

# Oral tolerance and Treg cells are induced in BALB/c mice after gavage with bovine $\beta$ -lactoglobulin

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## Keywords

allergic reaction; BALB/c mice; bovine  $\beta$ -lactoglobulin; IgE; induced Treg cells; oral tolerance.

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## Abstract

**Background:** Food allergy is considered as resulting from an impaired development or a breakdown of oral tolerance. We aimed to induce oral tolerance to the major cow's milk allergen bovine  $\beta$ -lactoglobulin (BLG) or corresponding trypsin hydrolysates (BLG-Try) and to investigate the mechanisms involved.

**Methods:** Wild-type BALB/cJ mice were gavaged on days 1–3 and 8–10 with different doses of native BLG (nBLG) or with nBLG-Try and were then sensitized on day 14 by i.p. administration of BLG in alum. Sensitization was assessed by measurement of BLG-specific antibodies in sera and of cytokines secreted by BLG-reactivated splenocytes. Elicitation of the allergic reaction was assessed by measurement of cytokines and mMCP-1 in sera collected 35 min after an oral challenge. Cellular and biochemical markers of the allergic reaction were also analysed in bronchoalveolar lavage fluids (BAL) collected 24 h after intra-nasal challenge. Analysis of the CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells in different organs obtained 3 days after gavage and *in vivo* depletion of CD25<sup>+</sup> cells before oral tolerance induction were then performed.

**Results:** Systemic sensitization and elicitation of the allergic reaction were totally inhibited in mice gavaged with 2 mg of nBLG whereas nBLG-Try was far less efficient. A high percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> cells were observed in BAL from tolerant mice, and a negative correlation between the number of eosinophils and the percentage of Foxp3<sup>+</sup> cells was evidenced. Efficient induction of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells after nBLG gavage and impaired oral tolerance induction after *in vivo* depletion of CD25 cells were then demonstrated.

**Conclusion:** For the first time, allergen-induced Treg cells that inhibited both the sensitization and the elicitation of the allergic reaction were evidenced in gavaged wild-type mice.

Regulatory T cells (Treg) are naturally produced in the thymus (nTreg) or induced in the peripheral tissues (iTreg). Both kinds are CD4<sup>+</sup>CD25<sup>+</sup> cells expressing the transcription factor forkhead p3 (Foxp3) and are actively involved in the maintenance of self-tolerance and immune homeostasis (1). Recent evidence has suggested a core mechanism for Treg suppressive function on antigen-presenting cells, involving

lymphocyte function-associated antigen (LFA)-1 and CTLA4. Auxiliary suppressive mechanisms involving IL-10, TGF $\beta$ , IL-35 and/or other mediators and mechanisms may also operate, depending on the environment and the type of immune response (1). Naïve T cells in the periphery can acquire Foxp3 expression and then Treg function. It is suggested that in naïve T cell, Foxp3 would hijack the transcription machinery for effector Th1, Th2 or Th17 cells, thus early converting them into Treg. Moreover, secretion of retinoic acid by CD103<sup>+</sup> DC in the lamina propria of the small intestine facilitates the differentiation of naïve T cells in Foxp3<sup>+</sup> cells (2). Such induced Treg cells, specific of an orally administered antigen, can then circulate and establish a systemic tolerance to this antigen. This phenomenon would

## Abbreviations

BAL, bronchoalveolar lavage fluids; BLG, bovine  $\beta$ -lactoglobulin; DMCT, Dunn's multiple comparison test; iTreg, induced Treg; MLN, mesenteric lymph nodes; nBLG, native BLG; nBLG-Try, trypsin hydrolysates of nBLG; Ova, ovalbumin; PP, Peyer's patches; Treg, regulatory T cells.

then largely contribute to the induction of oral tolerance. Food allergy, which is an increasingly prevalent disease with potential life-threatening clinical manifestations, is then considered as resulting from an impaired development of oral tolerance or a breakdown in existing oral tolerance (3).

Cow's milk allergy affects approximately 2.5% of young children and 0.4–0.9% of whole population (4–6). Severe forms are mainly immediate, IgE-mediated hypersensitivity reactions although T-cell-mediated delayed-type hypersensitivity to milk allergens is also observed (7, 8). Cow's milk allergic patients may be sensitized to various proteins, mainly bovine  $\beta$ -lactoglobulin (BLG) and casein (9). Although about 80% of infants allergic to milk seemed to become tolerant at 5 years of age, a lower rate of development of clinical tolerance has been more recently observed, mainly in patients with high milk-specific IgE levels in the first 2 years of life (10). Interestingly, patients that outgrew their cow's milk allergy demonstrated higher levels of circulating CD4<sup>+</sup>CD25<sup>+</sup> cells and decrease in BLG-induced *in vitro* proliferation of peripheral blood mononuclear cells (PBMC) as compared to patients who maintained clinically active allergy. Depletion of CD4<sup>+</sup>CD25<sup>+</sup> cells from PBMC of tolerant patients led to enhanced *in vitro* proliferation to BLG (11). Accordingly, Shreffler et al. further demonstrated that no functional defect of the Treg cells subset was detected in allergic individuals, but that a higher frequency of these specific Treg cells was associated with clinical tolerance. These cells were characterized as CD25<sup>+</sup>CD27<sup>+</sup>Foxp3<sup>hi</sup>CTLA4<sup>+</sup>CD127<sup>-</sup> T cells, and their proliferation was induced by casein in patients able to consume heated milk without allergic reaction. Conversely, their depletion enhanced *in vitro* allergen-specific effector T-cell proliferation (12), corroborating previous study in patients with IgE-mediated milk allergy (13). Altogether, these studies confirm that these Treg cells are functionally suppressive and then may be important *in vivo* for acquisition of clinical tolerance to milk.

