

# Regulatory T Cells Control Type I Food Allergy to Beta-Lactoglobulin in Mice

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## Key Words

Food allergy · Cow's milk · CD4CD25 regulatory T cells · Mast cells

## Abstract

**Background:** Regulatory T cells contribute to peripheral immune tolerance, yet their ability to control immediate-type hypersensitivity (ITH) reactions involved in IgE-mediated food allergy is still poorly documented. **Objectives:** We investigated in mice whether CD4+CD25+ regulatory T cells could control ITH to  $\beta$ -lactoglobulin (BLG), a major allergen in cow's milk. **Methods:** C3H/HeOJ mice were sensitized by repeated oral gavage with BLG plus cholera toxin as adjuvant and orally challenged with BLG alone to elicit allergic symptoms. Mice were treated with the anti-CD25 mAb (PC61) before sensitization. Oral sensitization (afferent phase of ITH) was assessed by production of BLG-specific serum antibodies and Th1/Th2-type cytokines by specific CD4+ T cells in mesenteric lymph nodes. ITH was elicited by oral BLG challenge (efferent phase of ITH) and we monitored symptom scores, numbers and function of intestinal mast cells and serum level of the mucosal mast cell protease mMCP-1. **Results:** Upon oral BLG challenge, orally sensitized mice developed only mild clinical signs. Anti-CD25 mAb-treated mice exhibited enhancement of both BLG-specific CD4+ T cell

priming with IL-4, IL-5, IL-13 and IFN- $\gamma$  production and total IgE, and BLG-specific IgE, IgG1 and IgG2a in serum. Anti-CD25 mAb treatment caused more severe symptoms upon BLG challenge, which correlated with enhanced serum levels of the mucosal mast cell protease mMCP-1. **Conclusions:** These data document that constitutive CD4+CD25+ regulatory T cells alleviate clinical signs of ITH to dietary BLG by modulating the priming of BLG-specific T and B cell responses during oral sensitization and enhancing mast cell degranulation.

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## Introduction

Cow's milk allergy (CMA) is the most frequent food allergy in children, affecting 2–3% of infants during the first year of life. The pathophysiology of CMA involves a wide range of IgE-mediated immediate-type hypersensitivity and T cell-mediated delayed-type hypersensitivity reactions [1]. CMA patients with IgE-mediated hypersensitivity have lower chances of outgrowing their allergy and occasionally develop allergies to other food antigens [2]. Unlike other food allergies, CMA in infancy spontaneously resolves around 3 years of age and is thus believed to result from impaired oral tolerance to major protein

antigens contained in cow's milk including casein,  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin.

Oral tolerance is a physiological mechanism, which prevents hypersensitivity reactions to dietary antigens and we have previously reported that it is mediated by CD4+CD25+ regulatory T cells (Treg) [3–5]. Treg comprise several subsets including, naturally occurring and thymus-derived CD4+CD25+ Treg expressing the transcription factor Foxp3 as well as antigen-induced CD4+CD25+Foxp3+ Treg, IL-10-producing (Tr1) and TGF- $\beta$ -producing (Th3) Treg cells generated in the periphery, which suppress CD4+ and CD8+ T cell responses and can control inflammatory and autoimmune diseases [6]. Interestingly, patients with X-linked immune dysregulation, polyendocrinopathy and enteropathy syndrome caused by a mutation in Foxp3 have a selective defect in constitutive CD4+CD25+ Treg and often develop atopic dermatitis, elevated serum IgE levels and food allergy [7–9]. Indirect evidence in patients with CMA favors a role of CD4+CD25+ Treg in the control of allergy. Beyer et al. [10] documented that in children with non-IgE-mediated CMA, active gastrointestinal disease is associated with high Th2- and low Treg-associated cytokines. Karlsson et al. [11] showed that children with delayed-type CMA who eventually outgrew their allergy had higher levels of circulating CD4+CD25+ Treg and decreased in vitro proliferative responses of peripheral blood mononuclear cells to bovine  $\beta$ -lactoglobulin (BLG), as compared to those who remained allergic. Moreover, depletion of CD25+ cells from peripheral blood mononuclear cells of tolerant children enhanced their in vitro proliferation in response to BLG, suggesting that CD4+CD25+ Treg play an important role in suppressing effector T cells generated after reintroduction of cow's milk [11]. More recently, Shreffler et al. [12] reported that Treg are important in the onset of clinical tolerance in CMA, by showing a higher frequency of proliferating casein-specific Treg in subjects who were able to consume heated milk without allergic reaction, than in CMA patients who reacted to heated milk. However, whether Treg can control oral sensitization to cow's milk antigens and development of immediate-type CMA remains to be addressed. Immediate-type CMA can lead to severe and potentially life-threatening anaphylactic reactions. As for other types of IgE-mediated food hypersensitivity, it is believed that immediate CMA proceeds in two phases. The afferent phase corresponds to sensitization and is induced upon first encounter with cow's milk protein allergens, leads to the priming of allergen-specific CD4+ Th2 cells and production of Th2 cytokines (IL-

4, IL-5, IL-13), which promote the differentiation of allergen-specific B cells and production of specific IgE. IgE binds to the high-affinity receptor for IgE (Fc $\epsilon$ RI) on mast cells and basophils. The efferent phase responsible for the allergic reaction is induced upon subsequent oral exposure to the same allergen, which by cross-linking mast cell-bound specific IgE leads to the rapid release of preformed and granule-stored allergic mediators, such as histamine, serotonin and mast cell proteases, which are key to the anaphylactic reactions [13, 14]. Several mouse models of immediate-type CMA have been developed in various strains of mice, including Balb/c and C3H using either intraperitoneal immunization with BLG in Alum [15] or oral immunization with BLG and cholera toxin (CT) as adjuvant and oral challenge with BLG alone [16, 17]. Interestingly, Frossard et al. [18], in a model of allergy induced by BLG/CT gavage, showed that tolerant mice, fed with BLG alone, could be distinguished from their allergic counterparts by a high production of IL-10 by Peyer's patch T cells, suggesting a role for Treg in the control of immediate-type CMA.

In the present study, we investigated the contribution of CD4+CD25+ Treg in CMA in C3H/HeOuJ mice orally sensitized and challenged with BLG. We showed that anti-CD25 mAb treatment before sensitization increases BLG-specific serum IgE, IgG1 and IgG2a antibodies, enhances Th1 and Th2 cytokine production by mesenteric lymph nodes CD4+ T cells. Moreover, Treg depletion resulted in more severe symptoms of systemic anaphylaxis together with enhanced levels of the mucosal mast cell protease-1 (mMCP-1) upon oral BLG challenge.

