscript are representative of three independent experiments. Average fluorescence intensity was quantified using ImageJ software (Rasband, W.S., ImageJ, US National Instutes of Halth, Bethesda, Maryland, http://rsb.info.NIH.gov/ij/, 1997–2008). Pearson's coefficient (Pearson's *r*) was calculated using ImageJ software with JACoP plugin [22]. A Pearson's $r > 0.6$ was considered significant.

2.9. Induction of EAE and treatment for suppression

EAE was induced by intradermal inoculation in both hind feet with 8 mg lyophilized whole bovine myelin emulsified (1:1) in PBS and complete Freund's adjuvant (CFA, Sigma–Aldrich Co., St. Louis, MO, USA). Group of animals randomly chosen between littermates received alternated six oral doses of 0.3 nmol LTBABC, LTBSC, LTB, ABC, LTB + ABC or vehicle (PBS-1.0 M urea) from the day of EAE challenge up to the 10th day post induction (dpi). In some experiments 0.15 nmol of LTBABC or 0.3 nmol ABC, LTB or the former LTBSC hybrid was administered to the animals. Not all groups were represented in all experiments. Clinical score of EAE was evaluated in a blinded fashion by an investigator from 0 to 24 dpi. Clinical signs (evident between 10 and 18 dpi) were graded as follows: 0, no evident clinical signs; 1, flaccid tail; 2, hind limb weakness; 3, definitive hindquarter paralysis and urinary

incontinence; 4, tetraparalysis; 5, moribund state or dead. For immunological analysis animals were sacrificed at 13 dpi. Rats were anesthetized with a mixture of xylazine and ketamine (10 and 65 mg/kg respectively, ip) and mesenteric lymph nodes (MLN), popliteal lymph nodes (PLN) and spleen were aseptically removed and placed in ice-cold RPMI 1640 medium.

2.10. Mononuclear cells culture

MLN and PLN were mechanically disrupted and cell suspensions of mononuclear cells (MNC) were filtered through a 70 μm cell strainer (Nylon membrane BD, Becton Dickinson, Argentina) and washed. Spleens were mechanically disrupted and cell suspensions were separated by Histopaque 1080 (Sigma–Aldrich, USA) gradient centrifugation and splenocytes were washed twice with RPMI 1640 containing 10% FCS. All cells were cultured in culture plates (Cell Star, Greiner-Bio-One) with RPMI 1640 medium (Sigma–Aldrich, USA) supplemented with 30 μg/ml gentamycin, 10% FCS (Natocor, Córdoba, Argentina) and 50 μM 2-mercaptoethanol at 37 °C in a humidified CO_2 incubator. Cellular viability was assessed by Trypan blue exclusion.

For cytokine determination MLN, PLN and spleen cells (1.5×10^6 cells/ml) were stimulated in culture in 24- or 48- well plates with 75 μg/ml of MBP for 48 h (INF-γ and IL-17) or 72 h (IL-10 and TGF-β). Supernatants were collected and kept at −70 °C until cytokine quantification.

2.11. Lymphocyte proliferation assay

MNC from PLN of animals treated with LTBABC, LTB, LTB + ABC, ABC or vehicle isolated at 13 dpi were stimulated in culture (1.5×10^6 cells/ml) in 96 well plates (Cell Star, Greiner-Bio One) with 75 μg/ml PBM, 75 μg/ml LTBABC or 0.25 μg/ml of concanavalin A (ConA, Sigma–Aldrich), for 72 h. Each well was pulsed with 1 μCi de [^{3}H]TdR during the last 18 h of culture. The radioactivity incorporated into cells was measured by standard liquid scintillation counting as previously described [15] and the results were expressed as stimulation index (SI) calculated as the ratio between the mean counts per minute (cpm) of stimulated and the cpm of unstimulated cells. A SI $\geqslant$ 2 was considered a positive response.

2.12. Cytokine analysis

All cytokines were measured in MNC culture supernatants by sandwich ELISA using High Binding plates (EIA, RIA 3690, Costar) 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). IL-17 was measured using the Ready-SET-Go kit from eBioscience (San Diego, CA, USA).

2.13. Flow cytometry analysis

CD4 and CD25 molecules were detected in freshly isolated MNC and splenocytes (1×10^6 cells) by incubating with the appropriate concentration of the conjugated monoclonal antibodies anti-CD4-FITC and CD25-allophicocyanin or the corresponding isotype controls (eBioscience, San Diego, CA, USA). For intracellular Foxp3 detection MNC were treated in culture (1.25×10^6 cell/ml) with 100 ng/ml of phorbol myristate acetate and 10 ng/ml of ionomicin (Sigma–Aldrich, USA) for 4 h, and then, 2 μM of monensin (eBioscience, San Diego, CA, USA) was added for 1 h. Cells that were stained with anti- CD4-FITC and CD25-allophicocyanin antibodies were fixed, permeabilized and stained with anti-Foxp3-PE according to the manufacturer's protocol (eBioscience, San Diego, CA, USA). Samples were acquired on a FACSCanto Flow Cytometer (BD, Buenos Aires, Argentina) and data were analyzed with Flowjo 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.

2.14. Delayed type hypersensitivity

Delayed type hypersensitivity (DTH) testing was performed in rats challenged for EAE and treated with 0.3 nmol LTBABC, LTBSC, LTB, ABC, LTB + ABC or vehicle (control) at 10 dpi. For this, rats received intradermal injection with sterile solutions of MBP in PBS in the right ear and the vehicle in the left ear according the procedure previously described [15]. The results were expressed as the difference in mm between the thickness of the right ear and the left ear measured with a digital caliper 24 h after the injection.

2.15. Statistical analysis

Statistical analyses were performed using the computer-based statistical GraphPad Prism V5.0. 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. Disease incidence was analyzed by χ^2 test. Differences in clinical parameters and percentage of body weight variation were analyzed by Kruskal–Wallis test. p values less than 0.05 were considered significant.

3. Results

3.1. Expression and biochemical characterization of recombinant proteins

Both LTBABC and LTB were expressed as inclusion bodies in *E. coli* BL21 (λDE-3) cells transformed with the respective expression vectors pET-LTBABC and pET-LTB and purified from washed inclusion bodies by Ni^{2+}-chelating affinity chromatography under denaturing conditions to 95% homogeneity. Denatured LTB chain separated by SDS–PAGE showed a major band of about 13 kDa [14] while LTBABC fusion protein appeared as a band of about 57 kDa (Fig. 2A, lane 1 and lane 5). Further identification of recombinant LTBABC was carried out by Western blot using antibodies that recognize synapsin epitopes (Fig. 2A, lane 6).

