

RESEARCH ARTICLE

Probiotic VSL#3-induced TGF- β ameliorates food allergy inflammation in a mouse model of peanut sensitization through the induction of regulatory T cells in the gut mucosa

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Scope: Among food allergies, peanut allergy is frequently associated with severe anaphylactic reactions. In the need for safe and effective therapeutic strategies, probiotics may be considered on the basis of their immunomodulatory properties. The aim of the present study was to investigate the immunological mediators involved in the effects of probiotic VSL#3 oral supplementation on Th2 inflammation and anaphylaxis in a mouse model of peanut allergy.

Methods and results: VSL#3 supplementation to peanut-sensitized mice was effective in ameliorating anaphylaxis and Th2-mediated inflammation, by promoting regulatory responses in the jejunum mucosa and in the mesenteric lymph node, as evaluated by ELISA, real-time PCR, histologic, and immunohistochemical analysis. Probiotic-induced TGF- β mediates its protective effects through the induction of regulatory T cells expressing FOXP3 and/or latency-associated peptide, as proven by in vivo blockade of TGF- β in VSL#3-treated mice with a neutralizing monoclonal antibody one day before challenge.

Conclusion: TGF- β , induced in the gut by VSL#3 supplementation, is capable of reducing the Th2 inflammation associated with food anaphylaxis in a mouse model of peanut sensitization. TGF- β acts through the induction/maintenance of regulatory T cells expressing FOXP3 and/or latency-associated peptide. Probiotics supplementation may represent an effective and safe strategy for treating food allergies in adult population.

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1 Introduction

The breakdown of oral tolerance in the gastrointestinal tract leads to a Th2-predominant immunologic reactivity to food proteins, resulting in the development of IgE-mediated food

allergy. Several factors have been hypothesized to contribute to the activation of the allergen-specific immune response in the gut, including increased intestinal permeability and defects in regulatory T cell mechanisms [1].

Food allergy has been estimated to affect around 6% of young children and 3–4% of adults in westernized countries [2] and has shown an increasing trend in the last decades in several regions throughout the world [3]. Among food allergies, peanut allergy (*Arachis hypogaea*) represents a major concern as it is frequently associated with severe anaphylaxis and life-threatening reactions, is highly prevalent in children, does not tend to resolve spontaneously and to persist throughout life with a high impact on the patient's quality of life [4–6].

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Abbreviations: DC, dendritic cell; **FACS**, fluorescence-activated cell sorting; **LAP**, latency-associated peptide; **MC**, mast cell; **MLN**, mesenteric lymph node; **PE**, peanut extract; **TB**, Toluidine Blue; **Treg**, T regulatory cell

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Furthermore, up to now the current standard for prevention and care of food allergy is still the strict allergen avoidance and the elimination of the triggering food from the diet, however accidental ingestion [7] is particularly difficult to avoid in the case of peanut allergens due to their hidden presence in trace amounts in many food products and crosscontamination during food processing. Therefore, the development of safe and effective therapeutic treatment aimed to restore immune tolerance is particularly needed [8].

There is evidence from epidemiologic and clinical studies that the increased prevalence of allergic diseases in developed countries is associated with reduced microbial exposure and alteration of microbial communities represented in the gut microbiota [9]. The composition and metabolic activity of the microbiota have been demonstrated to be crucial for both the development and the maintenance of immune homeostasis, as well as to affect the induction of immune tolerance [10,11]. Intervention strategies targeting the intestinal microbiota include the deliberate administration of probiotic bacteria. Probiotics are live microorganisms that confer a health benefit to the host when administered in adequate amounts [12]. Different bacterial strains or mixtures have been used in clinical trials to assess their protective effects in the context of allergic sensitization and allergic diseases, but results have been conflicting [13–15]. Possible mechanisms of their protective action include both the induction of regulatory dendritic cells (DC) and T cells and the skewing of the immune response from a Th2 to a Th1 polarization, together with the enhancement of the epithelial barrier function [9,16–18]. Nevertheless, the knowledge of cell and molecular mechanisms underlying probiotic-host interactions that shape host immune system in a protective setting is still incomplete. Mouse models of food allergy to clinically relevant allergens could be of help in providing information difficult to obtain in human studies. Recently, we demonstrated that oral therapeutic treatment with the probiotic mixture VSL#3 is effective in redirecting allergen-specific Th2-polarized immune responses towards Th1/T regulatory responses and in the protection against anaphylactic reactions induced by the allergen in a mouse model of food allergy to shrimp tropomyosin [19].

The aim of the present study was to investigate the immunological mediators involved in the gut in the protective effects of VSL#3 oral treatment on Th2 inflammation and anaphylaxis in a mouse model of food allergy to peanut. Immunological changes induced by probiotic treatment were studied in the jejunum mucosa and in the draining mesenteric lymph node.

2 Methods

2.1 Peanut protein extraction

Peanut extract (PE) was prepared by minor modification of published protocols [20]. Ground peanuts were defatted with

diethyl ether for 2 h and dried at room temperature (r.t.) overnight (o.n.). Protein extraction was made with 20 mM Tris buffer (pH 7.2) and the aqueous fraction was collected by centrifugation ($3000 \times g$ at r.t., for 30 min), dialyzed against distilled water, lyophilized, and stored at -20°C .

2.2 VSL#3 probiotic preparation

The probiotic VSL#3 was kindly provided by VSL Pharmaceuticals (Towson, MD) as a lyophilized mixture consisting of eight different Gram-positive organisms (*Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Bifidobacterium breve*, *Streptococcus salivarius* subsp. *thermophilus*) at the concentration of 3×10^{11} live total bacteria/g, without any excipient. The concentration of each strain is covered by patent.

2.3 Animals and in vivo experimental protocols

Eight-wk-old female C57BL/6 mice were purchased from Charles River Italy (Calco, Italy). Animals were housed in the Animal Care Unit of the Istituto Superiore di Sanità. Experimental protocol was authorized by the Decree n. 216/2010-B of the Italian Ministry of Health. Groups of 5–10 mice were treated according to the protocols described below, being kept on a peanut-free diet during the course of the study.

2.3.1 Mice sensitization

Mice were immunized by intragastric (i.g.) administration of 1 mg PE/mouse together with 10 μg /mouse cholera toxin (Gentaur, Brussels, Belgium) as adjuvant in total 400 μL of an isotonic bicarbonate solution, on days 0, 7, 14, 21 (Fig. 1). Blood samples from retro-orbital plexus were collected on days 0 and 25.

2.3.2 Allergen challenge and evaluation of anaphylaxis reaction

Sensitized mice were challenged one week after the last immunization by receiving one i.g. dose of PE (10 mg/mouse). The appearance of symptoms of systemic anaphylaxis was observed during 1 h after challenge. Symptoms were evaluated in the range 0–5 according to the scoring system reported in [21]. Serum samples were individually collected within 1 h after challenge to assess systemic histamine release. Faecal samples were also individually collected and processed as previously described [22], to evaluate local IgA antibody response.