## Materials and Methods

### *Mice*

Six- to eight-week-old female C3H/HeOuJ mice were purchased from Charles River Laboratories. All mice were housed in our specific pathogen-free facility and used at 6–10 weeks of age. All experiments were previously approved by the Animal Care and Use Committee according to institutional guidelines.

### *Experimental Model of Immediate-Type Hypersensitivity to $\beta$ -Lactoglobulin*

Mice were immunized 4 times at weekly intervals (on days 0, 7, 14 and 21), by intragastric gavage with 10 mg of BLG (Sigma-Aldrich) combined with 10  $\mu$ g of CT (Tebu-bio) or with CT alone. One week after the last immunization, all mice were challenged orally on day 28 with 50 mg of BLG alone or with PBS as control. To study the role of Treg, mice were injected intraperitoneally with 100  $\mu$ g of the anti-CD25 mAb (PC61) administered 3 and 1 day before the first immunization.

### *Clinical Symptoms of Food Hypersensitivity*

Clinical symptoms were monitored over 1 h after challenge. A cumulative symptom score was evaluated for scratching, lack of mobility and alteration of respiratory cycle, each based on a scale of 0–2, according to time of onset after challenge, duration and frequency of each symptom. The cumulative score was the addition of the three scores and ranged between 0 (no symptoms) and 6 (severe anaphylactic shock). Relative contribution of each symptom was calculated as the percentage of each symptom score to the maximal score of 2.

### *Measurement of Serum Antibodies*

Total IgE was measured in the serum of the sensitized mice by ELISA. Plates were coated with anti-mouse-IgE antibody (5 µg/ml, clone R35-72; BD-Bioscience) and incubated overnight at +4°C. Sera were added to the wells for 2 h at RT. The plates were washed in PBS Tween 0.05% and total IgE was detected with a biotin-conjugated rat anti-mouse IgE (R35-92; BD-Bioscience) and peroxidase-coupled streptavidin (Roche). Tetramethyl-benzidine (TMB; Sigma-Aldrich) substrate was added and incubated for 10–20 min. The reaction was stopped with H<sub>2</sub>SO<sub>4</sub> (12.5%) and optical density (OD) was read at 490 nm. OD values were converted into nanograms per milliliter using standard curves of purified IgE, κ anti-TNP Abs (BD-Bioscience) with the SoftMax software.

BLG-specific IgG1 and IgG2a were measured by ELISA. Briefly, Nunc MaxiSorp Elisa plates (VWR) were coated with 1 mg/ml BLG or with the irrelevant antigen ovalbumin (OVA; 1 mg/ml). Serial dilutions of sera were incubated for 3 h at RT. IgG1 and IgG2a antibodies were detected by sequential incubations with biotin-coupled rat anti-mouse IgG1 or IgG2a antibody (BD-Bioscience) and peroxidase-coupled streptavidin (Roche). The reaction was developed with TMB (Sigma-Aldrich) as substrate and stopped by addition of 12.5% H<sub>2</sub>SO<sub>4</sub>. OD was read at 450 nm by a SpectraMax ELISA reader equipped with the software SoftMax (Molecular Devices). The results were expressed as arbitrary units per milliliter as compared to pooled sera from BLG/albumin immunized mice used as an internal standard with 1,000 arbitrary units/ml. BLG-specific IgE was measured by sandwich ELISA as previously described [19]. Briefly, streptavidin-coated plates were coated with biotinylated BLG and incubated for 18 h at +4°C with 1:30 diluted sera. After washing, 100 µl of anti-IgE (clone LOME-3; Serotech) labeled with acetylcholinesterase were added for a further 18 h reaction at 4°C. Plates were then extensively washed and solid-phase bound acetylcholinesterase activity was determined by addition of 200 µl/well of Ellman's medium and the OD was read at 414 nm. Quantification of specific IgE was performed using standard curves performed using a BLG-specific IgE mAb (a kind gift from Dr. Granato, Nestlé, Lausanne, Switzerland).

### *In vitro Cytokine Production by Mesenteric Lymph Nodes CD4<sup>+</sup> T Cells*

Mesenteric lymph nodes (MLN) cells were isolated by enzymatic digestion using 1 mg/ml Collagenase (Sigma-Aldrich) and 1 µg/ml DNase (Roche) in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% HEPES, 2 mmol/l L-glutamine, 1% penicillin-streptomycin and 0.5% β2-mercaptoethanol (all from Invitrogen). Red blood cells were lysed with NH<sub>4</sub>Cl (0.83%). CD4<sup>+</sup> T cells were isolated by positive selection using a CD4 isolating kit (Miltenyi Biotec) and separated using LS columns according to

the manufacturer's instructions. The purity of cell suspensions was >97% as determined by FACS analysis. CD4<sup>+</sup> T cells (5 × 10<sup>5</sup> cells/well) were cultured for 3 days at 37°C in 96-well microplates together with irradiated spleen cells (2.5 × 10<sup>5</sup>/well) as APC in the presence of 1 mg/ml of BLG or OVA as control. Day 3 supernatants were harvested for cytokine titration.

### *ELISA Titration of IL-4, IL-5, IL-13 and IFN-γ*

Detection of IL-4, IL-5 and IL-13 in cell culture supernatants was performed using mouse ELISA kits, according to manufacturer's instructions (IL-4, IL-5 from AbCys; IL-13 from R&D Systems). Mouse IFN-γ was detected using the following procedure. ELISA plates were coated by overnight incubation at +4°C with the anti-IFN-γ capture mAb (clone R46 A2, 1 µg/ml). Cell culture supernatants were incubated for 2 h at RT. Purified mouse IFN-γ (R&D Systems) was used as standard. Bound IFN-γ was then detected by sequential incubations with a biotin-coupled rat anti-mouse IFN-γ mAb (BD-Bioscience), streptavidin-coupled alkaline phosphatase (Roche) and the substrate TMB (Sigma-Aldrich). The reaction was stopped by addition of 12.5% H<sub>2</sub>SO<sub>4</sub> and plates were read at 450 nm.

### *Titration of Serum mMCP-1*

Blood was collected 1 h after oral BLG challenge and serum levels of mMCP1 were determined using an Elisa Kit (Moredun Scientific Ltd.). ELISA was performed according to the manufacturer's instructions.