The recombinant synapsin ABC peptide expressed in *E. coli* strain BL21 Codon plus-RIL was partially purified by Ni^{+2}-Sepharose affinity chromatography from the soluble cell extract as a protein band of molecular mass about 48 kDa by SDS–PAGE (Fig. 2A, lane 3). Synapsin ABC peptide was further identified by anti-synapsin antibody in Western blot (Fig. 2A, lane 4).

To perform some selected experiments, LTBSC was expressed in inclusion bodies from *E. coli* BL21 (λDE-3) cells transformed with the expression vectors pET-LTBSC and purified from washed inclusion bodies by Ni^{2+}-chelating affinity chromatography according to conditions previously described [14].

Removing of denaturant from purified LTBABC, LTBSC and LTB was done by step dialysis and protein aggregation was estimated as OD_{450} nm. Decreasing denaturant concentration of protein solutions in dialysis buffer (PBS, pH 8.0) from 8 to 0.5 M increased turbidity probably due to aggregation of missfolded proteins but clearly LTBABC and LTB showed low turbidity development while LTBSC solutions showed the highest OD_{450} nm (Fig. 2B conditions A–D, H). Addition of 0.4 M L-arginine may increase refolding without affecting protein stability and redox environment provided by reduced/oxidized glutathione (GSH-GSSG) may potentially

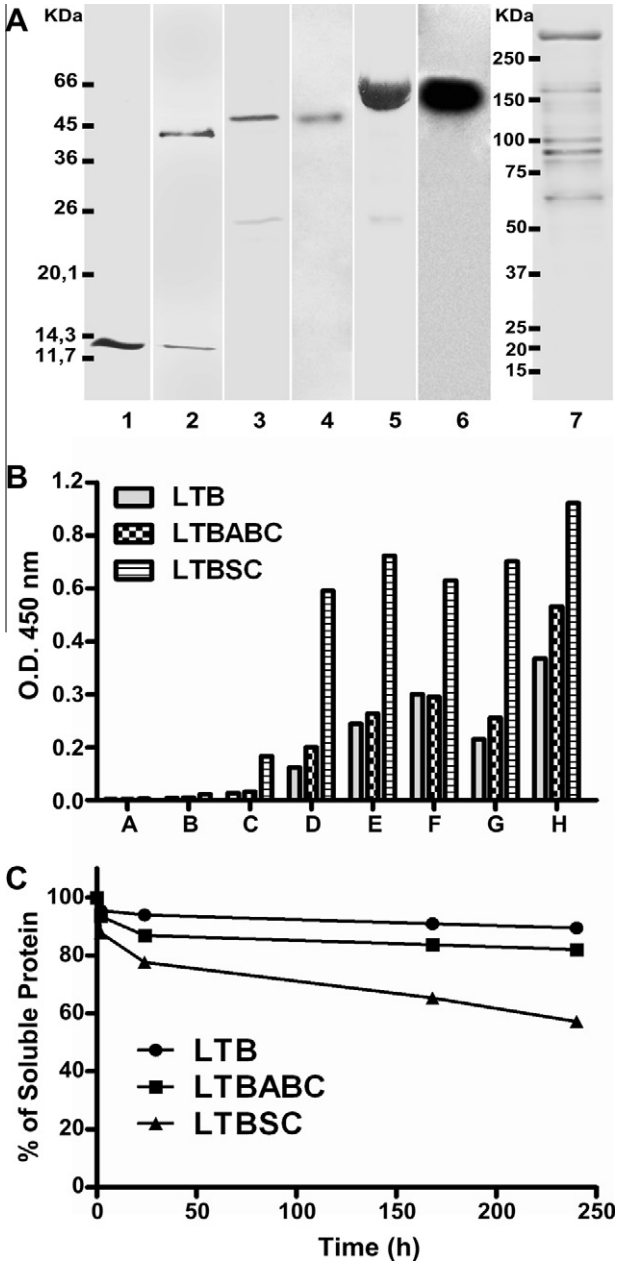

Fig. 2. Biochemical characterization of recombinant proteins. (A) SDS–PAGE and Western blot analysis of LTBABC and LTB expressed in *E. coli*. LTB purified from inclusion bodies was boiled under reducing conditions, electrophoresed on a 15% SDS–PAGE and stained with Coomassie Brillant Blue R-250 (lane 1); Western blot of refolded LTB treated for SDS–PAGE without reducing agent and heating. Nitrocellulose membranes were stained with anti-LT-I antibodies (lane 2); purified synapsin ABC peptide expressed in the soluble fraction of *E. coli*, electrophoresed on a 15% SDS–PAGE and stained with Coomassie Brillant Blue R-250 (lane 3); Western blot of the same sample detected with anti-synapsin antibodies (lane 4); LTBABC purified from inclusion bodies separated on 15% SDS–PAGE and stained with Coomassie Brillant Blue R-250 (lane 5) and the same sample analyzed by Western blot with anti-synapsin antibodies (lane 6). Western blot of refolded LTBABC treated for SDS–PAGE without reducing agent and heating electrophoresed on 6–17% SDS–PAGE and detected on nitrocellulose membranes with anti-LT-I antibodies (lane 7). 15–250 kDa were prestained markers (Bio-Rad). (B) LTBABC, LTB and LTBSC turbidity assessed as OD450 nm during refolding. Purified proteins (0.5 mg/ml) were dialyzed successively for 3 h at 4 °C against PBS pH 8.0 containing decreasing concentration of urea (A: 8 M urea, B: 3 M urea; C: 2 M urea, D: 1 M urea: H: 0.5 M urea) or 1 M urea containing L-arginine or oxidized/reduced glutathione (E: 0.4 M L-arginine; F: 2 mM:0.4 mM GSSG:GSH; G: 2 mM:0.2 mM GSSG:GSH). (C) Storage of LTBABC, LTB and LTBSC. Refolded proteins solutions (0.5 mg/ml) containing LTBABC (1 M urea), LTB (1 M urea) and LTBSC (2 M urea) were kept at 4 °C for 2, 24, 168 and 240 h, centrifuged at 20,000*g* and protein in supernatant was expressed as percentage of the starting protein concentration.

enhance native disulphide bridge formation [19,23]. Provision of these additives (Fig. 2B conditions E–G) to the dialysis buffer containing 1 M urea did not significantly affect LTB and LTBABC solubility nor contributed to prevent loss of solubility from LTBSC. Therefore, we selected refolding buffer containing1 M urea as standard refolding conditions for 0.5 mg/ml LTBABC and LTB, which resulted in a refolding yield about 86% for LTBABC and 96% for LTB. On the other hand, 2 M urea was selected as the refolding condition for LTBSC (refolding yield about 80%).