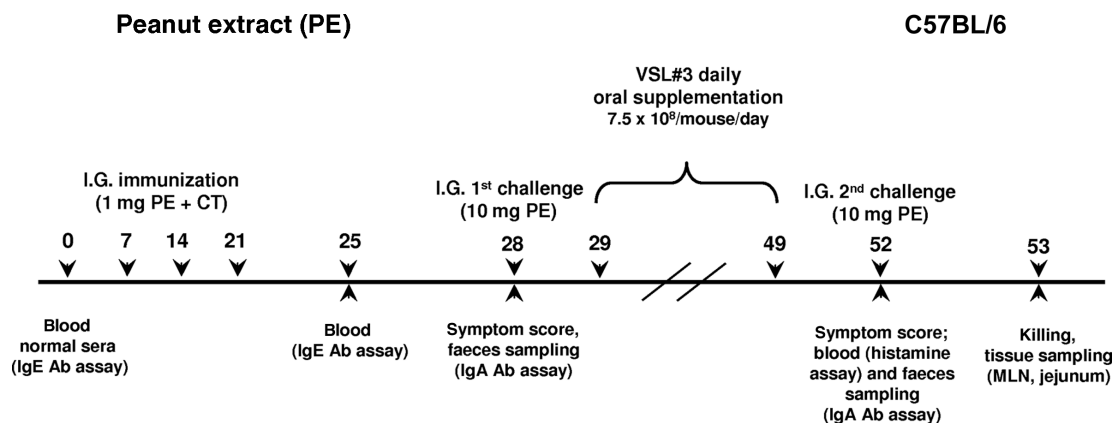


Figure 1. Experimental design of in vivo sensitization with peanut extract (PE) and probiotic VSL#3 supplementation. Groups of 5–10-week-old female C57BL/6 mice were treated during the study.

2.3.3 VSL#3 supplementation

From day 29 to day 49 sensitized mice were daily administered with 50 μ L/mouse of live VSL#3 mixture corresponding to 7.5×10^8 bacteria through the oral route [19]. Control groups received 50 μ L of sterile PBS. On day 52, mice were rechallenged as described above. Serum and faecal samples were collected as above. On day 53, mice were killed to collect mesenteric lymph node (MLN) and jejunum.

2.3.4 In vivo blockade of TGF- β

In a separate set of experiments, one day before the second challenge (day 51), VSL#3-treated mice were administered with the neutralizing anti-TGF- β 1, 2, 3 antibody derived from the hybridoma cell line 2G75A9 (kindly donated by Dr. J. Letterio, Dept. Pediatrics, Case Western Reserve University, Cleveland, OH) [23]. Distinct subgroups of mice were injected i.p. with 100 μ g of antibody in 200 μ L of sterile PBS per mouse, or with 200 μ L of PBS vehicle alone as control.

2.4 Measurement of histamine levels in sera

Histamine was assayed on postchallenge sera using an enzyme immunoassay kit (Immunotech, Marseille, France) according to manufacturers' instructions. Samples were analyzed at 1:10 dilution.

2.5 Evaluation of local IgA antibody response in faecal extracts

PE-specific IgA antibody response by individual mice was monitored by in-house ELISA on faecal extracts as previously described [22]. Plates (Greiner Bio-One, Frickenhausen, Germany) were coated with PE (20 μ g/mL), and faecal extracts were diluted 1:10. Results were expressed as arbitrary units,

which were calculated from a standard curve obtained with a pool of faecal extracts with high titre of PE-specific IgA.

2.6 In vitro stimulation of MLN cells: cytokine production and fluorescent-activated cell sorting analysis

Individual MLN cell suspensions were prepared from sensitized/treated mice after the second challenge with PE (day 53). Ex-vivo cells were maintained o.n. in culture without stimuli or cultured for 4 days with antigen stimulation (PE 50 μ g/mL), and then stained for fluorescent-activated cell sorting (FACS) analysis and cytokine production in culture supernatants.

FACS analysis was performed using antibodies for cell surface markers, including anti-MHC II class, anti-CD11c, and anti-CD103 (eBioscience, San Diego, CA). A FACScanto system (BD Biosciences, San Jose, CA) with BD FACSDiva software was used for analysis.

Culture supernatants were collected and stored at -20°C for cytokine detection. Levels of cytokines IL-10, TNF- α (BD Biosciences Pharmingen, San Jose, CA) and IL-13 (R&D Systems, Minneapolis, MN) were determined by sandwich ELISA kits, according to the manufacturers' instructions. Sensitivity for each assay was: 30 pg/mL (IL-10), 4 pg/mL (IL-13), 8 pg/mL (TNF- α).

2.7 Cytokine expression, histology, and immunohistochemistry in the jejunum

At the end of the experiment (d. 53), a two-centimetre section of the jejunum immediately distal to the duodenum was collected from individual mice [22]. Naïve mice were studied in parallel to evaluate basal levels. Three one-centimetre distinct fragments were cut to assess cytokine gene expression and protein production, and histology/immunohistochemistry.

Gene expression and protein production in the jejunum was evaluated by quantitative real-time PCR and ELISA assays in tissue homogenate, respectively, in two contiguous fragments of jejunum tissue as already described in Schiavi et al. [19]. The specific primers used in real-time PCR were: *TGF-β* forward: ACCGCAACAACGCCATCTAT; *TGF-β* reverse: GCACTGCTTCCCGAATGTCT; *IL-13* forward: AGACCAGACTCCCCTGTGCA; *IL-13* reverse TGGGTCCTGTAGATGGCATTG; *HPRT* forward: CTGGTGAAAAGGACCTCTCG; *HPRT* reverse: TGAAGTACTCATTATAGTCAAGGGCA. All the primers were used at the final concentration of 0.4 μM. Results for each gene have been analyzed by comparison to the HPRT values. Fold increases have been calculated according to the $2^{-\Delta\Delta C_t}$ method.

Content of TGF-β (BD Biosciences) and IL-13 (R&D Systems) in the jejunum extracts was evaluated by sandwich ELISA kits, according to the manufacturers' instructions. Sensitivity for TGF-β assay was 4.61 pg/mL and for IL-13 was 4 pg/mL. Results were expressed as pg/mg of total protein.

The third fragment was fixed o.n. in 10% buffered formalin, then transferred in sterile PBS and stored at 4°C for histological and immunohistochemical analysis. After processing and embedding in paraffin of jejunum samples, 3-μm-thick sections were stained with hematoxylin-eosin, Toluidine Blue (TB) for mast cell (MC) evaluation or processed for immunohistochemistry. Immunohistochemical evaluation of sections was performed essentially as reported elsewhere [24].

