

Lymphocytes in Peyer patches regulate clinical tolerance in a murine model of food allergy

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Background: Food allergy can lead to severe, potentially life-threatening anaphylactic reactions. To generate efficient strategies aimed at an active cure, a better understanding of the pathogenic mechanisms is strongly needed.

Objective: To investigate T-cell-related mechanisms of food allergy and tolerance to β -lactoglobulin (BLG) in gut-associated lymphoid structures.

Methods: β -Lactoglobulin-specific IgG1, IgG2a, and IgE in serum from mice anaphylactic to BLG were analyzed by ELISA and compared with those obtained in mice actively tolerized to BLG. The number of Ab-secreting cells in the spleen and in Peyer patches was determined by ELISPOT. The numbers of cytokine-producing cells after antigen-specific activation were measured by the same method. Furthermore, mesenteric lymph node cells and Peyer patches cells were transferred to naive mice, and Ab production as well as Ab-secreting cells were measured.

Results: Serum IgG1 and IgE Ab titers as well as IL-4-producing cell numbers were strongly increased in anaphylactic mice. IL-10 was found in Peyer patch cells from tolerant mice after BLG activation but not in anaphylactic mice. Peyer patch cells, in contrast to mesenteric lymph node cells, proliferated weakly in anaphylactic mice after antigen activation, and activation of Peyer patches was partially inhibited by tolerization.

Conclusions: Our data suggest a specific role for lymphocytes in Peyer patches in tolerance to BLG. Low IL-10 production in Peyer patches may favor symptoms of food allergy. (*J Allergy Clin Immunol* 2004;113:958-64.)

Key words: Food hypersensitivity, anaphylaxis, tolerance, T-lymphocytes, β -lactoglobulin, IL-10

Food allergy is a disease affecting approximately 2% to 5% of the population and is characterized by a breach in oral tolerance.¹ In human beings, elevated IgE antibodies as well as the presence of IL-4-producing, antigen-specific T lymphocytes suggest a T_H2-skewed mechanism. Although exact immunologic mechanisms eliciting food allergy are so far unknown, it is suspected that the

Abbreviations used

BLG: β -Lactoglobulin
CTX: Cholera toxin
MNC: Mesenteric lymph node cells
OVO: Ovomucoid
PPC: Peyer patch cells
SC: Spleen cells

“gut-related” immune system plays a key role in the pathogenicity of the disease.

Recently established mouse models of food allergy allow relevant in vivo studies of the mucosal immune system. In an initial study, Snider et al² sensitized young mice by intragastric feeding of a food antigen (hen egg lysozyme) together with the mucosal adjuvant cholera toxin (CTX). They could elicit symptoms similar to food-induced anaphylaxis after test gavage with antigens as well as a strong antigen-specific IgG1 and IgA but no IgG2a Ab response. This model has been further adapted to study allergy to various foods as well as potential options to cure food allergy.³⁻⁷ However, mechanisms responsible for loss of tolerance in food allergy have not been elucidated so far. By using this mouse model, we were able to demonstrate prevention of food allergy (ie, tolerance induction) by carrageenan, a high-molecular-weight galactose-based polysaccharide, with a decrease in antigen-activated proliferation of mesenteric lymph nodes cells (MNCs) and spleen cells (SCs).⁸ These observations lead us to the hypothesis that food allergy might be related to a particular type of immune response in one of the lymphoid compartments in or close to the gut.

METHODS

Mice

Four- to 5-week-old female C3H/HeOuJ mice were purchased from Charles River (L'Arbresle, France). The study was approved by the institutional review board for animal experiments.