### *Histological Staining of Mast Cells*

Paraffin-embedded sections (5 µm) of small intestines were stained with 1% toluidine blue as described [20]. Mast cells were counted in 50 microscopic fields from 3 sections per mouse. The results are expressed as the mean number of mast cells/field.

### *Statistical Analysis*

Statistical analyses were done using the Mann-Whitney test. A value of  $p < 0.05$  was considered statistically significant.

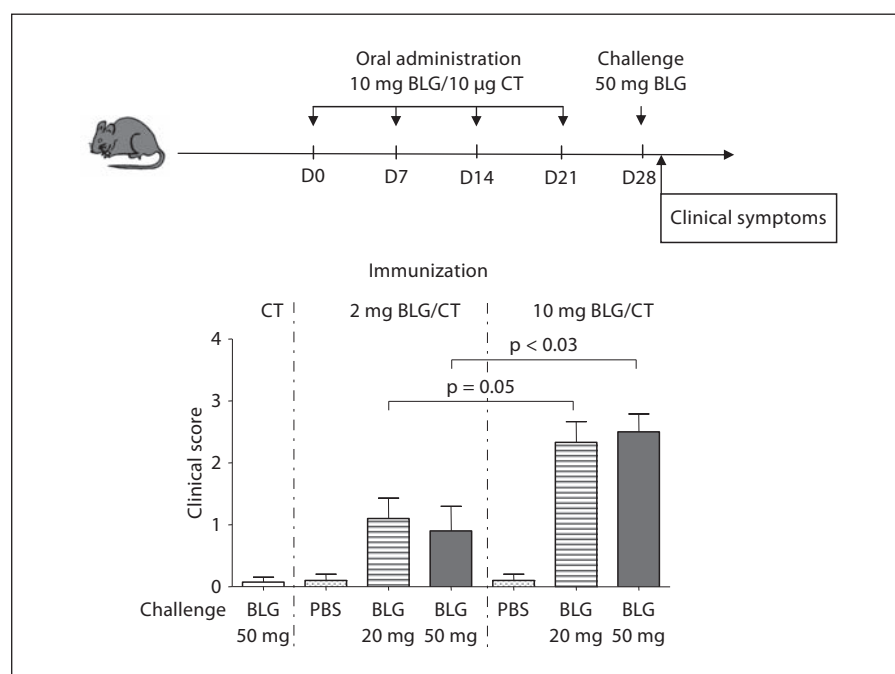
## **Results**

### *The Severity of Type I Allergy to BLG Depends on the Dose of BLG Used for Sensitization*

We adapted a previously described protocol of food allergy to BLG in C3H/HeOuJ mice (a strain susceptible to food allergy), after repeated oral administration of BLG with the mucosal adjuvant CT [18]. C3H/HeOuJ mice were sensitized by oral immunization 4 times at weekly intervals (days 0, 7, 14 and 21) with either 2 or 10 mg of BLG combined with 10 µg CT and challenged orally on day 28 with either 20 or 50 mg of BLG alone. Mice were observed for 1 h after challenge and the clinical symptoms were scored as described in Materials and Methods.

As expected, no clinical symptoms were observed in mice immunized with CT alone and challenged with BLG nor in BLG-immunized mice challenged with PBS

**Fig. 1.** ITH symptoms of type-I food allergy to BLG depend on the sensitizing dose of BLG. Mice were orally immunized on days 0, 7, 14 and 21 with either 2 (hatched bars) or 10 mg (grey bars) of BLG mixed with 10  $\mu$ g of CT or CT alone and challenged on day 28 with 20 or 50 mg of BLG alone. Clinical symptoms were scored for 1 h after challenge and expressed as a mean cumulative score ( $\pm$  SD). Data are representative of 1 of 2 experiments using 3–6 mice/group.



alone (fig. 1). Oral sensitization with 2 mg of BLG with CT induced only mild symptoms, irrespective of the dose of BLG used for challenge. Alternatively, mice immunized with 10 mg BLG exhibited clinical symptoms more pronounced, as compared to those immunized with 2 mg BLG, after challenge with either 20 mg of BLG (mean score:  $2.33 \pm 0.33$  vs.  $1.1 \pm 0.33$ ;  $p = 0.05$ ) or after challenge with 50 mg (mean score:  $2.5 \pm 0.28$  vs.  $0.9 \pm 0.4$ ;  $p = 0.03$ ). Yet, because challenge with 50 mg BLG gave more reproducible results (data not shown), mice were immunized with 10 mg BLG + 10  $\mu$ g CT and challenged with 50 mg BLG in all subsequent experiments. These data indicate that re-exposure to BLG in previously sensitized mice is necessary to induce immediate-type allergy to BLG and that the severity of allergic symptoms depends on the dose of BLG used for immunization.

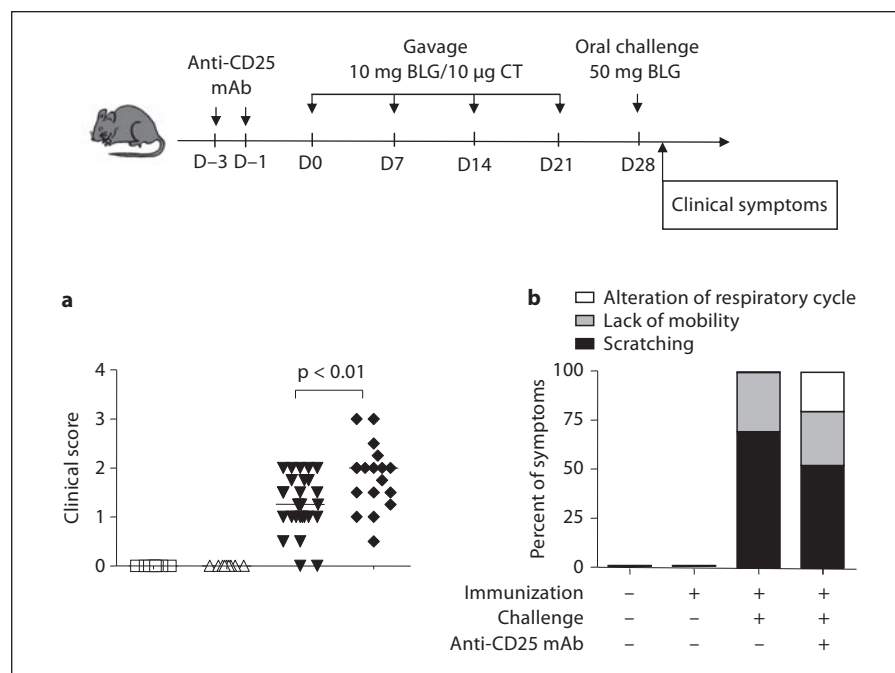
#### *Anti-CD25 mAb Treatment Increases the Severity of Food Allergy*