The following primary monoclonal (MAb) and polyclonal (PAb) antibodies were employed: a rat anti-human CD3 MAb (IgG1, clone CD3–12, MCA1477, AbD Serotec, Oxford, UK), diluted 1:10 in PBS; a rat anti-mouse FOXP3 MAb (IgG2a-kappa, clone FJK16S, 14–5773, e-Bioscience), diluted 1:50; a goat anti-human latency-associated peptide (LAP)/TGF-β1 PAb (AF-246-NA, R&D Systems), diluted 1:100; a rabbit anti-mouse TLR9/CD289 PAb (Creative Biomart, Shirley, NY), diluted 1:100. The following matched isotype controls have been respectively used: rat IgG1 MAb (clone YNB46.1.8, MA1–90035, Thermo Scientific Pierce Antibodies, Rockford, IL); rat IgG2a MAb (PA5–33212, Thermo Scientific Pierce Antibodies); normal goat polyclonal IgG control (AB-108-C, R&D Systems); normal rabbit polyclonal IgG (AB-105-C, R&D Systems). Biotinylated rabbit anti-rat IgG (BA-4000, Vector Laboratories, Burlingame, CA), rabbit anti-goat IgG (BA-5000, Vector Laboratories), and goat anti-rabbit IgG (E0432, DAKO, Glostrup, Denmark), were used as secondary antibodies at 1:200-dilution.

For evaluation, each tissue sample was subdivided in five areas. Three fields per area were randomly selected at 40HPFs, and all the cells stained by the primary antibody were counted and recorded.

2.8 Statistical analysis

Groups of 5–10 mice/treatment were evaluated throughout the study. Data were presented as means with SEM. The

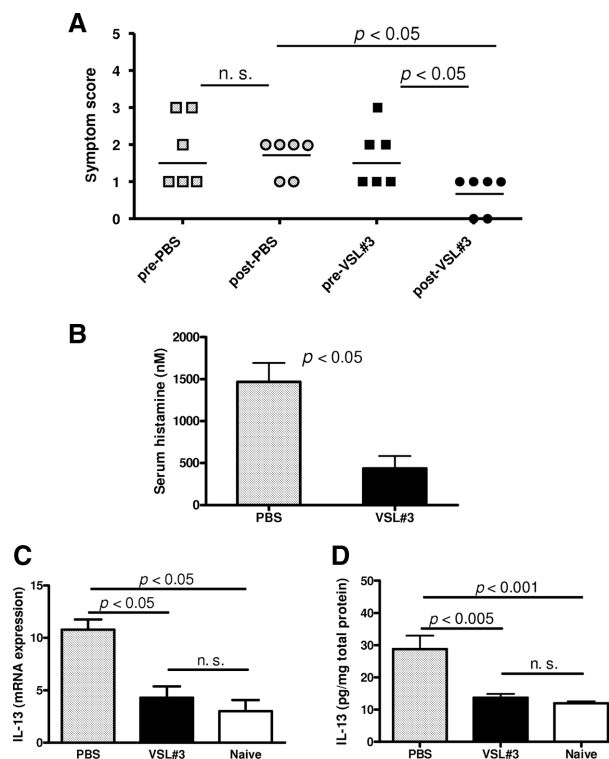


Figure 2. VSL#3 administration reduces systemic anaphylaxis and Th2 inflammation in the jejunum. (A) Symptom scores were recorded at the first (squares) and second (circles) challenge (pre- and posttreatment) in PBS- (gray symbols) and VSL#3-supplemented (black symbols) mice. Histamine was measured in serum samples collected at the second challenge (B); IL-13 mRNA expression (C) and IL-13 protein content in jejunum extracts (D) were evaluated. Values are presented as mean ± SEM from six individual mice/group, and are representative of three separate experiments.

Mann–Whitney *U* test (rank sum) or the Wilcoxon-matched pairs test when appropriate were used for the nonparametric analysis of differences between different experimental groups of mice. *p* values of less than 0.05 were considered statistically significant.

3 Results

3.1 Oral VSL#3 treatment attenuates anaphylaxis and Th2 inflammation in peanut-sensitized mice

We investigated whether the probiotic mixture VSL#3 was able to affect an in vivo established allergen-specific Th2 response, when administered by the oral route in a therapeutic setting to peanut-sensitized and challenged mice, as shown in Fig. 1.

PE-sensitized mice produced high PE-specific IgE levels (day 25, data not shown) and anaphylactic symptoms upon the first allergen challenge (day 28) with severity ranging from 1 to 3 as measured by the scoring system applied (Fig. 2A).

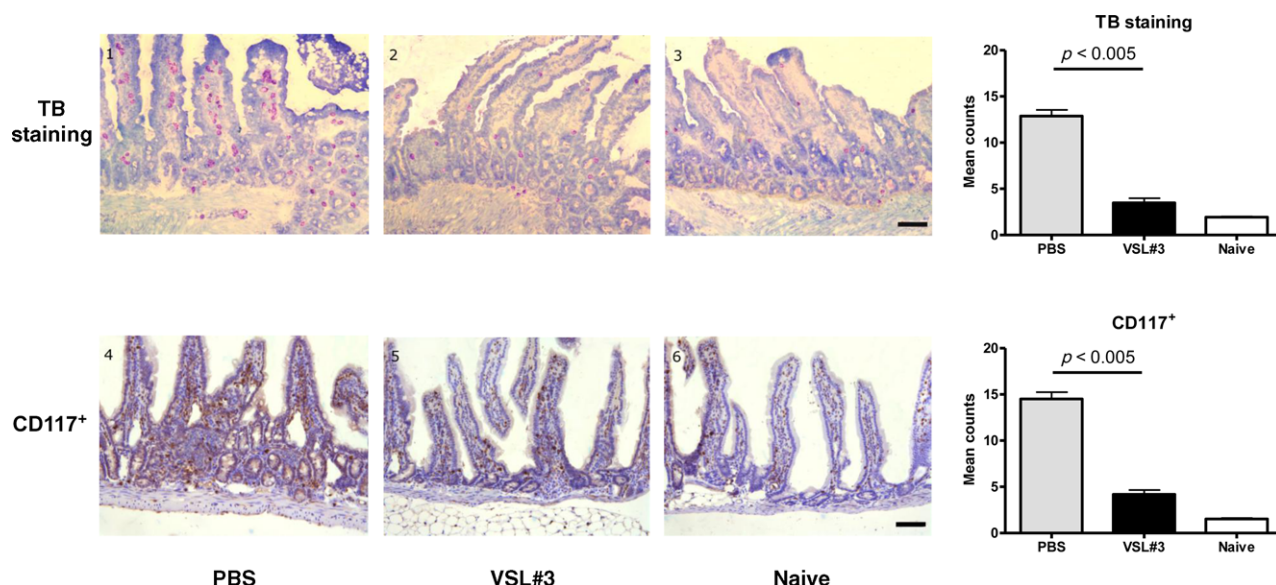


Figure 3. VSL#3 supplementation reduces MC infiltration and activation in the jejunum. Jejunum sections from sensitized mice treated with PBS or VSL#3 are compared with naïve controls. Photomicrographs ($\times 40$) of TB-stained (1–3) or anti-CD117 monoclonal antibody stained (4–6) paraffin sections of jejunum from representative mice are shown on the left (MC specific TB staining; immunohistochemistry (IHC): ABC method, Meyer's Haematoxylin counterstain; bar = 250 μ m). Aggregated data on mean counts obtained as described in Methods are reported on the right: values, representative of three separate experiments, are presented as mean $\pm$ SEM from six individual mice/group.