Induction of tolerance by some food proteins and analysis of the cellular mechanisms involved have been studied in various models. Notably, ovalbumin (Ova)-induced oral tolerance has been investigated in DO11.10 TCR mice, carrying TCR specific for Ova f(323–339) peptide, or after transfer of ovalbumin-specific T cells to recipient mice (14–17). The involvement of Treg cells in oral tolerance in normal, non-transgenic, mice was mainly investigated after *in vivo* depletion of CD4<sup>+</sup>CD25<sup>+</sup> cells (18, 19), but those cells were not demonstrated to be induced in BALB/c mice after gavage with Ova (16). In this study, we assessed the efficiency of oral tolerance induced by native BLG (i.e. with the conformational structure maintained by disulphide bridges) or corresponding BLG trypsin hydrolysates. To investigate whether or not the tolerization procedure enduringly activated the peripheral and not only the mucosal immune system, orally treated mice were further sensitized by the i.p. route and elicitation of the allergic reaction was assessed both at the gastrointestinal and respiratory levels. The implication of induced regulatory T cells in this model was then analysed.

## Material and methods

### BLG purification and hydrolysis

Native BLG (nBLG) was purified from raw milk using selective precipitation and chromatography as previously described (20, 21). Trypsin hydrolysis of nBLG was performed using trypsin (bovine pancreatic, Type XIII, 10 000–13 000 BAEE units/mg of protein, Sigma, St Louis, MO, USA) at a E/S ratio (m/m) of 1/25. Bovine  $\beta$ -lactoglobulin and trypsin were both solubilized in Tris 0.1 M buffer pH8. After 3 h at 40°C, reaction was stopped by adding TFA (0.2% final).

All proteins and trypsin hydrolysates were further characterized using reverse-phase high-performance liquid chromatography (RP-HPLC), mass spectrometry (MALDI-TOF, Voyager DE-Pro, Applied Biosystems, Courtaboeuf, France) and specific sandwich ELISA immunoassays (22). As trypsin hydrolysates contained residual undegraded BLG (about 1.1%), an additional chromatography was performed (Vydac C18 column, 300 Å, 250 × 22 mm). Purified trypsin hydrolysates of nBLG (nBLG-Try) then contained less than 0.01% of nBLG. Mass analysis of nBLG-Try demonstrated the presence of peptides f(21–40), f(41–60), f(61–69), f(92–124), f(101–124), f(92–135), f(92–138), f(102–124), f(142–148) and f(149–162) (PeptideMass EXPASY, <http://www.expasy.ch/tools/peptide-mass.html>). No or few amounts of peptides f(1–20) and f(71–91) were detected, as previously observed (23, 24). Additional peptide of 2780.2 Da MW was evidenced when using nondenaturing condition for mass analysis. It corresponds to the association of f(61–69) and f(149–162) linked by disulphide bridge.

Purified proteins and hydrolysates were dialysed against potassium phosphate buffer (100 mM and then 20 mM, pH7.4) and freeze dried. After solubilization in DPBS (Gibco, Invitrogen, Cergy-Pontoise, France), protein content was assayed by BCA following manufacturer's instructions (Pierce, Thermo Scientific, Rockford, IL, USA).

### Assessment of the efficiency of tolerance induction by BLG products

#### Mice

Specific pathogen-free BALB/cJ mice (3- to 4-week-old female, Centre d'Élevage René Janvier, Le Genest Saint-Isle, France) were housed in filtered cages under normal SPF husbandry conditions (autoclaved bedding and sterile water) and were acclimated for 2 weeks before immunizations. They received a diet deprived of animal proteins in which BLG was not detected using sensible and specific immunoassays (22). All animal experiments were performed according to European Community rules of animal care and with authorization 91–368 of the French Veterinary Services.

#### Administration of nBLG and nBLG trypsin hydrolysates and assessment of the effect on a further sensitization and elicitation of the allergic reaction

Native bovine  $\beta$ -lactoglobulin or corresponding trypsin hydrolysates (0.05–4 mg) in solution in DPBS were administered

to mice by intra-gastric gavage using an animal feeding needle (Popper & Sons, New York, NY, USA) on days 1, 2, 3, 8, 9 and 10. On day 14, mice were sensitized by i.p. administration of 5 µg of nBLG adsorbed on alum (Alhydrogel 3%, Superfos, Denmark, 1 mg/mouse). Mice sensitization was assessed by quantitative measurement of BLG-specific IgE, IgG1 and IgG2a antibodies (25) on individual serum samples collected from the retro-orbital venous plexus between day 33 and 36. Spleens were then removed under sterile conditions and pooled within groups to evaluate cytokine production under specific *ex vivo* re-stimulation. After spleen dilacerations, red blood cells were first lysed (180 mM NH<sub>4</sub>Cl, 17 mM Na<sub>2</sub>EDTA), and after several washes, the splenocytes were resuspended in RPMI-10 (RPMI supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 U penicillin and 100 µg/ml streptomycin) and incubated for 60 h at 37°C (5% CO<sub>2</sub>) in 96-well culture plates (10<sup>6</sup> cells/well) in the presence of BLG (20 µg/ml). Concanavalin A (1 µg/ml) was used as positive control and saline or irrelevant antigen (Ova, 20 µg/ml) as negative controls. After centrifugation (300 g, 10 min, +4°C), supernatants were collected and stored at -80°C until used for Th1/Th2/Th17 cytokine assays using BioPlex technology and mouse cytokines kit from BioRad, or for TGFβ assay (Cytoset™; Biosource International, Nivelles, Belgium), following provider's recommendations.