To determine whether regulatory T cells could control the magnitude of systemic symptoms of food allergy to BLG, C3H/HeO/J mice were treated with anti-CD25 antibodies before BLG immunization using a protocol previously shown to block the suppressive function of CD4+CD25+ Treg [21–23]. Analysis of MLN (data not shown) and nondraining lymph nodes (online suppl.

fig. 1, [www.karger.com/doi/10.1159/000323940](http://www.karger.com/doi/10.1159/000323940)) in several mice revealed that the anti-CD25 mAb treatment resulted in undetectable CD25-expressing CD4+ T cells (online suppl. fig. 2a) and a roughly 40% reduction of the frequency of CD4+Foxp3+ Treg (5 vs. 3% of total cells in control and anti-CD25-injected mice, respectively) (online suppl. fig. 2b) at day 0. CD4+CD25+Foxp3+ T cells then progressively recovered and reached a normal frequency within 7–10 days after mAb injection (data not shown), for example, in-between the first and the second oral immunization. Interestingly, analysis of gated CD4+ T cells revealed an overall decrease of the frequency of Foxp3+ T cells but a significant increase in the proportion of Foxp3+CD25– cells (online suppl. fig. 2d), indicating that, in addition to depleting some Treg, anti-CD25 mAb injection also downregulates or masks the CD25 molecule on their surface, leading to a block of their suppressive functions [21–23].

Anti-CD25 mAb treatment increases the severity of the allergic reaction induced by BLG challenge in previously sensitized mice (mean score:  $2 \pm 0.23$ ), as compared to that of untreated BLG-sensitized and BLG-challenged mice (mean score:  $1.26 \pm 0.10$ ,  $p < 0.01$ ; fig. 2a). Moreover, CD25 mAb-treated mice exhibited more severe symptoms, such as alteration of the respiratory cycle, that were not observed in untreated mice, which developed only scratching and lack of mobility, nor in naive

**Fig. 2.** Effect of anti-CD25 mAb injection of type I food allergy to BLG. Mice were orally immunized with 10 mg BLG/CT and challenged with either PBS (open triangles) or 50 mg BLG (black triangles), as described in figure 1. One group of BLG-immunized and challenged mice received 100  $\mu$ g of anti-CD25 mAb 3 and 1 day before immunization (lozenges). One group of naive mice was included as control (open squares). Data represent pooled values from 3 experiments using 4–7 mice/group. Cumulative clinical score (a) and frequency of each clinical sign (b) were assessed as described in Material and Methods.



animals (fig. 2b). These results indicate that CD4+CD25+ Treg are able to control the severity of systemic symptoms of food allergy to BLG.

#### Anti-CD25 mAb Treatment Increases BLG-Specific Th2/Th1 Cytokine Response in MLN

To determine whether Treg could affect oral T cell priming to BLG, we tested the outcome of anti-CD25 mAb injection on BLG-specific cytokine production by CD4+ T cells purified from MLN on day 28 (that is, at the time point when mice are challenged). Cytokines were measured in the supernatants of CD4+ T cells isolated from MLN and cultured in the presence of BLG or the irrelevant antigen OVA. CD4+ T cells from BLG-sensitized mice produced the Th2-type cytokines IL-4, IL-5 and IL-13 and low level of IFN- $\gamma$  (Th1 cytokine), after in vitro restimulation with BLG, but not OVA (fig. 3). CD4+ T cells from anti-CD25 mAb-injected and BLG-sensitized mice produced only minute levels of Th1 and Th2 cytokines after in vitro OVA stimulation. In contrast, anti-CD25 mAb treatment increased the production of IL-4, IL-5, IL-13 ( $p < 0.001$ ) as well as the secretion of IFN- $\gamma$  ( $p < 0.001$ ) by CD4+ T cells in response to in vitro restimulation with BLG (fig. 3). These data demonstrate that Treg negatively control T cell priming and suppress the production of both Th2- and Th1-type cytokines by CD4+ T cells in MLN during oral BLG sensitization.

#### Anti-CD25 mAb Treatment Enhances BLG-Specific IgE, IgG1 and IgG2a and Total IgE Serum Antibodies

We then studied the effect of Treg in the induction of humoral response. Total serum IgE and BLG-specific serum IgE, IgG1 and IgG2a were measured in BLG-immunized mice at day 28. As shown in figure 4, BLG-sensitized mice produced total and BLG-specific IgE, BLG-specific IgG2a and to a lesser extent IgG1. Anti-CD25 mAb injection significantly increased the serum level of total and BLG-specific IgE ( $p = 0.00005$  and  $p < 0.01$ , respectively) and IgG1 ( $p < 0.05$ ) as well as of IgG2a, although the latter increase did not reach statistical significance. These data demonstrate that Treg control allergen-specific B cell priming in response to oral BLG sensitization.

#### Effect of Anti-CD25 mAb Treatment on Gut Mast Cell in Allergic Mice

Since type I allergy is mediated by the release of pre-formed mediators contained in mast cell granules, the increased allergic symptoms induced by anti-CD25 mAb treatment could be attributed to an increased number of gut mast cells, an enhanced activation properties of gut mast cells or both. We thus investigated whether Treg can control the number and/or the activation of gut mast cells. To this end, paraffin-embedded sections of small intestine were stained with toluidine blue to detect connective

**Fig. 3.** Effect of anti-CD25 mAb injection on cytokine production by BLG-specific CD4<sup>+</sup> T cells. CD4<sup>+</sup> T cells were purified from MLN of anti-CD25 mAb-treated or untreated BLG-immunized mice on day 28 and restimulated in vitro with 1 mg/ml of BLG (black bars) or the irrelevant Ag OVA (white bars) in the presence of irradiated spleen cells as APC. Cytokines were titrated by ELISA in day-3 culture supernatants. Data represent means  $\pm$  SD obtained in triplicate wells using pooled CD4<sup>+</sup> T cells from 6 mice per group. Data are representative of 1 of 2 experiments.