Symptom scores recorded in mice subsequently allocated to the two experimental groups (pre-VSL#3 or pre-PBS) did not show significant differences. Mice were then supplemented for three weeks with daily oral administrations of VSL#3 or PBS as control. Three days after the last administration, mice were rechallenged and the anaphylactic reaction was monitored and scored as in the first challenge. Histamine levels were evaluated in serum samples collected during the anaphylactic reaction. VSL#3 supplementation significantly reduced the symptom score, in comparison with both the score recorded in the control mice treated with PBS (Fig. 2A, post-VSL#3 versus post-PBS), and the score recorded in the same mice before VSL#3 treatment (Fig. 2A, post-VSL#3 versus pre-VSL#3). Accordingly, serum histamine levels at the second challenge were significantly decreased in the VSL#3-supplemented group (Fig. 2B).

One day after the second challenge, mice were killed to evaluate changes induced in the gut tissue. The reduced severity of anaphylaxis after probiotic treatment was associated with a significant decrease of both gene expression and protein level of IL-13 induced in the jejunum by peanut sensitization (Fig. 2C, D), thus suggesting that VSL#3 supplementation was able to attenuate allergen-induced anaphylactic reactions and to suppress Th2-mediated allergic inflammation in the intestinal mucosa.

3.2 VSL#3 supplementation in peanut-sensitized mice downregulates inflammatory responses and promotes TGF- β production and regulatory responses

To further analyze the effect of *in vivo* probiotic treatment, we performed histological and immunohistochemical analysis of jejunum sections to evaluate MC infiltration and activation. A highly significant reduction of MC infiltration by TB histochemical specific staining, as well as reduced counts of CD117⁺ cells, was observed in the jejunum of VSL#3-treated mice, which show values not different from naïve mice (Fig. 3).

We also evaluated the potential of VSL#3 supplementation to affect cell environment in the gut-draining MLN of sensitized mice. The expression of the gut-associated marker CD103 was studied on MLN CD11c⁺/MHCII⁺ DC populations. We observed that peanut sensitization significantly increased the frequency of CD103⁺ DCs in MLN as compared with naïve mice (PBS versus Naïve: 39.77% $\pm$ 0.96 versus 12.82% $\pm$ 1.93; mean $\pm$ SEM, $p < 0.005$). VSL#3 supplementation further increased the frequency of CD103⁺ DCs in MLN (PBS versus VSL#3: 39.77% $\pm$ 0.96 versus 49.22% $\pm$ 3.35; mean $\pm$ SEM, $p < 0.05$) (Fig. 4A). Supernatants of *o.n.* culture of MLN cells without any stimulus contained significantly increased levels of IL-10 and decreased levels of TNF- α in the VSL#3-supplemented group as compared with PBS