In some experiments, an allergen challenge was performed to assess the effect of the oral administration of BLG products on the further elicitation of the allergic reaction. A boost administration of nBLG in alum was then performed 14–18 days after the first sensitization. In a first experiment, mice were then orally challenged with 10 mg of BLG 8 days after the boost injection. Th1/Th2/Th17 cytokines (MilliPlex, Merck Millipore, Molsheim, France) and mouse mast cell protease-1 (mMCP-1; Moredun Scientific Limited, Midlothian, UK) were then assayed on individual sera collected 35 min after oral challenge following provider's recommendations. In a second experiment, mice received an intranasal administration of 20 µg of BLG in 50 µl of DPBS, under light anaesthesia (Isoflurane Belamont, Nicholas Piramal Limited, London, UK) 6 days after the boost injection (26). Twenty-four hours after the challenge, mice were deeply anaesthetized by i.p. injection of 200 µl/mice of a cocktail of ketamine (15 mg/ml) and xylazine (2 mg/ml) (Imalgène 500; Merial, Lyon France; Rompum 2% Bayer Pharma, Puteaux, France). The trachea was cannulated, and bronchoalveolar lavage fluids (BAL) were collected in HBSS/EDTA 0.1 M (Gibco) and kept on ice. Total cells in BAL were counted using Viacount Reagent and EasyCyte Plus flow cytometer, both from Guava Technologies, following manufacturer's recommendation. Cellular composition of BAL was analysed as described in (27) using simultaneous labelling with 1 µg/10<sup>6</sup> cells of anti-CD3-PE-Cy5, B220-PE-Cy5, anti-CMHII-FITC, anti-CD11c-PECy7 (all from Pharmingen, Becton Dickinson (BD), San Jose, CA, USA) and anti-CCR3-PE (R&D Systems, Abingdon, UK). Acquisition and analysis were performed on Guava EasyCyte Plus cytometer using CytoSoft 5.1 software (Guava Technologies, Hayward, CA, USA). Another BAL aliquot

was stained using 1 µg/10<sup>6</sup> cells of anti-CD4-FITC (clone GK1.5, BD Pharmingen) and anti-Foxp3-PE using Foxp3 staining buffer set (Myltenyi Biotec, Paris, France) following provider's recommendation. All BAL were analysed individually and were initially blocked using 1 µg/10<sup>5</sup> cells of 2.4G2 antibody (anti-FcγIII/II receptor, FcR blocking reagent, BD) to avoid nonspecific binding. Aliquots of the remaining BAL were centrifuged and stored at -80°C until cytokine assays. IL-2, IL-4, IL-5, IL-10, IL-17, eotaxin, GM-CSF, IFN-γ and TNF-α were assayed on individual samples of BAL using BioPlex and kit from BioRad. TGFβ was also assayed (Cytoset™, Biosource International) following manufacturer's recommendations.

### Assessment of the Treg cells implication in BLG-induced oral tolerance

#### *Analysis of the CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells after gavage with BLG products*

Mice received one i.g. gavage with 2 mg of nBLG (*n* = 4) or the corresponding trypsin hydrolysate (*n* = 3) or DPBS as a control (*n* = 3). Three days later, mice were killed and mesenteric lymph nodes (MLN), Peyer's patches (PP) and spleen were removed and placed in DPBS. After dilacerations on 40 µm cell strainers (BD Falcon, Franklin Lakes, NJ, USA), cell suspensions were washed (500 g, 10 min, +4°C). An additional step was performed for spleen cells, consisting of red blood cell lysis, followed by 2 washes in DPBS. Cell pellets were finally resuspended in DPBS 1% BSA (Sigma). Cell count and CD4, CD25 (anti-CD25-PE-Cy5, clone PC61, BD Pharmingen) and Foxp3 staining were performed as previously described. Acquisition was performed on Guava EasyCyte Plus cytometer by acquiring 5000 events in a predefined FSC<sup>lo</sup>/SSC<sup>lo</sup> gate. Each organ from each mouse was treated individually. Percentage of CD25 and Foxp3-positive cells within CD4<sup>+</sup> population was then assessed using CytoSoft 5.1 software.

#### *In vivo depletion of Treg cells*

On days 1 and 2, 14 mice received i.p. injection of 100 µg of anti-CD25 antibody (functional grade purified clone PC61, eBiosciences, San Diego, CA, USA) (19, 28, 29), or equivalent amount of isotype control (functional grade purified rat IgG1 antibody, eBiosciences) both diluted in DPBS. As a control, 11 mice received DPBS. On days 3–5, 10, 11 and 12, 5–7 mice of each pretreatment group received 2 mg of nBLG or PBS by intra-gastric gavage. On day 16, all mice were sensitized as previously described. Bovine β-lactoglobulin-specific IgE, IgG1 and IgG2a antibodies were assayed on individual serum samples collected on day 38. Spleens were removed on day 40, and cytokines were assessed on reactivated spleen cells as previously described.

### Statistical analysis

All statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Normality distribution was first examined using

Shapiro–Wilk normality test before analysis of statistical significance with one-way ANOVA and Tukey's multiple comparison posttest. When data were not normally distributed, a nonparametrical test was performed, using Kruskal–Wallis test followed by Dunn's multiple comparison test (DMCT). Differences between experimental groups were regarded as significant when  $P \leq 0.05$ .

## Results

### Efficient tolerance is induced by gavage with 2 mg of nBLG for 6 days

A first set of experiment was performed to assess the efficiency of gavage with nBLG to protect against a further i.p. sensitization by nBLG in alum. Mice received either DPBS or 0.05 or 2 mg of nBLG on days 1, 2, 3, 8, 9 and 10 by i.g. gavage. All mice were then sensitized on day 14 by nBLG adsorbed on alum (26). As shown in Fig. 1A,B, mice that received DPBS displayed high levels of BLG-specific IgE and IgG1 antibodies in sera collected on day 36 and high IL-5 and IL-13 production by BLG-reactivated splenocytes, respectively. Conversely, mice receiving nBLG by gavage

demonstrated significantly lower levels of these antibodies and cytokines, whatever the dose considered. Notably, specific IgE and IgG1 responses and cytokine secretion were totally inhibited in mice receiving the highest dose (i.e. 2 mg) of nBLG. Concomitant Th1 (IgG2a, IFN $\gamma$ ) or Th17 (IL-17) BLG-specific immune responses were also efficiently inhibited, and not IL-10 nor TGF $\beta$  secretion was detected (data not shown). The same efficient tolerance on a further allergic sensitization was obtained using doses ranging between 2 and 4 mg/gavage, and the specificity of the induced tolerance was confirmed by sensitizing BLG-gavaged mice with an irrelevant allergen, i.e. the major peanut allergen Ara h 1 (data not shown).