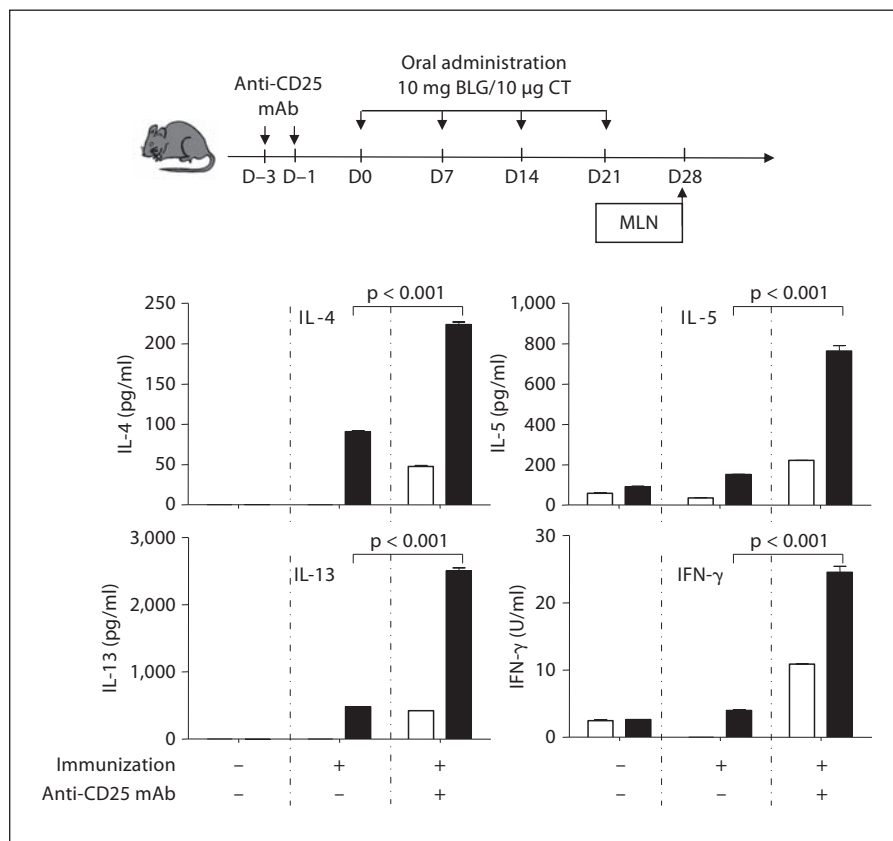
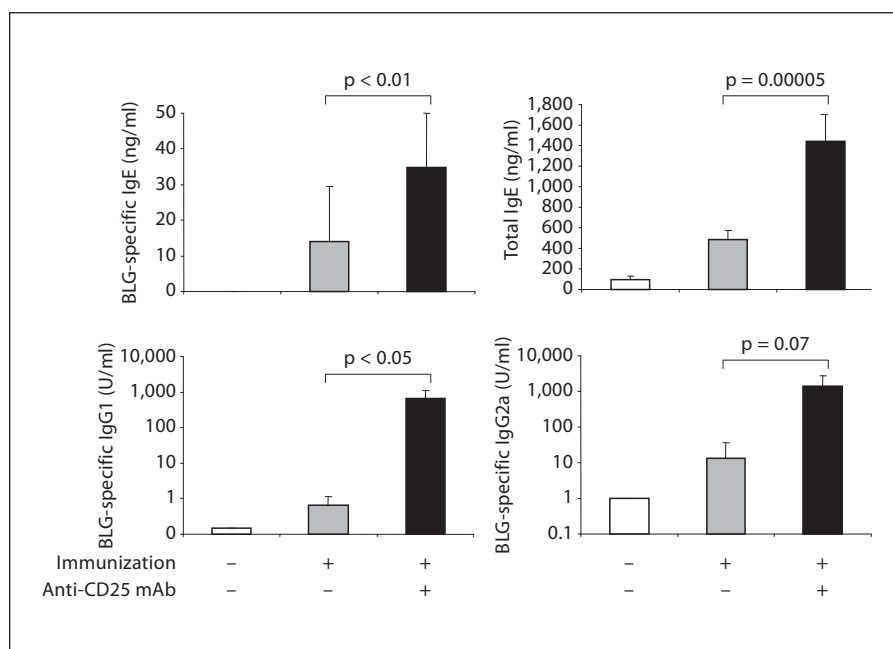


**Fig. 4.** Effect of anti-CD25 mAb injection on BLG-specific serum antibodies. Mice were either left untreated (white bars) or immunized with BLG + CT without (grey bars) or with (black bars) anti-CD25 mAb treatment before immunization. BLG-specific IgE (top panel), IgG1 (middle panel) or IgG2a (bottom panel) were titrated in the serum on day 28 by ELISA. Results are expressed as means  $\pm$  SD of arbitrary ELISA in U/ml (IgG1 and IgG2a) or ng/ml (IgE) for each group of mice (n = 5–7 mice).



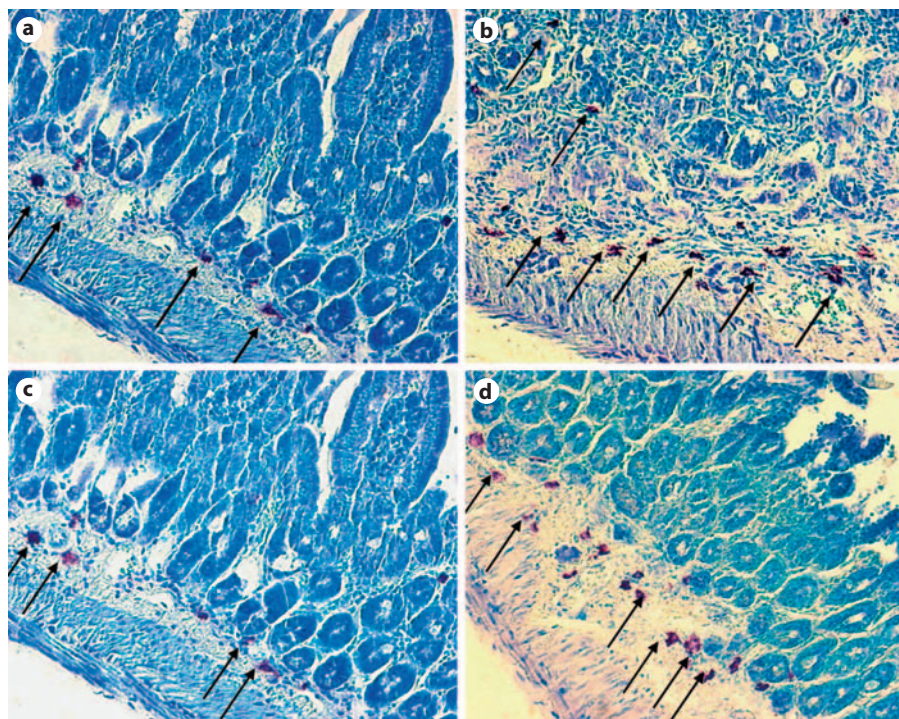
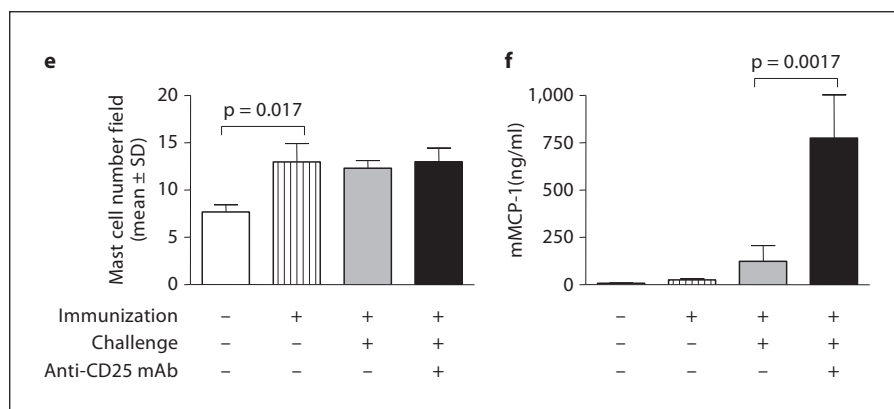