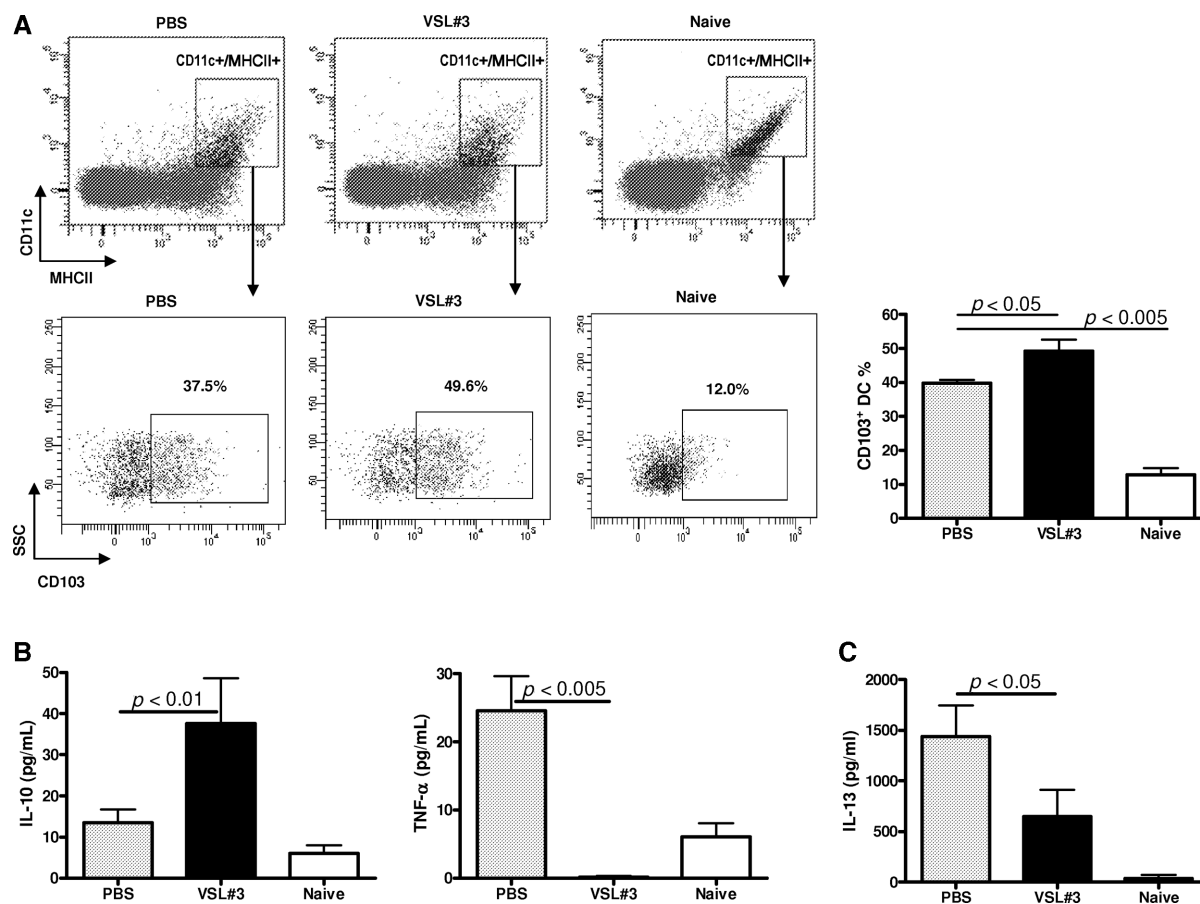


Figure 4. VSL#3 supplementation increases the frequency of regulatory CD103⁺ DCs, promotes IL-10 release and inhibit pro-inflammatory cytokines in the MLN. (A) Representative FACS diagrams for CD103 expression on gated CD11c⁺/MHCII⁺ DCs from MLN of one individual PBS- or VSL#3-treated mouse in comparison with one naïve mouse (left); aggregated frequency data (right) are reported for each experimental group. Cytokine production in culture supernatants has been evaluated after o.n. culture without stimuli (B), or after 4 day PE-restimulation culture (C). Bars represent mean $\pm$ SEM from six individual mice/group from one representative experiment out of four replicates with similar results.

controls (Fig. 4B). MLN cells were also *in vitro* restimulated 4 days with PE to assess antigen-specific T cell response. Antigen-induced IL-13 production was significantly reduced in probiotic-treated mice (Fig. 4C).

To further address the characteristics of the regulatory response promoted by probiotic supplementation, we studied TGF- β production in the inflamed jejunum of sensitized mice. Both TGF- β gene expression and protein production were significantly upregulated in jejunum mucosa of VSL#3-treated mice, as shown by quantitative real-time PCR analysis and protein assay in tissue homogenates, respectively (Fig. 5A). The upregulation of TGF- β in the gut was associated with a significant increase of IgA levels in the faecal extracts of probiotic-treated mice in comparison with control mice (Fig. 5B).

Immunohistochemical analysis of jejunum sections showed the induction of a regulatory pathway by VSL#3 supplementation. CD3⁺/FOXP3⁺ T cells were significantly

enriched in the jejunum of VSL#3-treated mice (Fig. 6). A significantly higher number of CD3⁺ T cells expressing LAP was also induced by VSL#3 treatment as compared with PBS controls (Fig. 6). Notably, these cells were arranged in subepithelial lymphoid aggregates, as found by histomorphological analysis in the jejunum tract of VSL#3-treated mice, which were not observed in PBS-treated or Naïve groups (Fig. 6 photomicrograph 2, arrow).

Toll-like receptor 9 (TLR9) was probably engaged in the crosstalk between VSL#3 probiotics and immune cells in the jejunum, as demonstrated by the significant upregulation of TLR9 expression in VSL#3-treated mice observed in the immunohistochemical analysis of jejunum sections (TLR9⁺ cells in PBS versus VSL#3 mice: 1.09 ± 0.32 versus 19.48 ± 0.51 ; mean counts $\pm$ SEM, $p < 0.005$; mean basal counts $\pm$ SEM in naïve mice: 0.50 ± 0.29).

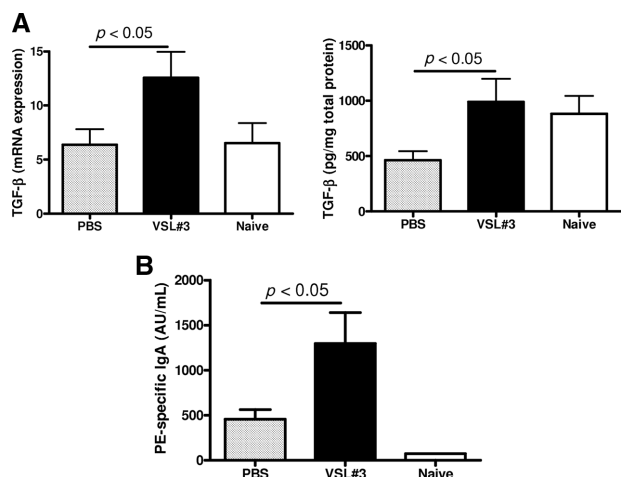


Figure 5. VSL#3 upregulates TGF- β and specific IgA production in the gut. (A) TGF- β mRNA expression and protein production have been evaluated in the jejunum mucosa at day 53. (B) PE-specific IgA antibody levels have been determined in faecal extracts collected during the 2nd challenge after probiotic or PBS treatment. Data are reported as mean $\pm$ SEM from six individual mice/group, and are representative of three separate experiments.

These data show that VSL#3 supplementation is able to downregulate the established Th2-driven intestinal inflammation in the jejunum of sensitized mice, and point to a central role played by VSL#3-induced TGF- β in the promotion of regulatory responses in the gut mucosa and in the draining LN.

3.3 In vivo blockade of TGF- β before challenge abrogates the anti-inflammatory effects induced by VSL#3 supplementation

To investigate the role of TGF- β in the protective effect of probiotic VSL#3, we used a neutralizing anti-TGF- β 1, 2, 3 monoclonal antibody that blocks TGF- β activity. In vivo administration of this antibody to VSL#3-supplemented mice one day before allergen challenge abrogated the capacity of probiotics to control anaphylaxis and Th2 inflammation, as indicated by histamine serum levels and IL-13 gene expression in the jejunum, respectively (Fig. 7A). Accordingly, histological and immunohistochemistry analysis showed a significant increase in MC infiltration and activation after TGF- β blockade (Fig. 7B).

Conversely, the administration of TGF- β -blocking antibody counteracts the regulatory response induced in the jejunum mucosa by probiotic supplementation (Fig. 8). Isotype staining controls with matched immunoglobulins showed negligible background not resembling specific staining (Supporting Information Fig. 1).

These results provide evidence that TGF- β induction mediates the protective effect of VSL#3 supplementation against

allergen-induced anaphylaxis and Th2 inflammation through the induction of regulatory T cells expressing FOXP3 and LAP.

4 Discussion

The main results of the present study demonstrate that TGF- β , induced in the gut by oral VSL#3 supplementation, is capable of reducing the Th2 inflammation associated with MC-driven food anaphylaxis in a mouse model of peanut sensitization. In fact, TGF- β blockade in VSL#3-treated mice before allergen challenge abrogates the capacity of probiotics to control anaphylaxis and Th2 inflammation, as indicated by histamine serum levels and IL-13 gene expression in the jejunum, respectively. Accordingly, histological and immunohistochemistry analysis shows a significant increase in MC infiltration and activation after TGF- β blockade.