In an additional experiment, mice were gavaged with PBS or nBLG (2 mg) and sensitized following the same protocol. Inhibition of BLG-specific IgE and IgG1 was checked on sera collected on day 35 (data not shown). A boost injection of nBLG in alum was performed, and all mice were then orally challenged with 10 mg of BLG. Th1/Th2/Th17 cytokines and mMCP-1 were then assayed in sera collected 35 min after the challenge as markers of the elicitation of an intestinal allergic reaction. Significant increase in IL-4, IL-5, IL-10, IL-12 and IL-17 and mMCP-1 was evidenced in sera

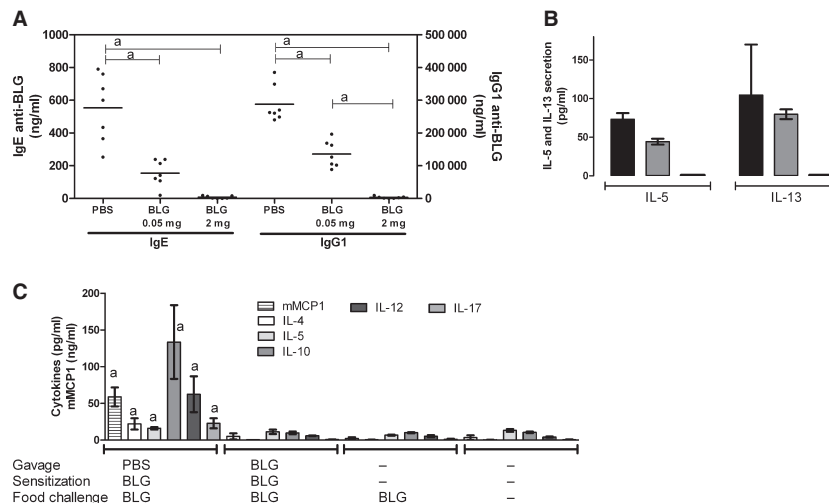


**Figure 1** Bovine  $\beta$ -lactoglobulin (BLG)-specific IgE and IgG1 antibody concentrations (A) and cytokine secretion by reactivated splenocytes (B) in mice gavaged with 0.05 mg or 2 mg nBLG prior to i.p. sensitization. (A) Seven mice received PBS, 0.05 or 2 mg of nBLG on days 1, 2, 3, 8, 9 and 10 by i.g. gavage and were then sensitized on day 14 by i.p. administration of 5  $\mu$ g of nBLG adsorbed on alum. Serum samples were collected on day 36, and BLG-specific IgE and IgG1 antibodies were quantified on individual serum samples, each assayed in duplicates. 'a' indicates  $P < 0.05$  using ANOVA and Tukey's multiple comparison test. (B) On day 38, spleens were removed, pooled per group, and splenocytes were reactivated *in vitro* for 60 h with 20  $\mu$ g of BLG, ConA (1  $\mu$ g/ml, not shown) or ovalbumin. IL-5 and IL-13 were assayed using mouse cytokine kit and BioPlex apparatus. BLG-specific secretion was obtained after nonspecific secretion

(ovalbumin-induced) subtraction. No statistical analysis was performed as results are expressed as means of duplicate determination on pools. Mice gavaged with PBS: black bars; BLG 0.05 mg: Grey bars; BLG 2 mg: empty bars. C. Cytokines and mMCP-1 in sera collected 35 min after an oral challenge: 5–6 mice were gavaged with PBS or 2 mg of nBLG as previously described. On days 14 and 36, all mice were sensitized by i.p. administration of 5  $\mu$ g of nBLG adsorbed on alum. Sensitized mice and five naïve mice were then orally challenged with 10 mg of nBLG, and sera were collected 35 min later to assess Th1/Th2/Th17 cytokines and mMCP-1 concentrations. Naïve mice not challenged were also considered. 'a':  $P < 0.05$  when compared to control group (naïve-challenged mice) using Kruskal–Wallis and Dunn's multiple comparison test.

from PBS-gavaged mice when compared to naïve or naïve- and BLG-challenged mice (Fig. 1C). Conversely, no cytokine or mMCP-1 was detectable in the sera from mice previously gavaged with nBLG.

#### Trypsin hydrolysates of BLG are less efficient for induction of oral tolerance

We then assessed the effect of the administration of whole tryptic hydrolysates produced from nBLG (nBLG-Try, 2 mg/gavage) on a further allergic sensitization with nBLG. As contamination as low as 2.5% of residual intact BLG in hydrolysates preparation can lead to partial tolerance induction whatever the efficiency of hydrolysates

(previous experiment), hydrolysates were highly purified and characterized before use. nBLG gavage was used as a control.

#### Effect of oral administration of tryptic hydrolysates on a further allergic sensitization

As previously observed, gavage with nBLG efficiently inhibited further BLG-specific IgE and IgG1 production (Fig. 2A, 'a') whereas nBLG-Try appeared to be less efficient. When the statistical comparison was performed using the Mann-Whitney test, PBS and nBLG-Try groups were significantly different for BLG-specific IgE production only, whereas BLG-specific IgG1 antibody levels were comparable (Fig. 2A, 'b'). Using the same test, BLG-specific IgE and