Oral sensitization and active tolerization to β -lactoglobulin

Mice in the sensitized group (food anaphylactic mice) were immunized at days 0, 7, 14, and 21 by gavage, after gastric intubation, with 20 mg β -lactoglobulin (BLG; Sigma, Buchs, Switzerland) and 10 μ g CTX (List Biological Laboratories, Campbell, Calif) in 0.2 mol/L NaHCO₃ (total, 0.4 mL). To have a group of mice fed with the antigen with objective signs of an immune response but tolerant to the

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food upon antigen challenge, another group of mice (tolerized mice) received 0.8 mg/mL BLG in their drinking water ad libitum for 4 weeks. The total amount of protein given (22.4 mg) was similar to the total amount of BLG given to the sensitized mice. To demonstrate that the tolerization procedure also enduringly activated the peripheral and not the mucosal immune system only, a group of tolerized mice was injected twice with 80 μ g ip BLG adsorbed to 1 mg alum at days 28 and 42.

Antigen challenge

On day 28, all mice were challenged by intragastric gavage with 100 mg BLG in 0.4 mL 0.2 mol NaHCO₃. Anaphylaxis was observed and graded by using a reaction score (0, no reaction, to 3, severe reaction or death) described in detail elsewhere.⁸ The core body temperature was measured by infrared at the ear before challenge and 30 minutes after gavage. After the animals were killed, blood was collected by cardiac puncture into EDTA-containing tubes, and plasma was obtained for histamine measurement by commercial ELISA kit (Immunotech, Marseille, France).

Mesenteric lymph nodes, Peyer patches, and spleen cell proliferation

Peyer patches were excised from the gut and incubated for 30 minutes in HBSS medium supplemented with 5 mmol EDTA (Life Technologies, Paisley, Scotland). Similarly, Peyer patches, spleens, and mesenteric lymph nodes were gently crushed and filtered through a 70- μ m nylon filter. Total MNC, SC, or Peyer patch cells (PPCs) was incubated on a nylon wool column at 37°C for 1 hour and followed by anti-MHC class II Abs (m5/114.15.2, ATCC, Rockville, Md) and by rabbit low-tox M complement (Cedarlane, Hornby, Ontario, Canada) to obtain an enriched population with >97% of CD3+ cells (verified by flow cytometry gated on viable [7AAD-] cells). CD3+ MNC, SC, or PPC (1.5 $\times$ 10⁵ cells/well) were then activated with antigen in presence of, respectively, irradiated total SC (for MNC and SC) or PPC (2 $\times$ 10⁵ cells/well). Spleen cells were previously incubated for 5 minutes in Tris-buffered NH₄Cl to remove red blood cells. The cell medium used was complete Dulbecco modified eagle's medium (Life Technologies, Basel, Switzerland) with 10% nonessential amino acids, 100 U/mL penicillin, 100 μ g/mL streptomycin, 2 mmol L-glutamine, 100 μ g/mL gentamicin, 10 nmol sodium pyruvate, 2 $\times$ 10⁻⁵ mol 2-ME, 1 μ g/mL polymyxin B (all from Sigma, St Louis, Mo) plus 10% FCS (Life Technologies, Paisley, Scotland). Either BLG or ovomucoid (OVO) as a control antigen was added at different concentrations (2.5 $\times$ 10⁻⁵ mol, 2.5 $\times$ 10⁻⁶ mol, 2.5 $\times$ 10⁻⁷ mol). The cells were pulsed on day 5 of culture with 1 μ Ci/well ³H-labeled thymidine (Amersham, Zurich, Switzerland) for 18 hours and harvested.

Enzyme-linked immunoassays for BLG-specific serum Ab

Sera were obtained for determination of BLG-specific IgE, IgG1, and IgG2a levels by a method adapted from Adel-Patient et al.⁹ MaxiSorp microtiter plates (Nunc, Roskilde, Denmark) were coated for 18 hours at room temperature with 250 ng/well streptavidin (Fluka, Buchs, Switzerland) followed by 300 μ L of a solution of polyvinylpyrrolidone K25 (Fluka) overnight. One microgram of biotinylated BLG was incubated for 3 hours, and diluted sera (1:6666 and 1:2222 for IgG1, 1:666 and 1:222 for IgG2a, 1:66 and 1:22 for IgE) in PBS plus 10% horse serum was added. For IgG1 and IgG2a Ab titer measurement, either 0.5 μ g/mL goat anti-mouse IgG1 or rat anti-mouse IgG2a peroxidase-labeled antibodies (Southern Biotechnologies, Birmingham, Ala) were incubated for 2 hours. For IgE measurement, a monoclonal rat anti-mouse IgE Ab (clone R35-72, BD Pharmingen, Heidelberg, Germany) followed by