Presence of assembled pentamers in refolded LTBABC and LTB was analyzed by SDS–PAGE followed by Western blot using anti-LT polyclonal antibodies. Sample of refolded LTBABC and LTB in 1 M urea (under non reducing conditions and without heating) showed assembled products. About 90% of the refolded LTB preparation showed a main 43–45 kDa- band while the monomer appears as a very minor band (Fig. 2A, lane 2). This refolded LTB preparation showed a molecular mass similar to that observed for soluble LTB purified from soluble fraction of cell lysate [14]. The difference between the calculated molecular weight and the theoretical molecular weight (64.2 kDa) has been attributed to the compact structure of the LTB pentameric ring, as previously observed in other recombinant expression systems [24]. On the other hand, refolded LTBABC preparation showed an oligomer with molecular mass higher than 250 kDa (probably pentameric) and some lower molecular weight-oligomers in Western blot detected with anti-LT-I antibodies (Fig. 2A, lane 7).

Stability of LTBABC, LTBSC and LTB solutions were tested upon storage as the ability of the protein to remain in the supernatant after centrifugation. Samples of LTBABC and LTB showed higher percentages of protein in the supernatant than comparative samples of LTBSC when solutions were stored from 2 to 240 h (Fig. 2C).

3.2. LTBABC and LTB recognize GM1

Refolded preparations of LTBABC and LTB were serially diluted and incubated with GM1-adsorbed polystyrene plates and the bound proteins detected with antibodies GM1-ELISA [14]. Both proteins showed similar ability to bind to the GM1 receptor, (Kd $\sim$ 5.4 nM and $\sim$3.8 nM, respectively) and closer to the CTB/LTB Kd ($\sim$1.2 nM) in this assay (Fig. 3A).

Since CHO-K1^{GM1+} cells also express GD1a on the membrane we also tested the ability of the refolded proteins to bind to this ganglioside in GD1a-ELISA. Purified LTBABC, LTB and standard LTB showed negligible ability to bind GD1a (Fig. 3A). Very low binding of LTB to GD1a respect to GM1 was previously observed in this system [25].

3.3. LTBABC and LTB bind and traffic into cultured cells

To further test biological activity of refolded LTBABC, LTB and LTBSC we examined their ability to bind to CHO-K1^{GM1+} cells. Both refolded LTBABC and LTB bound to CHO-K1^{GM1+} cells similarly with binding ability close to that of CTB. Cholera toxin/CTB binding specificity is almost restricted to the GM1 ganglioside [26]. Contrary to LTBABC, LTBSC showed poor binding ability with a binding curve suggesting a very low affinity for the GM1 receptor. The LTB standard (soluble LTB expressed in *E. coli*) [14] exhibited markedly greater binding avidity for CHO-K1^{GM1+} cells (Fig. 3B). Since broader oligosaccharide specificity was observed for LTB [27] from the results of GD1a-ELISA this ganglioside was discarded as a putative non-GM1 receptor for LTB.

Internalization of LTBABC, LTB and CTB into CHO-K1^{GM1+} cells was investigated by confocal fluorescent microscopy. All proteins were able to enter and traffic into CHO-K1^{GM1+} cells incubated at 37° C. Refolded LTBABC (Fig. 4C), LTB (Fig. 4B) and Alexa555-CTB (Fig. 4A) partially co-localized with Alexa 647-Tf, a marker of

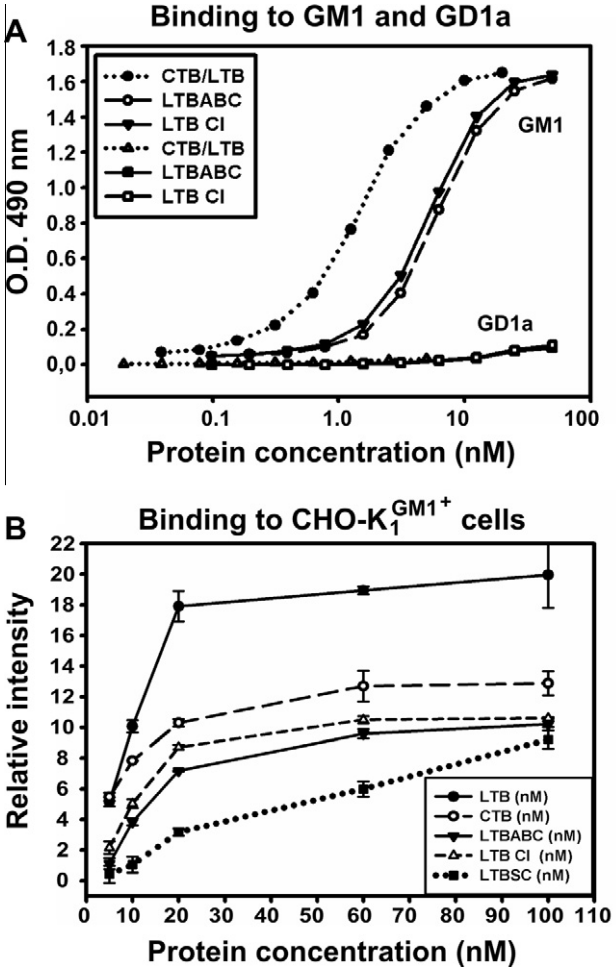

Fig. 3. Biological activity of recombinant proteins. (A) LTBABC and LTB bind to GM1. Binding of refolded LTBABC and LTB (LTB CI) to GM1 or GD1a precoated-microtiter wells (GM1 ELISA); CTB and soluble recombinant LTB were used for comparison. Bound proteins were detected with anti-LT-I or anti-CT antibodies/peroxidase conjugated-protein A and OPD/H2O2 as peroxidase substrate. The colorimetric signals were measured at 490 nm. (B) Binding of refolded LTBABC, LTBSC and LTB (LTB CI) to CHO-K1^{GM1+} cells. Dilutions of the proteins were incubated with CHO-K1^{GM1+} cells grown on 96 well- culture microplates in ice. Bound proteins were detected using rabbit anti-LT-I or anti-CT antibodies and secondary IR-800 coupled-mouse anti-rabbit IgG antibody. The infrared signal was measured in the Odyssey imaging system, Li-COR.