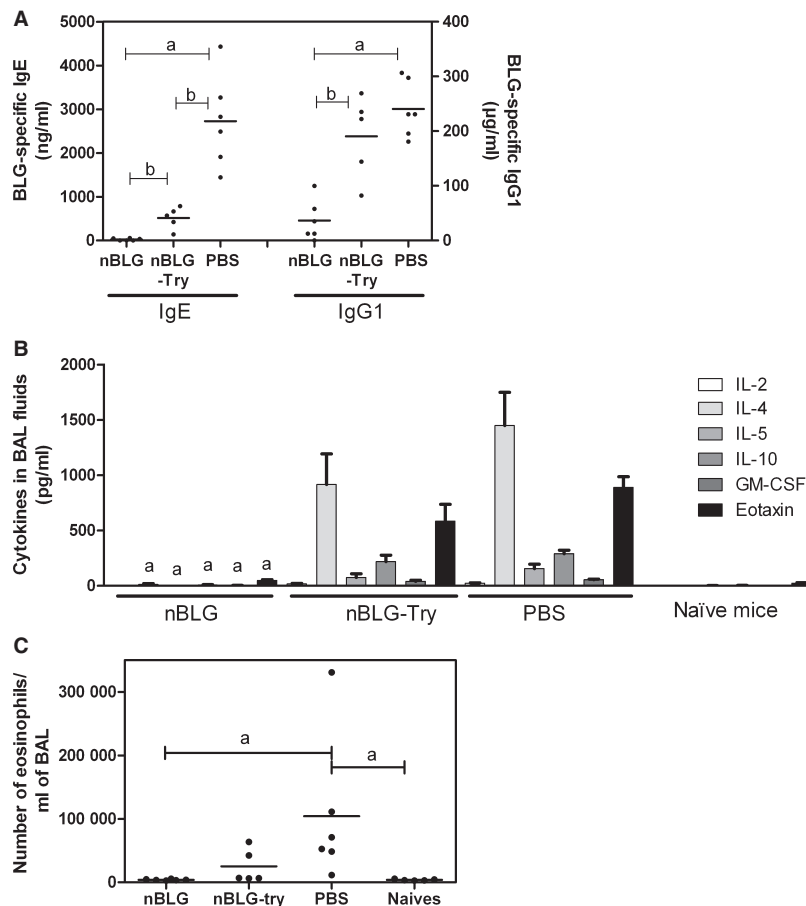


**Figure 2** Effect of i.g. administration of native bovine  $\beta$ -lactoglobulin (nBLG) or nBLG tryptic hydrolysates on a further sensitization and elicitation of the allergic reaction: Mice received PBS ( $n = 6$ ) or 2 mg of nBLG ( $n = 6$ ) or nBLG trypsin hydrolysates (nBLG-Try,  $n = 5$ ) on days 1, 2, 3, 8, 9 and 10 by i.g. gavage and were then sensitized on day 14 by i.p. administration of 5  $\mu$ g of nBLG adsorbed on alum. Five mice were not treated (naïve mice). A. Serum samples were collected on day 33, and BLG-specific IgE and IgG1 antibodies were quantified on individual serum samples, each assayed in duplicates. No specific antibody was detected in naïve mice (not shown). B. On day 36, a boost i.p. injection of nBLG in alum was performed, and mice were intra-nasally chal-

lenged with BLG on day 43. Naïve mice were also challenged with the allergen ('Naïve mice'). Bronchoalveolar lavage fluids (BAL) were collected 24 h later and cytokines assayed using BioPlex technology and reagents. Each individual BAL was assayed in duplicates. C. Eosinophil influx in BAL was assessed using flow cytometry (27) on BAL previously counted using ViaCount reagent. Total cell counts were the following (mean  $\pm$  SEM): 97276  $\pm$  18272 for naïve mice, 233825  $\pm$  112731 for PBS mice, 113111  $\pm$  5894 for nBLG mice and 175326  $\pm$  50345 for nBLG-Try mice. 'a' indicates  $P < 0.05$  using Kruskal-Wallis and Dunns multiple comparison post-test when compared to PBS group; 'b' indicates  $P < 0.05$  using Mann-Whitney comparison between specified group.

IgG1 antibody levels were found different between nBLG and nBLG-Try groups. The same results were obtained when mice were sensitized using BLG emulsified in incomplete Freund's adjuvant, which induced an immune response directed against a denatured form of the protein (26), instead of BLG adsorbed in alum (data not shown).

#### *Effect of oral administration of BLG or corresponding tryptic hydrolysates on a further allergic elicitation*

To assess the efficiency of the tolerance induced by gavage of BLG products on the elicitation of the allergic reaction at nonintestinal site, mice were further boosted and then challenged with BLG via the i.n. route. Although airway hyper-responsiveness was not assessed, relevant cellular and biochemical markers of the allergic reaction were measured in BAL collected 24 h later (26). The allergic reaction is elicited in controls, i.e. PBS-gavaged mice, as demonstrated by the releases of IL-4, IL-5, GM-CSF and eotaxin (Fig. 2B) and eosinophil influx (Fig. 2C) at the challenging site, i.e. in BAL. Conversely, gavage with nBLG prior to sensitization totally inhibited Th2 cytokine secretion and eosinophil recruitment after challenge. It is worth noting that this was neither associated with an increase in IL-10 secretion in BAL (Fig. 2B) nor with induction of TGF $\beta$  (not shown). After gavage with nBLG-Try, elicitation markers were comparable with those measured in the control PBS mice.

#### *Treg are presented at the elicitation site*

In parallel, the BAL collected after challenge were analysed for their content in regulatory cells. We then evidenced that percentage of Foxp3<sup>+</sup> cells within CD4<sup>+</sup> population is significantly higher in mice gavaged with nBLG than in PBS mice (Fig. 3A, a:  $P < 0.05$  Kruskal–Wallis and DMCT). Once again, nBLG-Try was less efficient than nBLG in this recruitment (b:  $P < 0.05$  between PBS and nBLG-Try groups using Mann–Whitney test). Interestingly, when considering all sensitized mice whatever the gavage performed ( $n = 17$ ), we found a strong negative correlation between the number of eosinophils and the percentage of Foxp3-positive cells in BAL (Fig. 3B, one-phase exponential decay,  $r^2 = 0.86$ ).