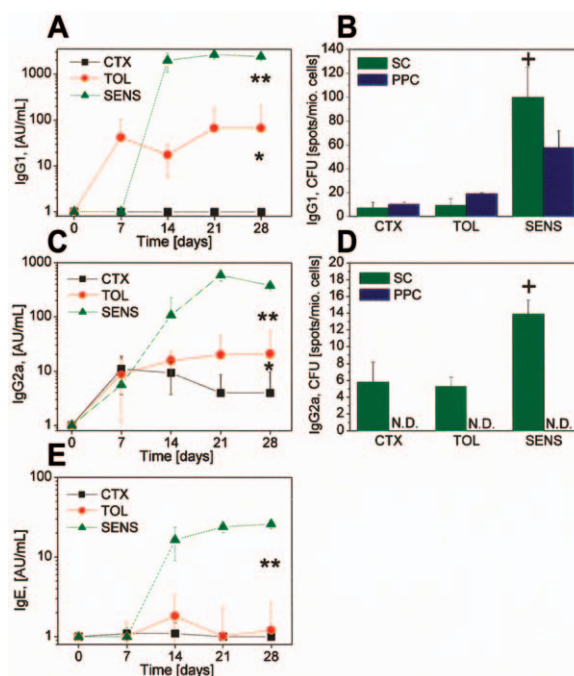


FIG 1. β -Lactoglobulin-specific serum IgG1 (A), IgG2a (C), and IgE (E) antibody titers by ELISA. Numbers of BLG-specific IgG1 (B) and IgG2a (D) antibody-producing cells counted by ELISPOT in spleen or in Peyer patches. Results are pooled from at least 3 different experiences. ND, Not detectable. ** $P < .001$, SENS (mice sensitized with β -lactoglobulin) vs CTX; * $P < .05$, TOL (mice tolerized with β -lactoglobulin) vs CTX; + $P < .05$, TOL vs SENS in SC; otherwise P = not significant.

peroxidase-coupled anti-rat Ab (Caltag, San Francisco, Calif) was added. Optical density was measured at 490 nm. All sera were tested in duplicate. Results were expressed as arbitrary units, with pooled sera from BLG plus alum-immunized mice used as a reference serum.

Antigen-specific Ab and cytokine production measured by ELISPOT

For the measurement of BLG-specific IgG1 and IgG2a antibodies, ELISPOT plates were coated with streptavidin overnight at 37°C, followed by addition of 1 μ g of biotinylated BLG for 3 hours. Lymphoblasts isolated on a Percoll 60%/66% gradient (Amersham, Zurich, Switzerland) were resuspended as 2 $\times$ 10⁶ in Iscove modified Dulbecco medium supplemented with penicillin, streptomycin, L-glutamine, 100 μ g/mL gentamicin, polymyxin B plus 5% FCS for 24 hours at 37°C, followed by overnight incubation at 4°C with anti-IgG1 and anti-IgG2a antibodies (Southern Biotechnology, Birmingham, Ala). In both procedures, cytokine and Abs ELISPOT, 100 μ L/well amino-ethyl-carbazole, were added for 10 minutes, and the spots were automatically counted by KS EliSpot 4.2.1 Software (Zeiss, Halbermoos, Germany) and expressed as cell-forming units per 10⁶ cells.