**Fig. 5.** Effect of anti-CD25 mAb injection on intestinal mast cells. The outcome of anti-CD25 mAb treatments on mast cells numbers (**a–e**) and function (**f**) were investigated. Paraffin-embedded cryostat section (5  $\mu$ m) of small intestine of either naive (**a**; white bars), BLG-immunized and PBS-challenged (**b**; hatched bars) or BLG-challenged (**c**; grey bars) or anti-CD25 mAb-treated BLG-immunized and BLG-challenged (**d**; black bars) mice were stained with toluidine blue to detect serosal mast cells (arrows). Mast cells were counted (3 sections/mouse, 30 microscopic fields/section, 4–5 mice/group). Mean ( $\pm$  SD) number of mast cells/field are shown in **e**. mMCP1 was titrated by ELISA in the serum of mice 1 h after challenge. Data represent means  $\pm$  SD of values pooled from 2 experiments ( $n = 4–6$  mice/group) (**f**).



tissue mast cells and serum levels of mMCP-1 were titrated to evaluate degranulation of mucosal mast cells.

Naive mice displayed a few toluidine blue-positive mast cells (mean =  $7.7 \pm 0.7$ ) located in the submucosa of the small intestine (fig. 5a, e). BLG-immunized mice exhibited on day 28 a higher number of mast cells (mean =  $13 \pm 2$ ) (fig. 5b, e), as compared to naive mice ( $p < 0.017$ ). This increase was similarly observed in PBS-challenged (fig. 5b, e) or BLG-challenged (fig. 5c, e) mice, showing that mast cell recruitment is induced by multiple oral BLG sensitizations and not by the challenge. Anti-CD25 mAb injection did not affect mast cell numbers in

BLG sensitized and challenged mice (fig. 5d, e). This indicates that mast cell recruitment in the gut is not affected by depletion of Treg during oral immunizations.

Alternatively, serum mMCP-1 was found in BLG-immunized mice 1 h after BLG challenge, but undetectable in either naive or BLG-immunized and PBS-challenged mice (fig. 5f), confirming that mast cell activation can only occur upon allergen challenge in sensitized mice. CD25 mAb treatment dramatically enhanced serum mMCP-1 levels in BLG-sensitized and -challenged mice (mean =  $773.8 \pm 228.6$ ) as compared to untreated mice (mean =  $123.4 \pm 82.56$ ) (fig. 5f).

These data indicate that mast cell are recruited into the small intestine during the oral sensitization phase and that Treg do not impact on mast cell recruitment but can downregulate mast cell degranulation.

## Discussion

In the present study, we showed that oral immunization of C3H/HeOuJ mice with BLG combined with the mucosal adjuvant CT allowed for the priming of BLG-specific CD4<sup>+</sup> T cells in MLN, that produce high levels of IL-4, IL-5 and IL-13 and low levels of IFN- $\gamma$ , compatible with a Th2-type response. Oral BLG sensitization also generated BLG-specific IgE, IgG1 and IgG2a indicating successful B cell priming. In addition, oral BLG challenge induced systemic symptoms of anaphylaxis associated with the release in the serum of the preformed mast cell mediator mMCP-1. These data are in line with previous studies reporting that immediate allergy to BLG can be generated by oral BLG challenge in C3H/HeOuJ orally immunized with BLG/CT [17, 24]. However, in our model we did not observed a drop in body temperature (data not shown) in anaphylactic mice and symptoms were only mild, as compared to previous reports [17, 24]. One possible explanation for these discrepancies may relate to the age of mice at the onset of sensitization (in our case 5–8 weeks, compared to 3 weeks in the study of Berin et al. [24]) or to the dose of BLG used for challenge (50 mg in our study, compared to 100 mg in the study of Frossard et al. [18]). Our experimental model of ITH to BLG mimics features of mild ITH to cow's milk in human and we thus believe it to be therefore well adapted for studying Treg contribution to the homeostatic control of immediate-type allergy.

Interestingly, we found that mice treated with anti-CD25 mAb before oral BLG sensitization developed more severe immediate systemic symptoms of anaphylaxis upon oral BLG challenge. This was associated with enhanced production of Th2 cytokines by MLN CD4<sup>+</sup> T cells, increased levels of BLG-specific serum IgE and IgG1 antibodies and enhanced release of the preformed mucosal mast cell mediator mMCP-1. Although anti-CD25 mAb treatment was initially considered to deplete Treg cells, several recent studies showed that such treatment induced functional inactivation of Treg by shedding the CD25 molecule rather than physically depleting Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> Treg [21–23], a finding in accordance with our own observation (online suppl. fig. 1). Previous studies have shown that Treg downregulate T

cell-dependent humoral responses [25, 26] and that a single injection of the anti-CD25 mAb PC61 was able to enhance specific IgG1, IgG2a and IgE responses up to day 28 following primary immunization with a protein antigen in CFA as adjuvant [25]. Treg were also reported to control development of Th2 responses in vivo and in vitro. Indeed, elimination of CD25<sup>+</sup> Treg can profoundly increase the Th2 response of Balb/c mice to *Leishmania* leading to an exacerbated infection [27]. In addition, Treg can inhibit the differentiation, IL-4 production and proliferation of Th2 cells in vitro [28]. Our findings are in line with a recent study showing that Treg depletion at the time of oral immunization with peanut extract with CT in C3H/HeOuJ mice enhanced the production of peanut-specific cytokines in spleen and MLN and antibodies in the serum [29]. This suggests that Treg can control oral sensitization to different types of food allergens.