#### **Induction of Treg cells after gavage with nBLG and nBLG tryptic hydrolysates**

We then analysed the Treg cells in the GALT and in spleen few days after the intra-gastric gavage with nBLG, nBLG-Try or PBS as a control. As shown in Fig. 4, percentage of CD25<sup>+</sup>Foxp3<sup>+</sup> cells within CD4<sup>+</sup> population were increased in MLN, PP and spleen from mice gavaged 3 days before with nBLG, whereas a significant increase was noticed only in MLN from nBLG-Try group.

#### **Effect of *in vivo* depletion of CD25<sup>+</sup> cells on oral tolerance induced by nBLG**

To confirm whether induced Treg cells were implicated in the tolerance induced in our experimental model, mice were pre-

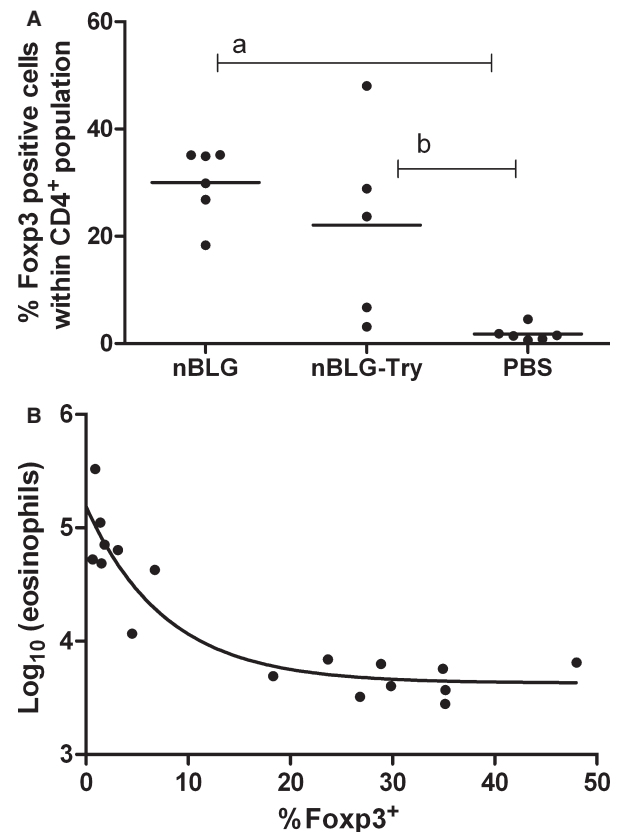


**Figure 3** Regulatory T cells are recruited at the elicitation site. (A) Foxp3-positive cells within CD4<sup>+</sup> population were assessed by flow cytometry on a Guava EasyCyte plus apparatus (see material and methods section) on bronchoalveolar lavage fluids (BAL) collected 24 h after an allergen challenge. (B) The logarithmic transformed number of eosinophils as a function of Foxp3-positive cells within CD4<sup>+</sup> population in the BAL was plotted for all individual mice included in the experiment, and one-phase exponential decay fit was performed using GraphPad prism software. 'a' indicates  $P < 0.05$  using Kruskal–Wallis and Dunns multiple comparison post-test when compared to PBS group; 'b' indicates  $P < 0.05$  using Mann–Whitney comparison between specified group.

treated with PC61 anti-CD25 antibody before tolerance induction by nBLG. Control groups received either an isotype control or PBS, and all mice were then experimentally sensitized. Figure 5A shows that within PBS-gavaged mice, BLG-specific IgE antibody production was significantly higher in the group pretreated with anti-CD25 antibody than in the PBS-pretreated group (a:  $P < 0.05$  nonparametric Kruskal–Wallis and DMCT). IL-5 and IL-13 secretion after BLG-specific reactivation were also enhanced in the PC61 group (Fig. 5D).

The effect of CD25<sup>+</sup> cells depletion on the efficiency of the tolerance induced by nBLG gavage was then analysed by comparing the BLG-specific IgE, IgG1 and IgG2a antibody productions within each pretreatment group. Bovine  $\beta$ -lactoglobulin-specific IgE were significantly reduced in nBLG-gavaged mice, whatever the pretreatment performed (Fig. 5A,

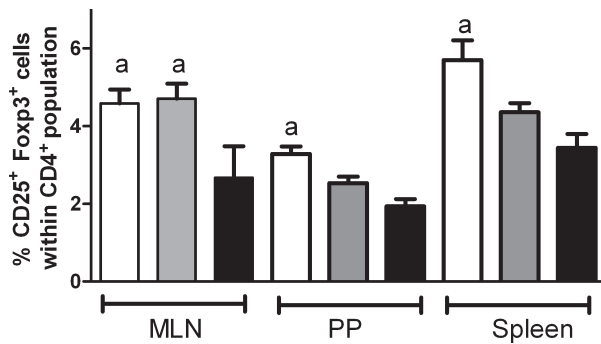


**Figure 4** Regulatory T cells are induced in MLN, Peyer's patches (PP) and spleen 3 days after i.g. administration of native bovine  $\beta$ -lactoglobulin (nBLG) products: CD25<sup>+</sup>Foxp3<sup>+</sup> cells within CD4<sup>+</sup> population was assessed by flow cytometry on MLN, PP and spleen cell suspensions. Tissues were obtained 72 h after one i.g. gavage with 2 mg of nBLG ( $n = 4$ , open bars) or corresponding trypsin hydrolysates ( $n = 3$ , grey bars), or with DPBS as a control ( $n = 3$ , black bars). The results are representative of 2 independent experiments. 'a' indicates  $P < 0.05$  when compared to PBS group using Kruskal–Wallis and Dunns multiple comparison post test.