For assessment of cytokine production, ELISPOT plates (Millipore, Bedford, Mass) were coated with either monoclonal antibodies against IL-4 (clone BVD6-24G2, BD Pharmingen, Heidelberg, Germany) or against IFN- γ (clone 01.E70.3B2, ATCC, Rockville, Md) (both at 0.5 μ g/well) or against IL-10 (clone JES5-2A5, BD Pharmingen, Heidelberg, Germany) (1 μ g/mL) overnight at 4°C followed by blocking with cell culture medium for 3 hours. Cells were added as 4 $\times$ 10⁵ CD3+ cells/well and 6 $\times$ 10⁵ irradiated total

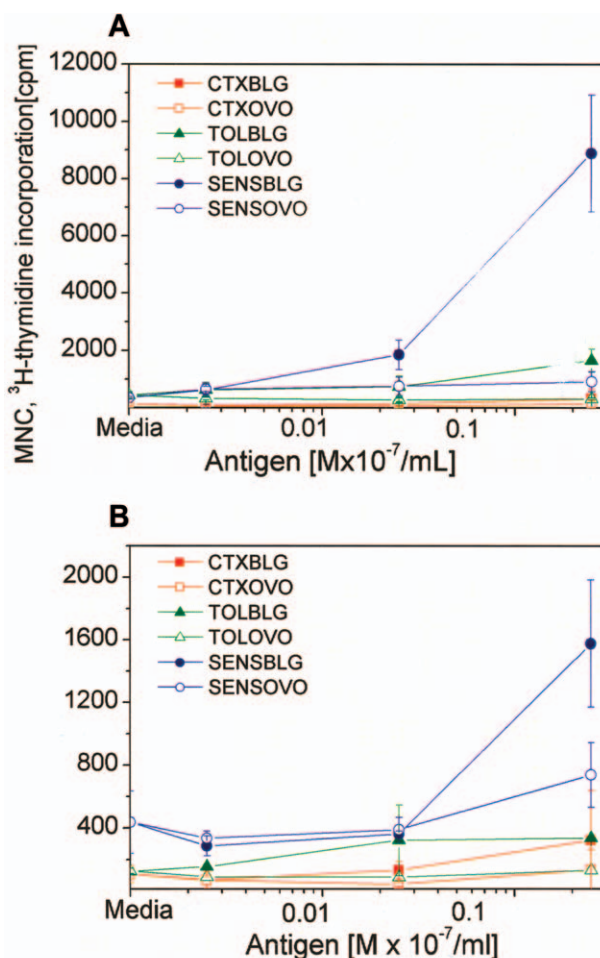


FIG 2. Antigen-activated MNC (**A**) or PPC (**B**) proliferation. CD3⁺ cells were isolated from CTX mice and activated with BLG (CTXBLG) or OVO (CTXOVO), BLG or OVO-activated cells from TOL mice (mice tolerized with β -lactoglobulin) (TOLBLG and TOLOVO), or BLG or OVO-activated cells from SENS mice (mice sensitized with β -lactoglobulin) (SENSBLG and SENSOVO). Results shown are from pooled cells in one representative experiment.

cells as antigen-presenting cells in the presence of 2.5×10^{-5} mol BLG. The plates were incubated for 3 days for measurement of IFN- γ and 4 days for IL-4 and IL-10, at 37°C. Corresponding biotin-labeled Abs against IL-4 (clone BVD4-1B11, BD Pharmingen, Heidelberg, Germany), IL-10 (clone SXC-1, BD Pharmingen, Heidelberg, Germany), or IFN- γ (clone AN 18.03.C12, ATCC, Rockville, Md) at 0.1 μ g/well were added for overnight incubation at 4°C followed by streptavidin horseradish peroxidase at 1:1000 for 2 hours. Initial experiments showed identical comparative results with either SC or MNC in both Ab or cytokine measurement. However, better yields of Ab titers in SC and of cytokines in MNC were obtained. To limit the number of mice per experiment, we had to choose to restrict the measurement of Ab to SC and to MNC for cytokines experiments.

Adoptive transfers

After isolation of CD3⁺ cells from the mesenteric lymph nodes, the spleen, or the Payer patches, the cells were resuspended in autologous mouse serum and PBS (1:1) at a concentration of 50×10^6 cells/mL. Ten mio cells were injected through the tail vein to a 4-week-old naive C3H/HeO/J mouse. Forty-eight hours after the cell transfer, the mice were boosted by an intragastric gavage of 20 mg of BLG.