perinuclear recycling endosomes (RE) at 60 min (Pearson's *r*: LTBABC 0.74 ± 0.047; LTB 0.83 ± 0.03 and Alexa555-CTB 0.75 ± 0.04). The three proteins co-localized with RE in above 90% of cells (Fig. 4D). At longer time (120 min) less amount of the three proteins still remain at the RE compartment, and the fluorescent signals were lower than at 60 min (results not shown).

3.4. Treatment with LTBABC ameliorated disease severity

Rats were challenged with myelin in CFA (0 dpi) and fed with LTBABC (0.3 or 0.15 nmol) or 0.3 nmol of LTB + ABC, LTB, ABC, LTBSC or vehicle on alternated days from 0 to 11 dpi. Only rats that received 0.3 nmol of LTBABC showed decreased disease severity as indicated by lower mean maximum clinical score, cumulative score and disease index. On the contrary, disease incidence, day of onset or length of acute EAE showed no difference among experimental groups. EAE development is associated with weight loss about 15% during the acute stage. Treatment with 0.3 nmol LTBABC also diminished mean body weight loss to 9.8%. Lower dose (0.15 nmol) of LTBABC had no effect on all these parameters. Similarly, LTBSC, LTB + ABC nor the separated peptides ABC or LTB had protective effects on EAE (Table 1).

3.5. LTBABC diminished T cell reactivity against MBP

EAE is characterized by T cell mediated reactivity directed to myelin antigens among them the encephalitogenic MBP. EAE as well as DTH are triggered by antigen-specific CD4$^+$ T cells of the Th1 cytokine- phenotype [28]. We investigated the effect of the oral treatment with 0.3 nmol of LTBABC, LTBSC, LTB, ABC and LTB + ABC or vehicle on DTH against MBP at 10 dpi. DTH was significantly lowered in rats treated only with LTBABC respect to the vehicle-treated EAE group (Fig. 5A).

MBP specific-proliferation of PLN cells from rats treated with 0.3 nmol LTBABC, LTB, ABC and LTB + ABC or vehicle was also quantified at 13 dpi (acute stage). Proliferation of MNC was abrogated only in LTBABC treated-rats respect to control animals. On the contrary, similar positive levels (SI > 2) of MNC proliferation of vehicle and the other experimental groups were stimulated by MBP (Fig. 5B). In some experiments MNC from rats treated with 0.3 nmol LTBSC were included but proliferation stimulated by MBP was not different from control (results not shown). On the other hand, MNC from all groups of rats did not proliferate when were stimulated with LTBABC and similar SI were observed in response to Con A stimulation (Fig. 5B). Taken together, these observations suggest that LTBABC treatment modulated MBP specific cellular reactivity.

3.6. LTBABC treatment modulated cytokine profile

EAE is driven by Th1 and Th17 inflammatory responses in rat models [16,29]. To determinate whether LTBABC is able to modulate proinflammatory Th1 (IFN-γ) and Th17 (IL-17) cytokine secretion, rats were challenged for EAE and treated with LTBABC (0.3 nmol, high dose or 0.15 nmol, low dose), 0.3 nmol LTB + ABC, LTB, ABC or vehicle as indicated in Materials and methods. Animals were euthanized at 13 dpi and MNC from MLN, PLN and spleen were isolated and stimulated in culture with MBP. IFN-γ, IL-17, IL-10 and TGF-β were determined in culture supernatants. Even when the mean levels of IFN-γ in the three lymph organ of the group treated LTBABC were lower than the levels measured in the control group, and the reverse situation was found for IL10, differences between concentrations of each cytokine were not statistical significant among experimental groups (Supplementary Fig. 1). Then, we calculated the ratio of IFN-γ and IL10 levels for each individual and we observed that the IFN-γ/IL10 ratios were diminished in all lymphoid nodes of the LTBABC group respect to those of the vehicle treated- group (Fig. 6A). TGF-β concentrations in MLN were higher only in rats treated with LTBABC (Fig. 6B). Treatment with 0.3 nmol LTBABC did not decrease levels of proinflammatory IL17 secreted respect to control group in the lymphoid organs investigated (Fig. 6C). Similarly, lower dose of LTBABC (0.15 nmol) or the other recombinant proteins tested had no effect on any cytokine profile in the lymphoid nodes analyzed (Fig. 6).

3.7. LTBABC treatment expanded regulatory T cells

Since regulatory T cells (Treg) cells are major regulators in the priming and effector functions of T cells we determined the proportion of CD4$^+$CD25$^+$Foxp3$^+$ cells (functional Treg cells) in PLN, MLN and spleen from rats treated with 0.3 nmol LTBABC, LTB + ABC or vehicle at 13 dpi. The population of CD4$^+$CD25$^+$Foxp3$^+$ cells was expanded in PLN and MLN from rats treated with LTBABC but the percentages of spleen Treg cells among experimental groups were similar (Fig. 7).