The first evidence that TGF- β could be able to directly control MC function was provided in 1994 [25], demonstrating in an in vitro system that TGF- β downregulates the expression of *c-kit* gene (influencing MC development, migration, and activation) and its cell surface product in murine hematopoietic progenitors including promastocytes. *c-kit* (CD117) expression in the jejunum tract observed in our mouse model of food anaphylaxis is significantly reduced by probiotics, while the in vivo TGF- β blockade before allergen challenge restores high level of CD117 expression. This trend is paralleled by the modulation of typical markers of Th2 inflammation, such as histamine release in the serum and IL-13 expression and production in the gut mucosa. Immunoregulation of MC function (in terms of mediator synthesis and release) by TGF- β has been also reported for human intestinal MC [26]. Accordingly, our histological observations in mouse jejunum with TB staining indicate a significant reduction of TB positivity in VSL#3-supplemented mice in comparison with PBS controls, completely reverted by TGF- β blockade.

Our results also provide evidence that the TGF- β -mediated protective effect of probiotic treatment acts through the induction of regulatory T cells expressing FOXP3 and LAP. CD4⁺FOXP3⁺ inducible T regulatory cells have been very recently demonstrated to in vitro inhibit MC function and to attenuate established MC-driven skin contact hypersensitivity after in vivo transfer, through TGF- β 1-dependent mechanisms [27]. In our mouse model of food sensitization and anaphylaxis, VSL#3 supplementation upregulates the expression and production of TGF- β in the jejunum, as well as a regulatory pathway characterized by a significant CD3⁺ T cells enrichment, expressing FOXP3 and/or LAP as suggested by immunohistochemical analysis of contiguous jejunum sections. Although both FOXP3 and LAP expression on T cells appear to be significantly upregulated by VSL#3 administration and downregulated by TGF- β blockade, we cannot distinguish by this technique if they represent two separate regulatory populations or if a double positive T regulatory cell

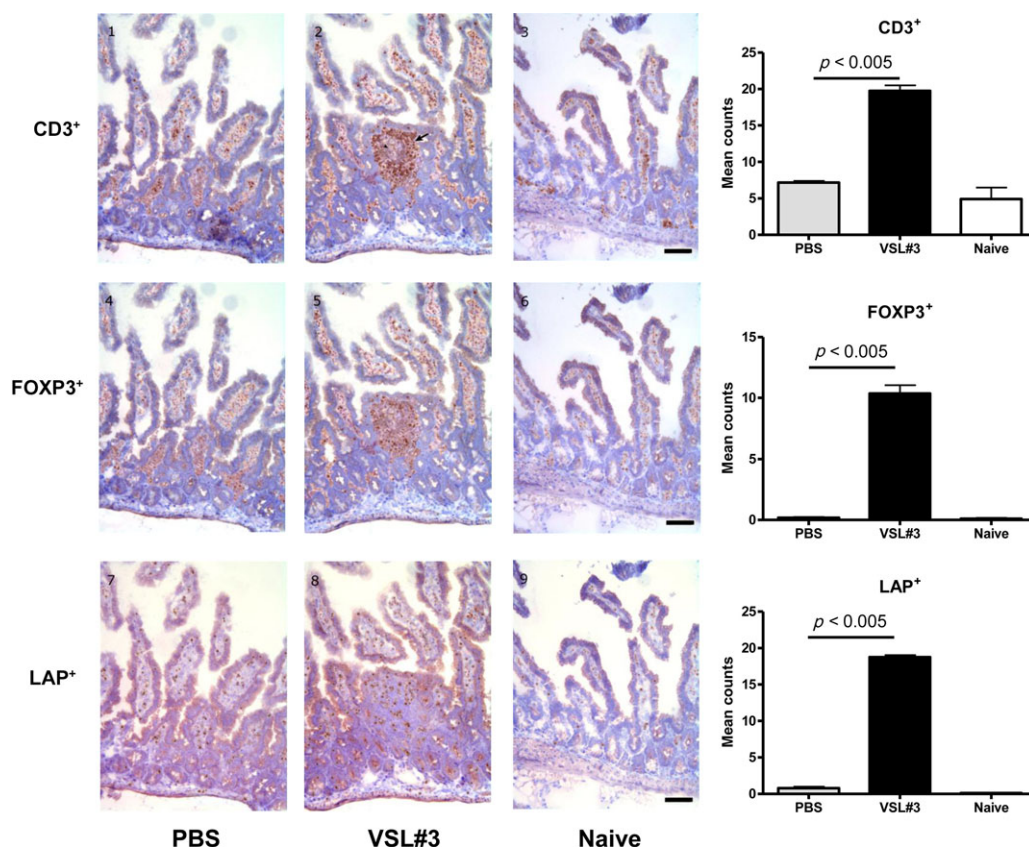


Figure 6. VSL#3 supplementation induces a regulatory pathway in the jejunum. Jejunum sections from sensitized mice treated with PBS or VSL#3 are compared with naïve controls. Photomicrographs ($\times 40$) of paraffin sections of jejunum from representative mice stained with anti-CD3 (1–2–3), anti-FOXP3 (4–5–6), anti-LAP (7–8–9) monoclonal antibodies are shown on the left (IHC, ABC method, Meyer's Haematoxylin counterstain; bar = 250 μ m). A characteristic follicular organization of CD3⁺ T cells can be observed in VSL#3 treated mice (2), characterized by a well-defined coronal area (arrow), and a "clear" CD3[−] center-follicular area (arrowhead). No tertiary lymphoid structures were observed in PBS (1), and naïve controls (3). Aggregated data on mean counts obtained as described in Methods are reported on the right: values, representative of three separate experiments, are presented as mean $\pm$ SEM from six individual mice/group.

(Treg) coexpressing FOXP3 and LAP (FOXP3⁺ Treg activated by TGF- β) is also involved in the probiotic effect observed in our system.

LAP⁺ T cells induced in the colon by VSL#3 oral supplementation have been shown by our group in a mouse model of recurrent Th1 TNBS colitis [28] and in humans with ileal-pouch-anal anastomosis for ulcerative colitis [29]. CD4⁺ LAP⁺ inducible Treg populations have been demonstrated to be involved in suppressing inflammation in a TGF- β -dependent manner in mouse experimental models of colitis and autoimmune diseases [28, 30–33]. Next, LAP expression by activated mouse and human FOXP3⁺ Tregs has been shown by Andersson et al. [34] and Tran et al. [35].

More recently, the involvement of LAP⁺ T cells in the regulation of allergic responses has been demonstrated by Duan et al. [36], reporting the generation of such cells in response to TGF- β and their capacity to suppress both the induction and effector phases of asthmatic lung inflammation in the mouse. Our results demonstrate for the first time that the

induction of LAP⁺ T cells (coexpressing or not FOXP3) in the jejunum mucosa by oral administration of probiotics is associated with the significant reduction of anaphylaxis symptoms and Th2 inflammation markers in a mouse model of food allergy. These effects are mediated by probiotic-induced TGF- β (as demonstrated by their complete abrogation after in vivo TGF- β blockade), which in turn sustains in vivo FOXP3⁺ Treg induction/maintenance and surface LAP expression, as described by Marie et al. [37] and Gabrysova et al. [38], and Oida et al. [39], respectively.