Statistical analysis

Values between groups were analyzed by the Wilcoxon rank signed test for nonparametric unpaired data. The Fisher exact test was used as a binomial test for two independent samples (anaphylaxis scores).

RESULTS

Only mice orally sensitized to BLG in presence of CTX have anaphylaxis in response to BLG challenge

Three groups of mice were constituted, mice orally sensitized to BLG in the presence of CTX, mice tolerized to BLG with continuous feeding in the absence of an adjuvant, and a control group of mice gavaged with CTX alone. None of the animals from the tolerized and the CTX group (0/11) were anaphylactic upon oral challenge with BLG on day 28. However, all mice orally sensitized with BLG in presence of CTX (11/11) exhibited an anaphylaxis score ≥ 2 ($P < .001$). β -Lactoglobulin-induced anaphy-

laxis correlated with a significant drop in body temperature ($2.6^{\circ}\text{C} \pm 0.7^{\circ}\text{C}$ vs $0.3^{\circ}\text{C} \pm 0.3^{\circ}\text{C}$ in tolerized mice; $P < .05$). Histamine levels after challenge also correlated with clinical signs of anaphylaxis. None of BLG-sensitized mice reacted after a challenge with OVO used as a control antigen.

This first set of experiments confirmed the clinical reactivity of the mice actively sensitized, as well as the clinical tolerance of the mice receiving BLG in absence of the adjuvant (tolerized group).

Food allergy is characterized by an increased production of IgG1, IgG2a, and IgE, predominantly by spleen cells

A strong increase in BLG-specific serum IgG1 (Fig 1, A), IgG2a (Fig 1, C), and IgE (Fig 1, E) were observed in the sensitized group only. The rise was observed beginning 7 days after the first oral sensitization, with a plateau at 28 days for IgG1. Although a significant increase in IgG1 and IgG2a but not IgE was also observed in the tolerized group, the levels were lower than in the sensitized group. Specific Ab in the CTX group remained undetectable. The frequency of IgG1 and IgG2a Ab-secreting cells from the spleen and of IgG1-producing cells from Peyer patches was higher in sensitized mice than in control mice and tolerized mice (Fig 1, B and D). In correlation with low levels of circulating BLG-specific IgE Ab, no IgE-secreting cells could be detected in either lymphoid structure (results not shown). These results indicate that food-induced anaphylaxis correlates with a strong increase of antigen-specific IgG1 and IgE and a moderate increase of IgG2a Ab, in contrast to tolerized animals who had only a moderate increase of IgG1. Experiments with MNC showed a similar pattern, with a lower frequency of Ab-producing cell spots (not shown), suggesting that the spleen is the major site of Ab production.

Lymphocyte proliferation to BLG in mesenteric lymph nodes and Peyer patches is only observed in sensitized mice

Lymphocyte proliferation with CD3+ MNC in the presence of BLG (Fig 2, A) was higher in the sensitized group than in the tolerized and the control groups. A low proliferation was seen with BLG in PPC of sensitized mice (Fig 2, B). These results show that antigen-induced cell proliferation was stronger in sensitized than in tolerized animals in MNC and PPC.

IFN- γ , IL-4, and IL-10 production in MNC and PPC by ELISPOT is specifically related to allergy or tolerance

Spontaneous and antigen-induced IFN- γ , IL-4, and IL-10-secreting cell numbers were higher in CD3+ MNC (Fig 3, A, C, and E) from sensitized animals compared with CTX control mice ($P < .05$). Numbers of BLG-induced IFN- γ but not IL-4 or IL-10-secreting cells were