Mast cells are crucial effectors of anaphylaxis and are present in the small intestine. We found that BLG sensitization alone induced mast cell recruitment in the serosa of the small intestine and that serum release of mMCP-1, a hallmark of mucosal mast cell degranulation [14, 30], occurred only upon oral BLG challenge. Anti-CD25 mAb-treated BLG-sensitized and BLG-challenged mice develop more severe symptoms of anaphylaxis than untreated mice, and this was associated to higher levels of serum mMCP-1, without detectable change in mast cell numbers, suggesting an impact of Treg on mast cell activation rather than on mast cell recruitment. van Wijk et al. [29] also reported that mice injected with anti-CD25 mAb before sensitization exhibited augmented mMCP-1 release upon oral peanut extract challenge, although they did not document whether this resulted in more severe clinical signs of anaphylaxis. It is unlikely that these effects resulted from a direct effect of Treg on mast cells. Indeed, Treg numbers were restored to normal values within 10 days after anti-CD25 injection, that is, at the time of the second oral immunization. Therefore, it is most likely that the mechanism behind enhanced anaphylaxis and mMCP-1 production in anti-CD25-treated mice upon BLG challenge results from increased amounts of BLG-specific IgE leading to more efficient Fc $\epsilon$ RI-dependent mast cells degranulation upon IgE cross-linking by BLG. Nonetheless, we found that anti-CD25 mAb injection just before BLG challenge increased mMCP-1 release in the serum (online suppl. fig. 2), indicating that Treg can also control mast cell degranulation during the efferent phase of immediate allergy, independently of their capacity to control induction of the adaptive T and B cell immune response. This is in line with a recent study

showing that CD4+CD25+ Treg can directly decrease mast cell function by inhibiting calcium-dependent release of  $\beta$ -hexosaminidase in vitro and downregulate passive anaphylaxis in vivo via a mechanism dependent on OX40-OX40 ligand interactions [31]. Unfortunately, we cannot definitely conclude regarding the potential of Treg to control the efferent phase of BLG type I allergy, since injecting the anti-CD25 mAb before challenge resulted in abrogation of clinical symptoms (online suppl. fig. 3), possibly due to the elimination of other CD25+ cells contributing to the symptoms.

The nature and mode of action of Treg which control immediate CMA remain to be defined. Treg [32] and IL-10 [33] have been reported to control mast cell activation by inhibiting Fc $\epsilon$ RI expression. We recently documented

that a subset of allergen-induced Foxp3+ICOS+ Treg producing IL-10 was endowed with the most potent suppressive function on contact dermatitis in mice [34]. Interestingly, we also observed that infants with type I allergy to cow's milk have a selective defect in a subset of Foxp3+ICOS+ Treg [in prep.]. Whether these ICOS+ Treg also contribute to spontaneous remission in immediate CMA remains to be elucidated to open up new avenues for specific immunotherapy.

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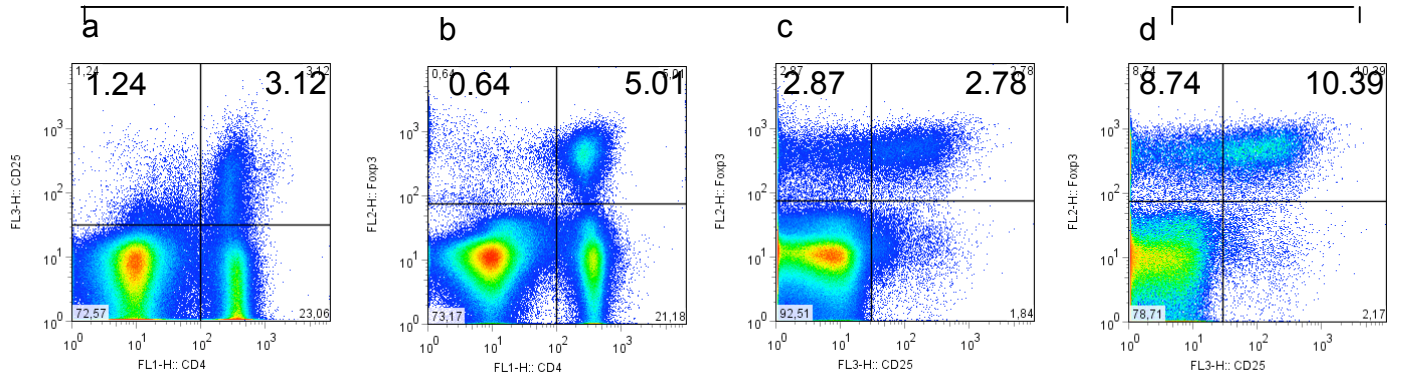
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## Supplementary Fig 1