The regulatory activity of probiotics is likely to occur via modulation of local DCs. Intestinal DCs expressing CD103 are specialized to activate latent TGF- β and are able to induce FOXP3⁺ Tregs [40, 41]. As already shown by Wang et al. [42], VSL#3 oral supplementation is able to modify the distribution and phenotype of DC subsets in the intestinal mucosa and MLN. We focussed our attention on CD103⁺ DCs in MLN, due to their known capacity to induce Tregs [43], and found that VSL#3 treatment significantly increased the

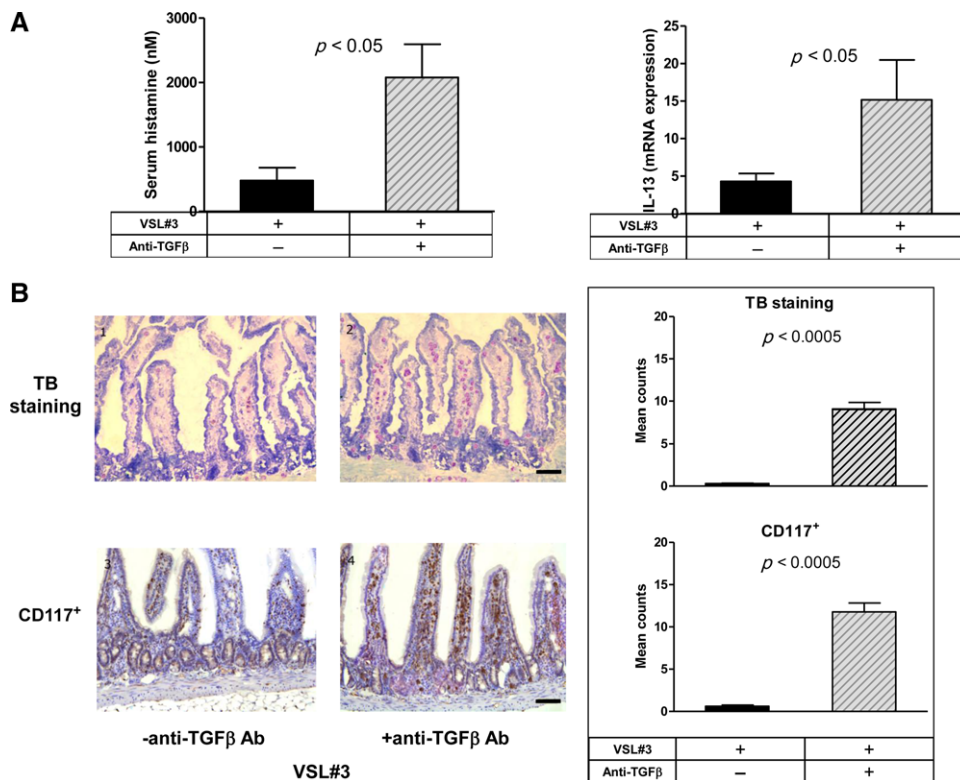


Figure 7. Probiotic-induced TGF- β is essential for the inhibition of Th2 inflammation. Mice sensitized and treated with VSL#3 were subjected to in vivo TGF- β blockade one day before challenge. (A) Serum histamine levels and IL-13 mRNA expression in the jejunum mucosa have been evaluated at day 52 and 53, respectively. (B) Photomicrographs ($\times 40$) of TB-stained (1–2) or anti-CD117 monoclonal antibody stained (3–4) jejunum sections from representative mice are shown on the left (MC-specific TB staining; IHC: ABC method, Meyer's Haematoxylin counterstain; bar = 250 μ m). Aggregated data on mean counts obtained as described in Methods are reported on the right. Bars in (A) and (B) represent mean $\pm$ SEM from five individual mice/group in one out of two separate experiments.

frequency of such DC subset in MLN, as assessed by ex vivo FACS analysis, in comparison to PBS controls. These DCs are expected to have tolerogenic properties, as suggested by the significant increase in IL-10 and significant decrease in TNF- α secretion after o.n. culture of MLN cells without any stimulus. Indeed, the restimulation of MLN cells four days with PE to assess the effects on antigen-specific T cell responses, yielded a significant reduction of IL-13 production in the VSL#3-supplemented group, thus supporting the capacity of MLN CD103⁺ DCs to preferentially induce regulatory responses able to control an established Th2 inflammation.

In the complex pathways driving the probiotic modulation of DCs [44], Toll-like receptors (TLRs) play a fundamental role. As evidenced in our results, the significantly higher immunohistochemical expression of TLR9 in the jejunum mucosa of VSL#3-treated mice demonstrated that TLR9 was probably engaged in the crosstalk between probiotics and immune cells. Data obtained in our food allergy system confirm those reported in a murine model of experimental colitis by Rachmilewitz et al. [45], and comply with the general observation that gut flora DNA plays a major role in intestinal homeostasis through TLR9 engagement by critically controlling the balance between regulatory and effectors T cells [46].

Probiotics-immune cells crosstalk in the jejunum is also associated with a peculiar pattern of lymphocyte recruitment and organization in the gut mucosa. In our system, VSL#3

supplementation induced a different pattern of lymphocyte recruitment, characterized by the formation of subepithelial lymphoid aggregates, absent in PBS or Naïve groups of mice. Although these structures have been mainly associated to chronic inflammation and infection in the gut and to the differentiation of IgA-producing cells in the gut lamina propria [47], more recent observations highlight the contribution of intestinal bacterial commensals to this process [48, 49], by communicating with the local immune system to generate adaptive lymphoid tissues and maintain intestinal homeostasis [50]. Our finding of such lymphoid aggregates in the VSL#3-supplemented group can be related to the significantly increased levels of specific anti-PE IgA detected in this group.

In conclusion, we have shown that oral supplementation with VSL#3 probiotic mixture significantly ameliorates anaphylaxis symptoms and reduces established Th2 inflammation in a mouse model of peanut allergy. We also demonstrated, for the first time in this system, that these effects are associated with the induction/maintenance of T regulatory cells expressing LAP and/or FOXP3, and dependent on TGF- β induced by VSL#3.

These findings obtained with VSL#3 probiotic mixture provide further evidence that oral supplementation of probiotic mixtures may represent an effective and safe strategy for treating food allergies in adult population, for which no established and effective cures based on the pathogenetic mechanisms are available.