*b*:  $P < 0.05$  nonparametric Kruskal–Wallis and DMCT). However, BLG-specific IgG1 (Fig. 5B) and IgG2a (Fig. 5C) were not significantly decreased after nBLG gavage within the PC61 pretreatment group, whereas these decreases were significant in PBS and isotype control pretreatment groups (*b*:  $P < 0.05$  Kruskal–Wallis and DMCT). Moreover, the BLG-specific IgE, IgG1 and IgG2a antibodies were significantly higher in the PC61-pretreated/nBLG-gavaged mice

than in the PBS-pretreated/nBLG-gavaged mice (Fig. 5A–C, *c*:  $P < 0.05$  nonparametric Kruskal–Wallis and DMCT). In parallel, IL-5 and IL-13 secretions were decreased by 79.3% and 75%, respectively, in the PC61 group, whereas 92–100% of inhibition was observed in the other groups (Fig. 5D).

## Discussion

Induction of tolerance by food proteins has been extensively studied in various models. Notably, Ova-induced oral tolerance has been mainly investigated in DO11.10 TCR mice, carrying TCR specific for peptide Ova f(323–339) (14–16). Mucosal tolerance to Ova was also efficiently achieved in BALB/c mice after several gavages with 1–20 mg of protein (18, 30). After gavages with high dosages of Ova, both sensitization and elicitation of the allergic reaction were repressed (30). BLG has also been used as a tolerogenic protein. Frosard et al. (31) demonstrated that administration of a total of 22.4 mg of BLG in the drinking water for 4 weeks efficiently prevented further i.p. sensitization in C3H/HeOuJ mice. Pecquet et al. (32, 33) demonstrated that oral tolerance to BLG was efficient in BALB/c mice after a single administration of more than 2.5 mg of protein per g of animal, mainly in weaning mice. In the present study, tolerance was efficiently and totally achieved by administering 2 mg of nBLG for 6 days, and partial tolerance was observed at the 0.05 mg dose. In the schedule of administration we used, the efficient doses are then lower than those required in previous studies. Moreover, the induced tolerance was systemic, i.e. efficient after an i.p. sensitization, and allowed the efficient inhibition of the elicitation of the allergic reaction after challenge at the intestinal

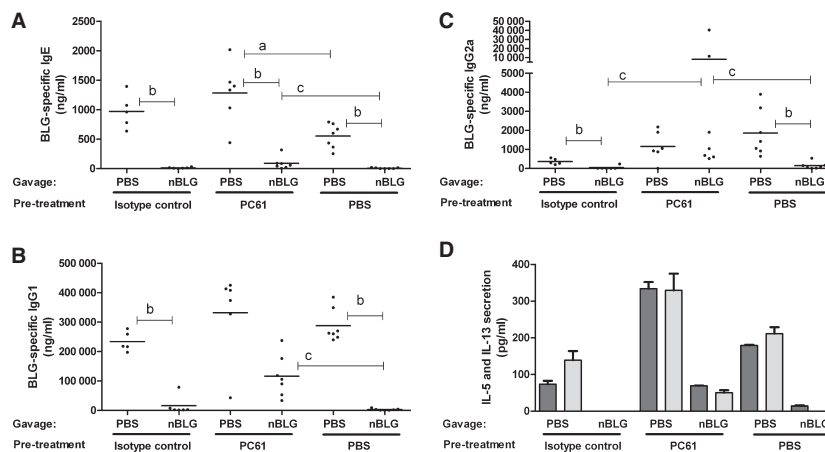


**Figure 5** Regulatory T cells are involved in the oral tolerance induced by native bovine  $\beta$ -lactoglobulin (nBLG): Mice received PC61 anti-CD25 antibody ( $n = 14$ ), rat IgG1 isotype control ( $n = 11$ ) or PBS ( $n = 14$ ) on days 1 and 2. Five to seven mice per group were then submitted to i.g. gavage with 2 mg of nBLG or PBS on days 3–5 and 10–12 and were then sensitized on day 16 by i.p. administration of 5  $\mu$ g of nBLG adsorbed on alum. (A) Serum samples were collected on day 36, and BLG-specific IgE and IgG1 antibodies were quantified on individual serum samples, each assayed in duplicates. No specific antibody was detected in naïve mice (not

shown). 'a', 'b' and 'c': see results section. (B) On day 38, spleens were removed, pooled per group, and splenocytes were reactivated *in vitro* for 60 h with 20  $\mu$ g of BLG, ConA (1  $\mu$ g/ml, not shown) or ovalbumin. IL-5 (black bars) and IL-13 (open bars) were assayed using mouse cytokine kit and BioPlex apparatus, all from Biorad, following provider's recommendation. BLG-specific secretion was obtained after subtraction of nonspecific secretion. No statistical analysis was performed as results are expressed as means of duplicate determination on pools.

or the respiratory level. Although administration of whole tryptic hydrolysates of nBLG allowed the decrease in BLG-specific IgE antibody levels, as previously observed (32), BLG-specific IgG1 and the markers of the allergic reaction were not significantly reduced in these mice when compared to control group.

The cellular mechanisms involved in BLG-induced oral tolerance were then further studied. Interestingly, we observed for the first time that CD4<sup>+</sup>Foxp3<sup>+</sup> cells are present at the site of the elicitation of the allergic reaction in both nBLG- and nBLG-Try-gavaged mice and that the percentage of these cells were inversely correlated with eosinophil number in BAL. Thus, this suggests that inhibition of the elicitation of the allergic reaction involved the inhibition of the allergic sensitization, i.e. IgE antibody production, but also an active phenomenon implicating iTreg cells at the challenging site. This is in opposition with the depletion study by van Wijk et al. (19), suggesting that CD4<sup>+</sup>CD25<sup>+</sup> T cells are not directly involved in controlling degranulation of mast cells after oral challenge of BALB/c mice i.g. sensitized to peanut. However, our results are consistent with observations demonstrating that asthmatic children show quantitative and functional impairment of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in BAL when compared to nonasthmatic children (34). Moreover, transfer of Ova-specific CD4<sup>+</sup>CD25<sup>+</sup> cells from DO11.10 mice to Ova-sensitized BALB/c mice resulted in the presence of those cells in airway lumen and lung of recipient mice after Ova challenge. This transfer allowed to significantly decrease airway hyperreactivity, eosinophil and Th2 cells recruitment, and Th2 cytokine secretion in challenged recipient mice (17). The mechanism involved in this latter study was evidenced to be IL-10 dependant. Although we did not evidence an increase in IL-10 production in BAL, additional blocking experiments are required to conclude on the involvement or not of IL-10 in our experimental model. However, in our model of elicitation of the allergic reaction, only one i.n. challenge is performed, in contrast to the daily challenge for 6 days with aerosolized Ova in precited study. In our model, mast cells are actively involved as demonstrated by immediate release of histamine and leucotrienes (26), leading to eosinophils and Th2 cell recruitment. The underlying mechanism of suppression observed could then result from a direct inhibition of mast cell degranulation by induced specific Treg, possibly through OX40-OX40L interaction (35). Interestingly, BLG-Try gavage also induced Treg cells that should then be less efficient in their suppressive function after challenge with the entire protein.