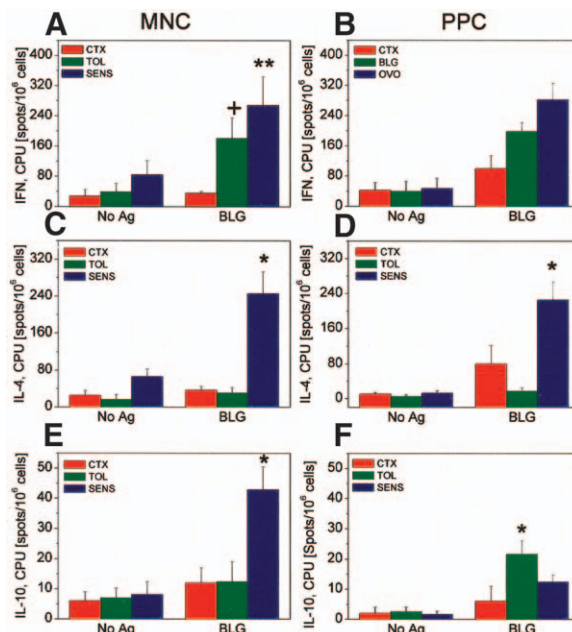


FIG 3. Numbers of unstimulated and BLG-specific IFN- γ (A and B), IL-4 (C and D), and IL-10 (E and F) producing cells counted by ELISPOT. Results are pooled from 3 different experiments. * $P < .05$, TOL (mice tolerized with β -lactoglobulin) vs SENS (mice sensitized with β -lactoglobulin); ** $P < .05$, SENS vs CTX; + $P < .05$, TOL vs CTX; otherwise P = not significant.

higher in both the tolerized and sensitized groups than in the CTX group (Fig 3, A). In tolerized animals, the number of antigen-induced IL-4 and IL-10-secreting cells were significantly lower than in sensitized animals ($P < .05$). A similar pattern of cytokine production was observed in CD3+ PPs for IFN- γ (Fig 3, B) and for IL-4 (Fig 3, D). In contrast, antigen-induced IL-10-producing cells were significantly increased in tolerized mice when compared with the group anaphylactic to BLG ($P < .05$) (Fig 3, F). These results suggest a characteristic cytokine secretion pattern in mice, depending on their sensitivity to food and the originating lymphoid compartment (MNC or PPC), with an increased production of IL-10 in PPC from BLG-tolerant mice as the most prominent feature.

Tolerization is an active process that cannot be fully reverted by subsequent sensitization

Specific IgG1 and IgE levels were significantly lower in mice first tolerized and then sensitized (ip) twice (pretol) when compared with mice only sensitized (ip) (nontol) (Fig 4, A, B, and C). Similarly, T-cell proliferation in SC and MNC after activation with BLG was decreased (Fig 4, D and E). This set of experiments confirms that oral tolerance could not be reverted by our sensitization protocol. Oral tolerance corresponds thus to an active process characterized by a poorly reversible inhibition of specific Ab production and T-cell reactivity.