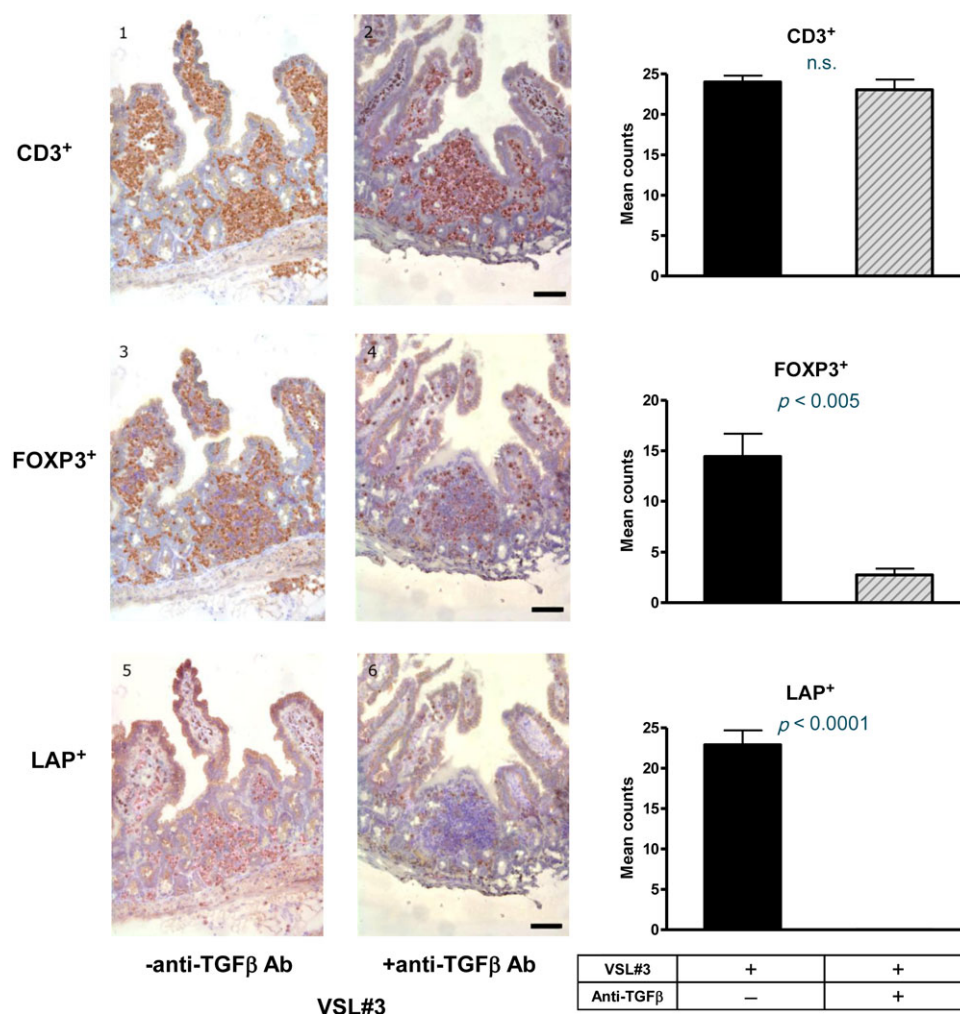


Figure 8. Probiotic-induced TGF- β is critical for the protective regulatory responses evoked by VSL#3 treatment. Mice were treated as described in Fig. 7. Photomicrographs ($\times 40$) of jejunum sections from representative mice stained with anti-CD3 (1–2), anti-FOXP3 (3–4), anti-LAP (5–6) monoclonal antibodies are shown on the left (IHC: ABC method, Meyer's Haematoxylin counterstain; bar = 250 μ m). Aggregated data on mean counts obtained as described in Methods are reported on the right: bars represent mean $\pm$ SEM from five individual mice/group in one out of two separate experiments.

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The authors have declared no conflict of interest.

5 References

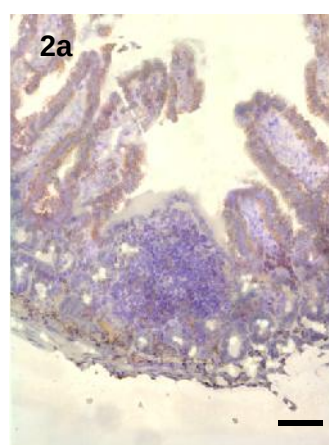
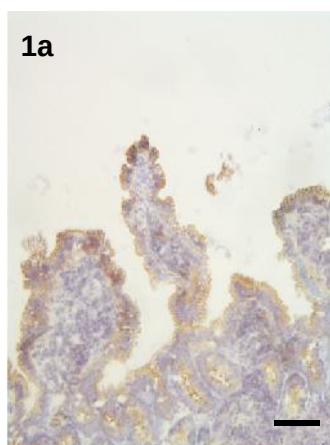
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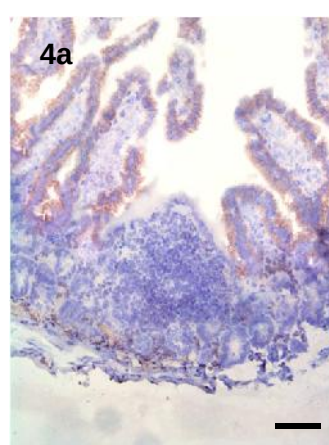
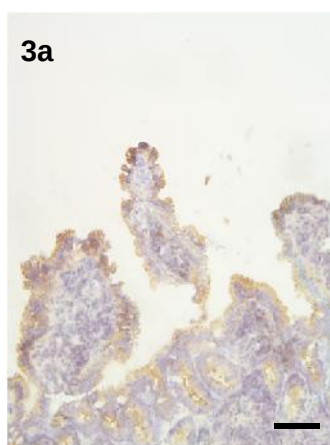
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Supplemental Figure S1

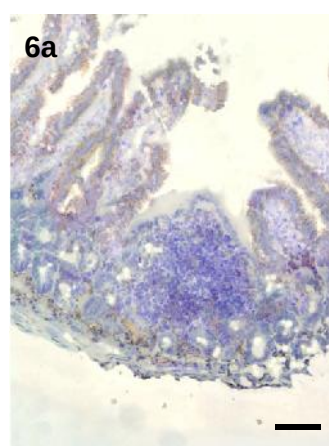
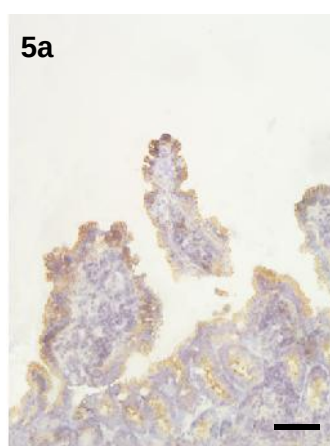
**Rat IgG1
(anti-CD3
Isotype control)**



**Rat IgG2a
(anti-FOXP3
Isotype control)**



**Goat IgG
(anti-LAP
Isotype control)**



VSL#3	+	+
Anti-TGFb	-	+