Induction of CD4<sup>+</sup>CD25<sup>+</sup> cells after antigen feeding has been evidenced mainly in Ova TCR transgenic mice or in BALB/c mice transferred with naive Ova-specific T cells (KJ1-26<sup>+</sup>) (14–16). Interestingly, Ova TCR transgenic mice-fed BSA shows a slight but nonsignificant increase in KJ1-26<sup>+</sup>CD25<sup>+</sup> cells within CD4<sup>+</sup> population of MLN, spleen and PP, but CD4<sup>+</sup>CD25<sup>+</sup> cells could not be evidenced in BALB/c mice-fed Ova (16). This suggests the difficulty to evidence antigen-specific CD4<sup>+</sup>CD25<sup>+</sup> when using non-Ova-specific transgenic cells. However, in the present study, using

wild-type BALB/cJ mice, we did observe significant increase in CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells in GALT and spleen 3 days after nBLG gavage. This induction was limited to the MLN after administration of nBLG trypsin hydrolysates. CD4<sup>+</sup>CD25<sup>+</sup> cells were no more significantly evidenced 10 days after BLG gavage in the different tested organs (not shown).

Altogether, these results thus suggested that gavage with nBLG efficiently induced Treg at the local (MLN and PP) and systemic (spleen) levels, then allowing efficient inhibition of further sensitization and elicitation of the allergic reaction. Conversely, gavage with trypsin hydrolysates of nBLG allowed only local induction of Treg (MLN) and partial inhibition of sensitization. Although increased at the challenging site, nBLG-Try-induced Treg did not prevent the elicitation of the allergic reaction. Thus, this suggests that trypsin hydrolysis of nBLG reduces its tolerating potential. These results are in line with those obtained in rat by Fritsché et al. (36), demonstrating that a standard cow's milk formula or a partially hydrolysed whey formula can induce oral tolerance whereas extensively hydrolysed formula cannot. Moreover, hydrolysis of whey cow's milk formula reduced its immunogenicity (37). Altogether, this suggests that extensive hydrolysis would destroy most of the BLG T-cell epitopes and then greatly limit the initiation of an immune response, either effector or regulatory. It is then worth noting that extensively hydrolysed cow's milk, better tolerated by cow's milk allergic patients, would then not favour the induction of an oral tolerance in these patients.

To further confirm the role of iTreg in nBLG-induced oral tolerance, we performed *in vivo* CD25 cell depletion before induction of tolerance. In accordance with results obtained in C3H/HeOuJ mice using peanut as allergen (19) and in BALB/c mice using Ova (18), we also demonstrated that oral tolerance to BLG is impaired in CD25<sup>+</sup> T-cell-depleted animals, confirming the implication of induced Treg cells in this model of BLG-induced tolerance. However, CD4<sup>+</sup>CD25<sup>+</sup> T-cell depletion did not totally impaired the induced tolerance in our study, as in (18), suggesting either that Treg cells are not the only effector in the BLG-induced tolerance or that depletion is not complete so residual induction of Treg cells can occurred during oral tolerance procedure. The first hypothesis is reinforced by the synergic effect of CD4<sup>+</sup>CD25<sup>+</sup> depletion and TGFβ neutralization to totally impair oral tolerance induction (18), which was not assessed in our study. On the other hand, the second hypothesis is also comforted by the fact that only partial deletion occurred after i.p. injection of 200–400 µg of anti-CD25 PC61 antibody (18, 28, 29). Moreover, accelerated splenic Treg repopulation from peripheral CD4<sup>+</sup> have been demonstrated, for example, after acute malaria infection (38), suggesting that *de novo* generation of Treg cells after gavage with BLG may occur despite PC61 treatment. Conversely, sensitization level was higher in CD4<sup>+</sup>CD25<sup>+</sup> T-cell-depleted mice when compared to PBS-pre-treated mice. These results further demonstrated that Treg cells induced at the same time as effector cells following immunization allow to regulate the levels of sensitization and then to maintain immune homeostasis by

avoiding excessive immune response against nonself-antigen (19, 28, 39).

In conclusion, our results demonstrated for the first time the induction of iTreg in GALT and spleen after gavages of BALB/cJ mice with a food allergen, i.e. BLG. Those cells contribute to the inhibition of further systemic sensitization to BLG. Moreover, both inhibition of the allergic sensitization and active suppression of effector cells by BLG-induced Treg cells at the challenging site allow the inhibition of the elicitation of the allergic reaction. Conversely, trypsin hydrolysis of BLG significantly reduced its tolerating potential.

## Conflict of interest

K. Adel-Patient conceptualized and realized or participated to all the *in vivo* and *in vitro* experiments, analysed the cell populations and writes the paper; S. Wavrin participated in animal studies and cell analysis; H. Bernard produced and characterized the different proteins and hydrolysates; N. Meziti and S. Ah-Leung produced and characterized the different proteins and participated in animal studies; J.-M. Wal conceptualized the experiments and help in writing the paper. All authors concur with the submission and do not have conflict of interest.

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