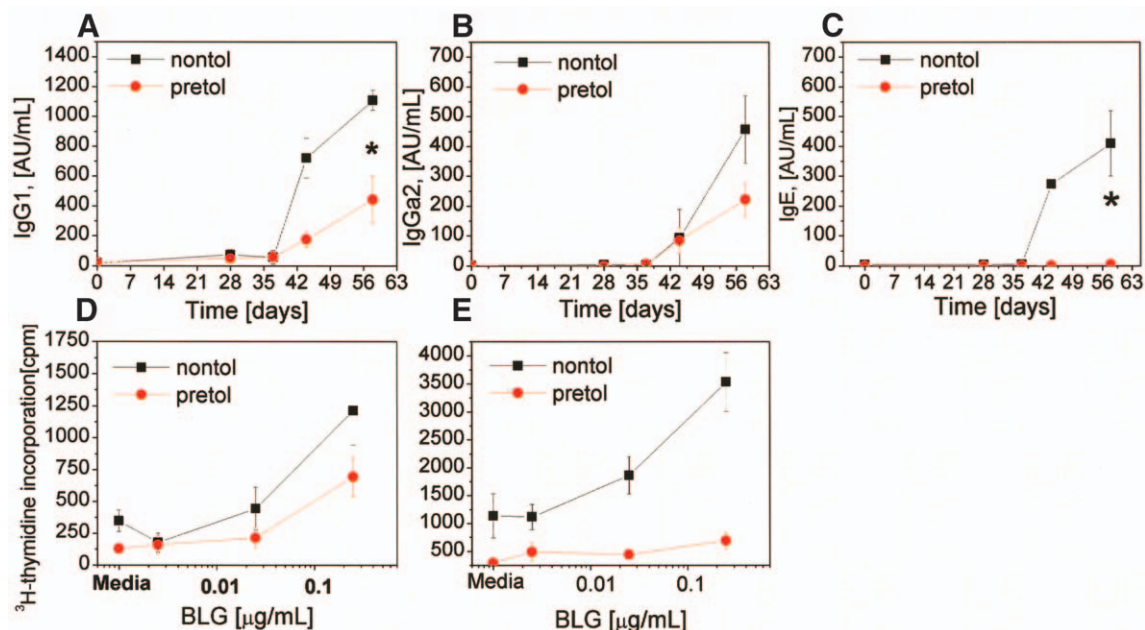


FIG 4. Naive mice (*nontol*) or mice previously orally tolerized to BLG (*pretol*) were subsequently sensitized (ip) with BLG adsorbed to alum. BLG-specific serum IgG1 (A), IgG2a (B), and IgE (C) antibody titers from pretol and nontol mice. Antigen-activated proliferation of cells from pooled SC (D) or MNC (E). * $P < .05$, nontol vs pretol.

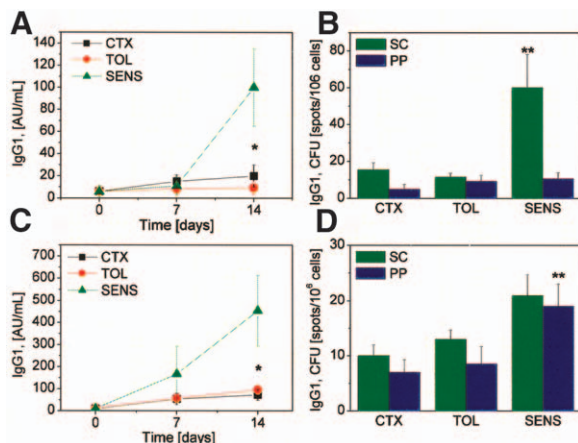


FIG 5. β -Lactoglobulin-specific serum IgG1 antibody titers after adoptive transfer of MNC (A) or PPC (C). Numbers of IgG1 antibody-secreting cells counted by ELISPOT in SC or PP after adoptive transfer of MNC (B) or PPC (D). Results are pooled data from two different experiments. * $P < .01$, SENS (mice sensitized with β -lactoglobulin) vs TOL (mice tolerized with β -lactoglobulin); ** $P < .001$, SENS vs TOL.

Adoptive transfer of MNC and PPC elicits specific Ab response

Only cells from sensitized mice were able to elicit a BLG-specific Ab production 14 days after cell transfer (Fig 5, A and C). After adoptive transfer of CD3+ MNC from sensitized animals, BLG-specific IgG1-producing cells were predominantly located in the spleen and were present in a significantly higher number after transfer of cells from sensitized than from tolerized mice ($P < .001$)

(Fig 5, B), whereas after transfer of CD3+ PPC, BLG-specific Ab-producing cells were found in both the spleen and the Peyer patches. These experiments show that only T cells from sensitized mice but not from tolerized mice induce specific IgG1 Ab production in recipient mice. Furthermore, it is demonstrated that MNC in contrast to PPC induce specific IgG1 Ab-forming cells in the spleen but not in Peyer patches of recipient mice.