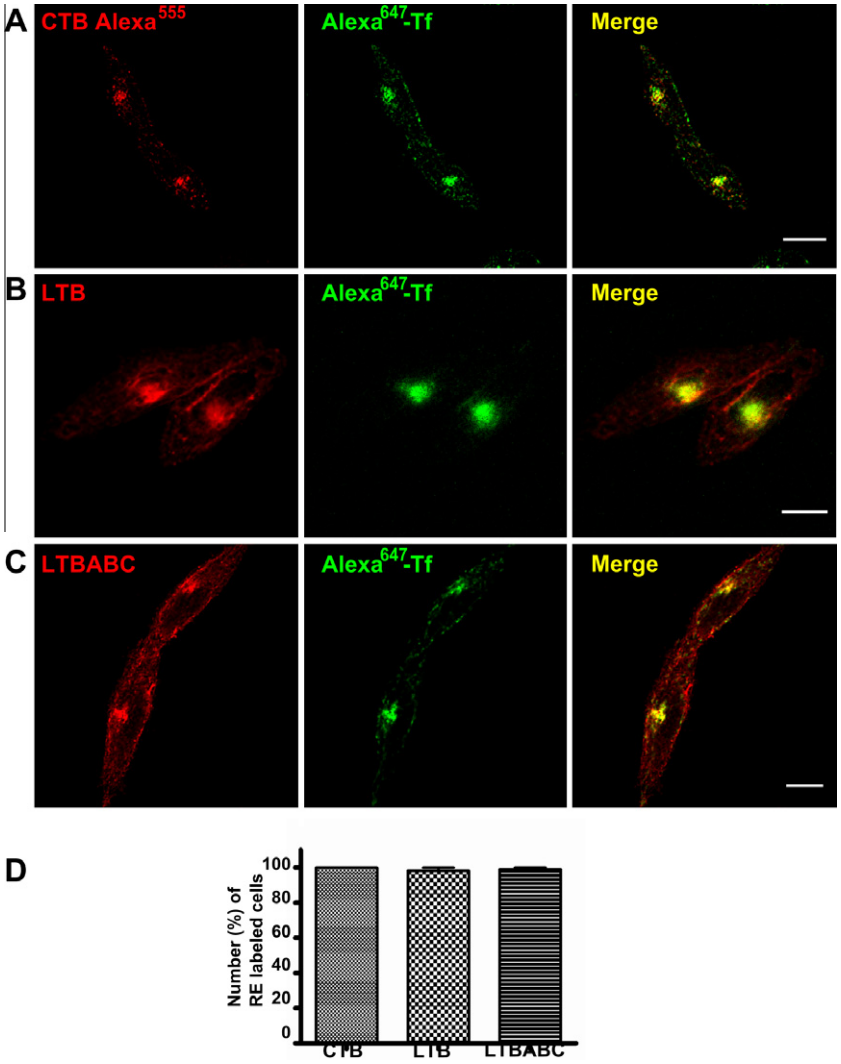

Fig. 4. Internalization of LTBABC, LTB and CTB in CHO K1^{GM1+} cells. CHO K1^{GM1+} cells were incubated in ice to inhibit intracellular transport and then CTB-Alexa555 (A), LTB (B) or LTBABC (C) were added and incubated in ice during 30 min to allow membrane binding. Next, cells were transferred to 37 °C, in the presence of Alexa 647-Tf (Alexa^{647}-Tf, pseudo-colored green) to allow internalization for 60 min and fixed. Proteins internalized were detected with anti-LT-I antibodies and Cy3 coupled-anti rabbit IgG as secondary antibody. The merged confocal images (yellow) present in LTBABC, LTB and CTB-Alexa555 (red) with Alexa 647-Tf (green) indicated partial co-localization of internalized LTBABC, LTB and CTB with Tf-positive recycling endosomes (RE). Representative confocal sections 0.7 μm taken parallel to the coverslip are shown. Scale bars: 10 μm. (D) Number of cells (%) showing recombinant protein/antibody to LTB (anti LT-I) or CTB-Alexa555 at the RE after 60 min of endocytosis. Recycling endosomes were identified by Alexa 647-Tf signal. The values are mean ± S.E.M. from three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Effect of oral treatment with the recombinant proteins on EAE clinical signs.

Group	Disease incidence[d]	Day of onset	Maximum weight loss (%)	Length of disease (days)	M.M.C.S[a]	Cumulative score[b]	Disease index[c]
Vehicle	9/9	11.86 ± 0.30	15.18 ± 0.88	5.20 ± 0.20	3.36 ± 0.18	11.52 ± 0.80	100.40 ± 8.87
LTBABC 0.3 nmol	8/9	12.40 ± 0.34	9.82 ± 1.05*	4.80 ± 0.22	2.40 ± 0.20*	7.50 ± 0.72*	64.87 ± 6.05*
LTBABC 0.15 nmol	9/9	11.60 ± 0.37	12.20 ± 1.47	5.50 ± 0.17	2.95 ± 0.16	11.25 ± 0.66	96.75 ± 4.58
LTB CI 0.3 nmol	9/9	12.06 ± 0.37	12.16 ± 0.89	4.90 ± 0.26	2.96 ± 0.13	11.31 ± 0.58	105.80 ± 7.68
LTB CI 0.3 nmol + ABC 0.3 nmol	9/9	11.94 ± 0.40	14.83 ± 1.14	5.40 ± 0.15	3.28 ± 0.18	11.84 ± 0.96	101.20 ± 9.25
ABC 0.3 nmol	9/9	11.44 ± 0.33	14.44 ± 0.90	5.30 ± 0.18	3.19 ± 0.14	11.91 ± 0.88	106.20 ± 9.55
LTBSC 0.3 nmol	9/9	12.01 ± 0.41	12.32 ± 1.22	4.90 ± 0.21	2.98 ± 0.23	10.93 ± 0.91	94.20 ± 8.29

Animals were fed with the indicated amount of protein or vehicle alone at 0, 2, 4, 6, 8 and 10 days after the active EAE induction with bovine myelin in complete Freund's adjuvant. The data are compiled from three separate experiments in which animals were observed for clinical sign development during 24 days after EAE induction (dpi). Results are expressed as mean ± S.E.M. Differences in clinical parameters and percentage of body weight variation were analyzed by Kruskal–Wallis test and disease incidence was analyzed by χ^2 test $^*p < 0.05$, treated group *vs.* vehicle.

[a] M.M.C.S, mean maximum clinical score is the sum of the highest clinical score (1–5) achieved by each rat during the clinical phase of the disease divided by the number of rats that got sick in that group.

[b] Cumulative score, sum of scores of days 10–18.

[c] The disease index was the sum of the daily clinical score for each animal throughout the experimental period divided by the day of onset of EAE clinical signs × 100.

[d] Disease incidence was also analyzed in rats from this table plus rats included in other experimental protocols with the following results: EAE 23/23, LTBABC 0.3 nmol 20/23, LTBABC 0.15 nmol, LTB 21/21, LTB + ABC 22/23, ABC 21/21. No statistical differences between treated and control groups by the χ^2 test.