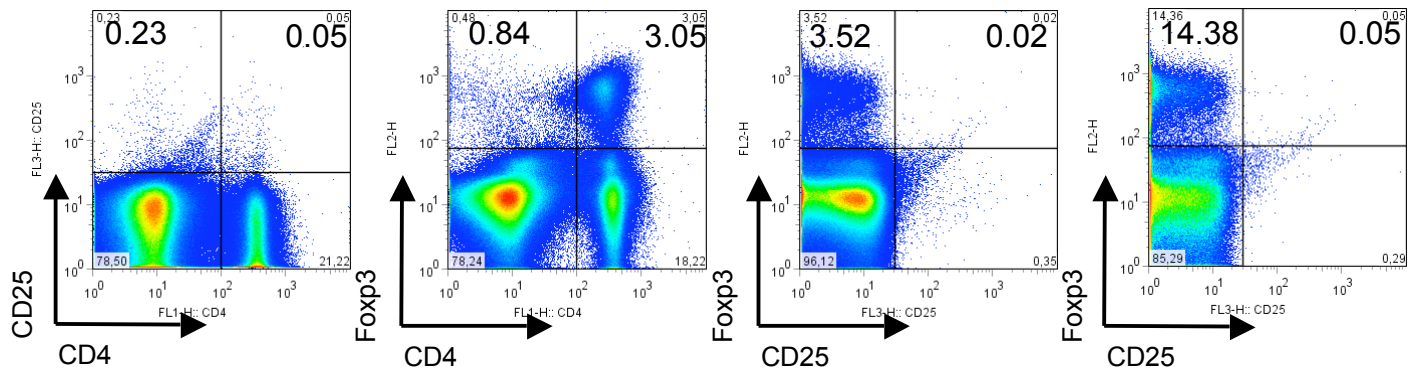
Without treatment

Total cells

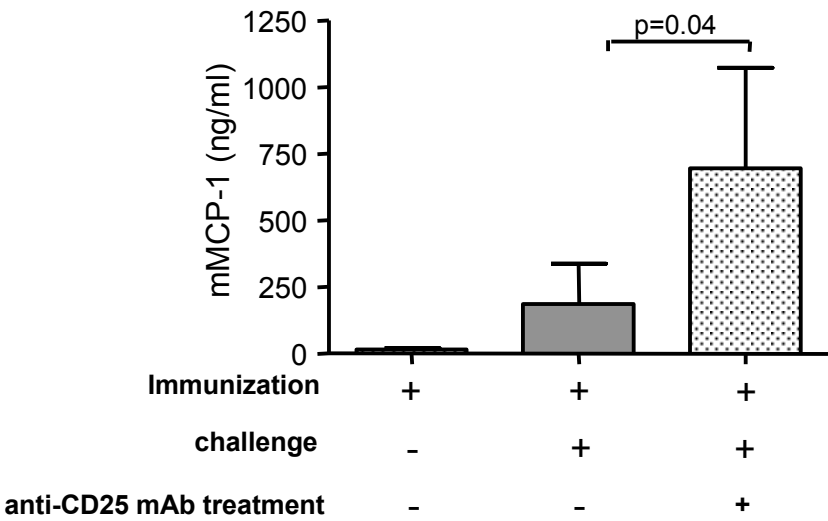
CD4+ cells



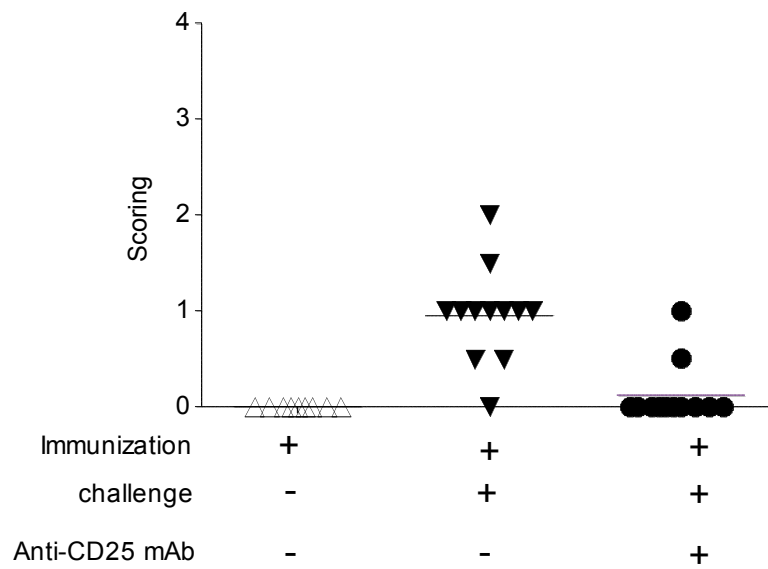
Anti-CD25 mAb treatment



Supplementary Fig 2



### Supplementary Fig 3



## **SUPPLEMENTARY MATERIAL**

### **Materials and Methods**

#### **Flow cytometry staining:**

Axillary and inguinal lymph nodes were removed from mice one day after the last treatment with anti-CD25 mAb. Lymphocytes were prepared by enzymatic digestion as described before and incubated with FACS medium (PBS, 2% SVF, 0,05% mM EDTA, and 0,01% NaN<sub>3</sub>) for at least 15 minutes. Cells were distributed in 96-wells plates and Fc- $\gamma$  receptors were blocked using 50  $\mu$ l of 2.4G2 mAb (supernatant of hybridoma culture) and incubated for 15 minutes at +4°C. After washing with FACS medium, cells were incubated with FITC anti-CD4 and PerCPcy5.5 anti-CD25 mAbs (both from BD Biosciences). After washing with FACS medium, cells were resuspended in fix/permeabilisation medium (eBioscience, France) and incubated for 1.50 h at +4°C. After washing with permeabilisation medium, cells were incubated with rat anti-mouse Fc $\gamma$ -receptor antibody (supernatant from the hybridoma 2.4G2) for 15 minutes at +4°C. Anti-Foxp3-PE was added and incubated for 30 minutes at +4°C. FACS analysis was performed using a FACS Canto (Becton Dickinson) and the data were analysed with the FlowJo software .

### **Figure legends**

#### **Supplementary figure 1: Efficacy of anti-CD25 mAb treatment**

Mice were injected with anti-CD25 mAb twice 2 days apart as described in the legend of figure 2 and the frequency of Treg in lymph nodes 1 day later was compared to that of normal untreated mice. Cells were prepared from inguinal and axillary lymph nodes and stained for CD4, CD25 and Foxp3 as described in the supplementary materials and methods. Expression of CD4, CD25 and Foxp3 on total lymph node cells is shown (a-c), as well as the expression of CD25 versus Foxp3 on gates CD4<sup>+</sup> cells (d).

**Supplementary figure 2: Anti-CD25 mAb injection before challenge enhances serum mMCP1.**

Mice were orally immunized with 10 mg BLG + CT and challenged with either PBS (*white bar*) or BLG (*grey bar and black bar*). One group of BLG-sensitized mice received 100 µg of anti-CD25 mAb one day before BLG challenge (*black bars*). mMCP-1 was titrated by ELISA in serum 1 hr after BLG challenge. Data are representative of 1 out 2 experiments using 4 mice per group.

**Supplementary figure 3: Anti-CD25 mAb injection before BLG challenge abrogates clinical symptoms of ITH**

Mice were orally immunized with 10 mg BLG/ CT and challenged with either PBS (*open triangles*) or 50 mg BLG (*black triangles*), as described in the Figure 1's legend. One group of BLG-immunized and -challenged mice received 100 µg of anti-CD25 mAb on day 25 and 27 , e.g. just before the BLG challenge (*black circles*). The cumulative clinical score was determined as described in Material and Methods. Data represent pooled values from 2 independent experiments using 4-7 mice/group.