DISCUSSION

Food allergy is a breach in oral tolerance, an extensively studied subject for several decades. However, the effect of oral tolerance on the immune system was mostly studied by means of delayed hypersensitivity tests.^{10,11} The mouse model of food allergy now widely used allows correlation of clinical scores of food-induced anaphylaxis with parameters of sensitization.^{2,4,5,8} To interpret parameters obtained in the food allergy model, we chose a mouse model fed BLG to achieve clinical tolerance in a way similar to previously published studies.¹²⁻¹⁴ In sensitized mice, we observed a predominantly T_H2 -type immune response (with increased food-specific IgE and IgG1 antibodies and concomitant increase IL-4 and IL-10 production). Similar Ab and cytokine profiles have been found in human beings with food allergy. In addition, the T_H1 cytokine IFN- γ was induced by sensitization (Fig 3, A and B), as observed in other models of murine anaphylaxis.¹⁵⁻¹⁷ We could also show that early feedings with BLG lead to active tolerization, with a specific pattern of cytokine secretion in MNC and PPC (presence of IFN- γ in the absence of IL-4). We believe that the two groups of

mice studied here accurately reflect, respectively, food allergy and oral tolerance to foods as observed in human beings, thus supporting the study of mechanisms involved in food allergy in the gut-related immune system in mice.

Previous studies, both in mice and human beings, have shown that antigen-specific lymphocytes from peripheral blood or from gut-associated lymph node structures could be activated^{8,15,18-20}; however, to our knowledge, no other studies have examined the site of serum Ab production in food allergy so far. β -Lactoglobulin-specific Ab-secreting cell numbers in the spleen and in Peyer patches were determined by ELISPOT (Fig 3). Although allergen sensitization in the presence of CTX takes place in the lamina propria as well as in Peyer patches,^{21,22} the highest numbers of IgG1-secreting cells, after cell enrichment for lymphoblasts on a Percoll gradient, were found in the spleen. For IgG2a or IgE, either no or low numbers of Ab-secreting cells were found in Peyer patches. As antigen-specific T cells have been isolated from both mesenteric lymph nodes and Peyer patches (Fig 3), it remained unclear if antigen-specific T-lymphocytes activate B cells exclusively in PPCs or if B-cell activation in the presence of BLG-specific T cells also occurs in peripheral organs such as the spleen. Adoptive transfer experiments further showed that the transfer of CD3+ SC was inducing BLG-specific Ab production only in the spleen, whereas PPC were inducing Ab production in both the spleen and Peyer patches. Taken together, these results suggest that antigen priming may take place in Peyer patches and that CD3+ cells with a tropism to the spleen are responsible for activating Ab production. These findings correlate with a recent report by Mora et al,²³ showing that oral antigens selectively induce effector/memory gut-homing T cells in Peyer patches.

Tolerization by BLG feedings indeed revealed an active immunologic process. Despite subsequent intraperitoneal sensitization, IgG1 Ab levels remained decreased and IgE secretion was abrogated below the levels of detection, whereas some decrease in IgG2a was also observed. These findings are in accordance with previously published studies.²⁴

Most strikingly, a distinct cell cytokine secretion pattern, depending on the lymphoid site, was observed in tolerized mice. In contrast to sensitized mice, IL-4-secreting cells were seen neither in MNC nor in PPC, whereas an increased number of BLG-specific IL-10-secreting cells was seen in Peyer patches. IL-10 is secreted by a subset of regulatory T cells^{25,26} and is a cytokine involved in modulation of tolerance.²⁷ Thus, low IL-10 in Peyer patches might be a key factor in the development of food allergy. Preliminary characterization of these IL-10-producing, antigen-specific PPC show secretion of TGF- β in the absence of significant IL-2. Furthermore, no definite expression of CD25 could be identified.

Transfer of CD3+ MNC and PPC induced significant IgG1 Ab secretion measured in the serum of recipient mice. However, even with higher numbers of cells transferred (up to 30×10^6 cells), anaphylaxis could not be elicited after antigen challenge, and no antigen-specific,

cytokine-secreting cells could be demonstrated by ELISPOT in lymph node cells from recipient mice. Additionally, we transferred total MNC or PPC but again without eliciting anaphylaxis in recipient mice. These experiments suggest that the T cells may only be one player responsible for eliciting an allergic response to the food.

In summary, we showed that clinical tolerance to foods is an active mechanism involving IL-10-secreting cells in Peyer patches. Whether IL-10 may modulate food allergy in human beings needs to be further studied.

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