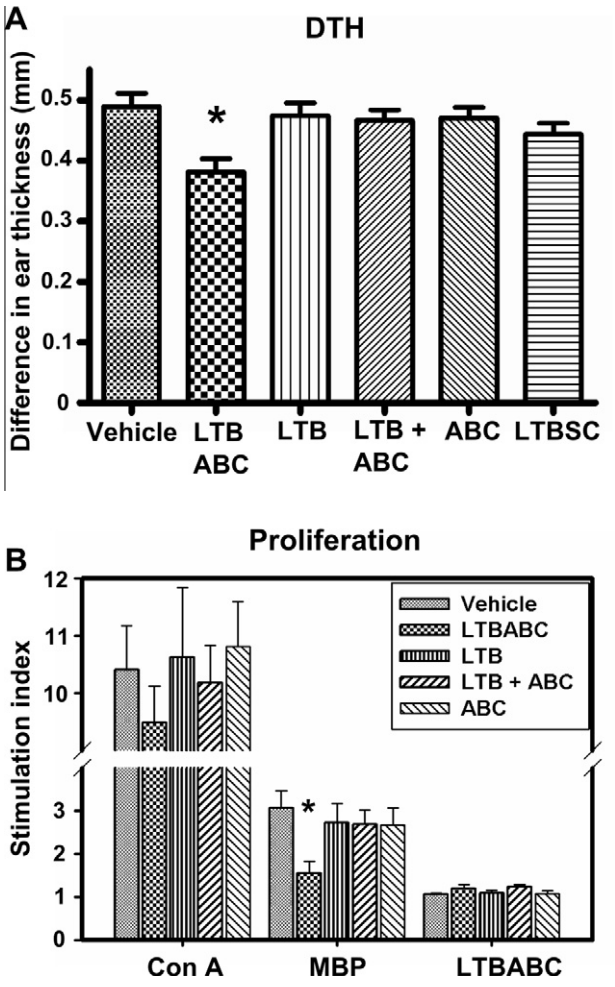

Fig. 5. Modulation of MBP-specific T cell responses by LTBABC. (A) Rats were orally treated with six doses of 0.3 nmol of recombinant proteins LTBABC, LTB, LTB + ABC, ABC, LTBSC or vehicle from the day of EAE challenge up to 10 dpi. DTH was tested in rats at 10 dpi 24 h after injection with MBP in the left ear and the vehicle in the right ear. Swelling was expressed as the difference of thickness (mm) between the left and right ear. (B) Rats were orally treated with six doses of 0.3 nmol of recombinant proteins LTBABC, LTB, LTB + ABC, ABC or vehicle from the day of EAE challenge up to 10 dpi. MNC were isolated from popliteal lymph nodes of animals during the acute period (13 dpi) and stimulated in culture with MBP, LTBABC and Con A. The proliferative responses are indicated as stimulation index (SI). Significant differences of treated group *vs.* vehicle are indicated by $^{*}p < 0.05$. Data correspond to the mean value ± S.E.M. of one experiment of three performed each with four animals per group.

4. Discussion

This work demonstrates that the hybrid LTBABC expressed in *E. coli* exhibited great biological activity to recognize the GM1 ganglioside and effectively ameliorated clinical signs of EAE. Reduction of clinical parameters was associated with modulation of MBP-specific Th1 inflammatory response, elevation of TGF-β as well as expansion of Treg cells. We previously showed that preventive administration of the hybrid LTBSC consisting of the well conserved synapsin C domain and LTB promoted EAE suppression. LTBSC diminished the T cell reactivity against MBP due to a functional cross reactive recognition of MBP specific T cells and synapsin [15,16]. EAE inflammatory reactions are directed to myelin antigens but it is apparent that the synapsin ABC domain could be recognized by some MBP-reactive T cells. It was recently shown that synapsin ABC domain (not the DE domains) was recognized by selected MBP-reactive T cell clones isolated from animals challenged with MBP with induction of proliferation and secretion of several cytokines [30].

We showed in this study that both LTBABC and LTB expressed in *E. coli* cells could be purified from inclusion bodies with great homogeneity. Most importantly, both proteins assembled in oligomeric species and exhibited great affinity for the GM1 ganglioside in a solid phase- immunoassay (GM1-ELISA). The Kd calculated for the recombinant proteins indicated that LTBABC and LTB exhibited only 4 times less affinity respect to CTB/LTB. Additionally, these molecules were more resistant to aggregation than LTBSC, showing greater solubility upon refolding protocols and storage (Fig. 2B and C). Further, cell binding studies clearly showed improved LTBABC biological activity respect to LTBSC. It was early demonstrated that GM1 recognition is relevant for the LTB adjuvanticity and modulation of lymphocyte subsets [31,32]. The LTBABC chain contains a comparatively short N-terminal sequence corresponding to the A and B domains followed by the hydrophobic well conserved- synapsin C domain. The A-B peptide may function as an extended and flexible hinge linker peptide between LTB and the C domain that potentially contributes to increase flexibility of the LTBABC chain allowing better folding and solubility of the molecule and therefore improving its biological activity.

Notably, refolded LTBABC and LTB showed CHO-K1^{GM1+} binding ability close to that of CTB which is considered a molecular marker of GM1 on membranes. However, the presence of oligomers lower than 250 kDa in the refolded LTBABC preparation most likely do no bind GM1 and hence, may account for some lower LTBABC binding to cells when compared to CTB binding to CHO-K1^{GM1} cells and in GM1-ELISA. On the contrary, LTB possess a broader binding specificity than CTB and hence may recognize some extra receptors other than GM1 on cell membranes [27] this would contribute to the highest cell binding capacity exhibited by soluble recombinant LTB. Based on the results of the GD1a-ELISA this disialoganglioside expressed on CHO-K1^{GM1+} cell membranes [22] could not be considered a putative receptor of standard LTB in CHO-K1^{GM1+} cell membrane, but the nature of LTB non-GM1 receptors was not further investigated.

Internalization experiments with refolded LTBABC and LTB in CHO K1^{GM1+} cells using CTB as marker for GM1-mediated intracellular transport showed that these molecules trafficked to the recycling endosome compartment at 60 min of endocytosis. However, Iglesias-Bartolomé et al. [33] observed that Alexa 555 conjugated-CTB was localized in the late endosome and Golgi compartments in CHO K1^{GM1+} cells. It has been earlier observed that when GM1 is in lipid-rafts the glycosphingolipid is responsible for transport into these compartments [34]. Since our results showed that LTBABC and LTB followed similar traffic and intracellular localization to CTB we assumed that discrepant observations may be related to the status of our cells.

More importantly, administration of the recombinant LTBABC preparation from EAE induction (0–10 dpi) effectively diminished clinical signs of EAE but LTBSC could not mimic this effect, probably due to poor biological activity. Oral administration of LTBABC to rats ameliorated disease severity and body weight loss during the EAE acute stage. These LTBABC effects support the notion of the well known properties of LTB/CTB as antigen carrier and mucosa adjuvant observed in other experimental autoimmune conditions [11]. Our results suggest that LTB, an atoxic toxin subunit, is responsible for efficient targeting of the coupled ABC peptide mediated by GM1 receptor recognition on intestinal cells. This could be considered since the administration of the separated LTB + ABC molecules or the ABC peptide alone could not suppress EAE or modify cytokine secretion.

EAE is mediated by Th1 and Th17 reactions [16,29]. We demonstrated in this study that LTBABC diminished *in vivo* cellular

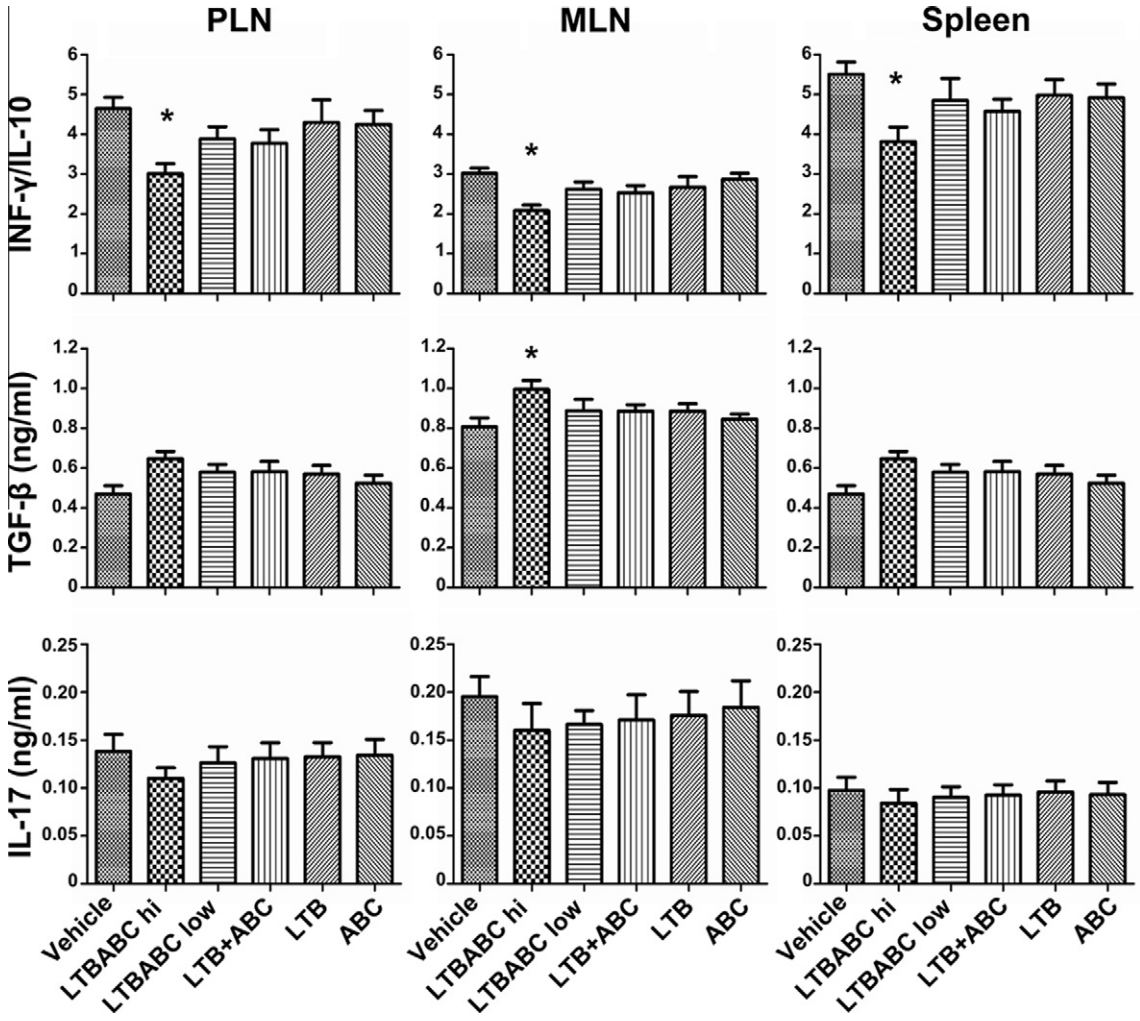

Fig. 6. Cytokine levels secreted by mononuclear cells in the experimental groups. Rats that were orally treated with six doses of 0.3 nmol of recombinant proteins LTBABC (hi), LTB, LTB + ABC, ABC, 0.15 nmol LTBABC (low) or vehicle starting the day of EAE induction up to 11 dpi. Cells from drenant popliteal lymph nodes (PLN), mesenteric lymph nodes (MLN) and spleen were isolated at 13 dpi and stimulated in culture with myelin basic protein. IFN-γ, IL-10, TGF-β and IL-17 were determined in culture supernatants by ELISA. Significant differences of treated group *vs.* vehicle are indicated by $^{*}p < 0.05$. Data correspond to the mean value ± S.E.M. of one experiment of three performed each with four animals per group.

immune response against MBP as shown by a significantly lower DTH. Additionally, *ex vivo* inhibition of lymph node MNC proliferation elicited by MBP suggested immunomodulation of cellular inflammatory pathways in EAE. To further investigate the immunological mechanism of the LTBABC therapeutic effect we determined the peripheral status of proinflammatory and modulatory cytokines at the acute stage of disease. Based on the diminished IFN-γ/IL10 relationship in PLN, MLN and spleen of LTBABC treated rats may be suggested a modulation of Th1 possibly by a shift towards a Th2 or Treg IL-10 mediated-secretion. However, similar IL17 levels were observed in lymph nodes from all experimental groups. Detection of differential cytokine expression between groups may be hampered by the low concentrations of this cytokine found in PLN in this study. Previously we also observed decreased percentages of inguinal lymph nodes $CD4^{+}IL\text{-}17^{+}$ T cells and IL-17 levels between 12 and 15 dpi in our model [16]. Further, the lack of peripheral IL-17 proinflammatory modulation by LTBABC observed in this study led us to examine Th17 and Th1 cytokine secreting-cells in the CNS. Preliminary results showed that percentages of both, $CD4^{+}IL\text{-}17^{+}$ and $CD4^{+}$ IFN-γ^{+} cells infiltrating SNC were diminished only in the group treated with LTBABC.

The levels of TGF-β were increased in MLN from LTBABC treated rats. Upregulated TGF-β expression in a major inductive site of the GALT was implicated in the specific type 3 helper reaction (Th3) elicited by preventive oral tolerance induction shown in our previous work [16]. The hypothesis of a putative generation of Treg cells secreting TGF-β at MLN in animals treated with LTBABC should be tested experimentally in future studies. As previously reported, bystander oral vaccination with antigen induced potent secretion of TGF-β by Treg cells capable of protecting from EAE [35].

One striking feature that strongly suggests the efficacy of the EAE suppression by LTBABC was the induction/expansion of Treg cells *in vivo*. Natural and induced $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells are critical mediators of peripheral tolerance. $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells modulate autoaggressive inflammatory $CD4^{+}$ T cells in autoimmune disorders [36]. We found that oral treatment only with LTBABC expanded $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells in relevant sites such as drenant PLN and MLN mucosal inductive sites. In this study Treg cells may be the principal T cell subset responsible for protection *via* IL-10 as it was shown by others [37]. The present results suggest that introducing the antigen through the GALT may be crucial for tolerance induction since the gut mucosa is a preferential site for differentiating Treg cells [38]. Our results also agree

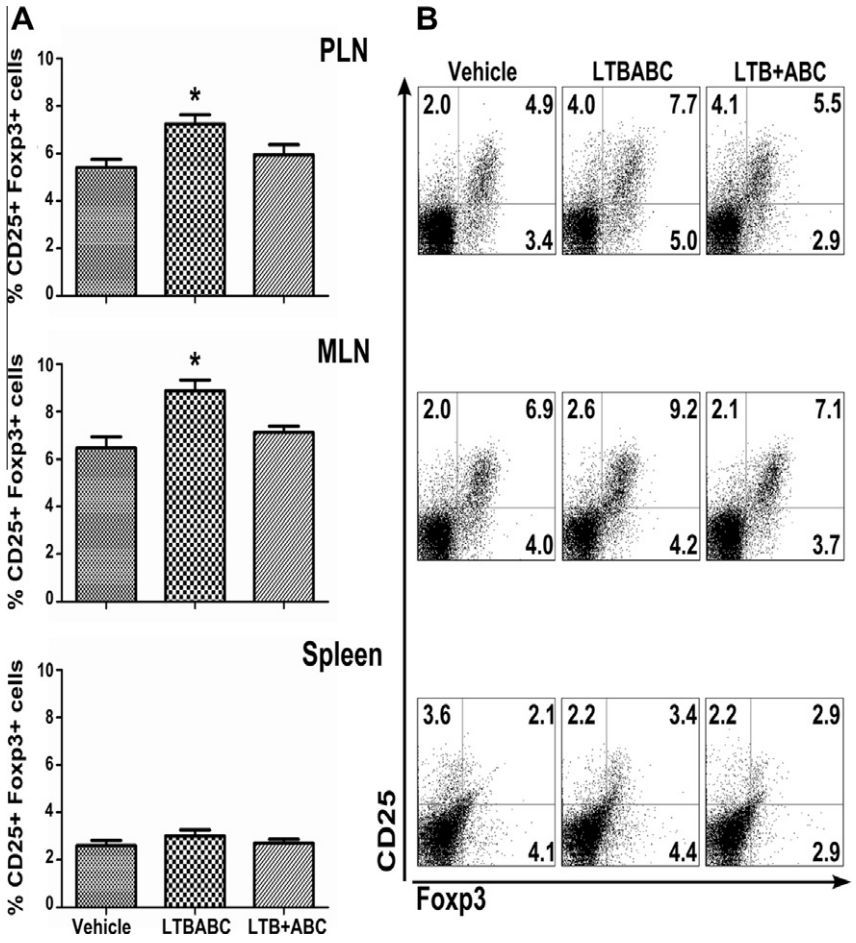

Fig. 7. LTBABC expanded $CD4^+CD25^+$ $Foxp3^+$ regulatory T cells. Rats were orally treated with 0.3 nmol of recombinant proteins LTBABC, LTB + ABC or vehicle at alternated days from the day of EAE induction up to 11 dpi. Popliteal (PLN), mesenteric lymph nodes (MLN) and splenic cells were obtained at 13 dpi and stained on the surface with anti-CD4 FITC, anti-CD25 APC and intracellularly with anti-Foxp3 PE and then analyzed by flow cytometry. (A) Relative percentages of $CD4^+Foxp3^+$ cells within the $CD4^+$ cell population from PLN, MLN and spleen from group of animals that received the different treatments. (B) Representative dot plots showing CD25 and Foxp3 expression on gated $CD4^+$ T cells from an individual from each experimental group. Numbers in each quadrant indicate the percentage of cells that were positive for the CD25, Foxp3 and both CD25 and Foxp3 antibodies. Each value represents the mean ± S.E.M. Significant differences are indicated for treated group *vs.* vehicle: $^*p < 0.05$. Results in (A) and (B) correspond to one experiment of two performed with 3 animals per group.

with studies showing that oral tolerance induced by low doses of CTB-antigen conjugates expands Treg cells, upregulates IL-10 and TGF-β and reduces levels of IFNγ and other Th1-associated cytokines [12,39,40].

5. Conclusion

Our observations indicate that the LTBABC hybrid can be a useful therapeutic tool to ameliorate EAE by immune modulation mediated by Treg cells. Nevertheless, additional studies are necessary to better characterize the effect of LTBABC on inflammation at the target organ of EAE.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cellimm.2012.11.012.

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