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Original Article

Exposure to High Doses of Lipopolysaccharide during Ovalbumin Sensitization Prevents the Development of Allergic Th2 Responses to a Dietary Antigen

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Abstract: Food allergies are driven by aberrant T helper (Th) 2 cells. Lipopolysaccharide (LPS) influences the development of Th2-mediated diseases, but its role in food allergy and tolerance remains unclear. To address this issue, we established mouse models presenting allergic or tolerant responses to ovalbumin (OVA). Mice sensitized with crude OVA developed Th2 responses including acute diarrhea, increases in serum OVA-specific IgE, dominant production of serum OVA-specific IgG1, increases in Th2-type cytokines and proliferation of mast cells in duodenal and colonic tissues. Sensitization of mice with crude OVA and LPS abrogated Th2-type responses observed in allergic mice. The level of OVA-specific proliferation in mesenteric lymph node $CD4^+$ T cells was comparable in allergic and tolerant mice, indicating that the tolerance is not caused by anergy and apoptosis of antigen-primed T cells. Expression of Th1- and Th2-type cytokines was suppressed in whole spleen cells and/or purified spleen $CD4^+$ T cells of tolerant mice, indicating that the tolerance was not caused by the shift from Th2 to Th1. On the other hand, interleukin (IL)-10, a regulatory cytokine produced by regulatory T cells, was upregulated in whole spleen cells and purified spleen $CD4^+$ T cells of tolerant mice. Furthermore, spleen $CD4^+$ T cells from tolerant mice suppressed the growth of $CD4^+$ T cells from DO11.10 mice in co-culture. These results indicate that tolerance is induced in allergic mice by simultaneous exposure to LPS during sensitization with OVA and that a population of T cells producing IL-10 plays an important role in the tolerance induction. (DOI: 10.1293/tox.27.2014-0023; J Toxicol Pathol 2014; 27: 205–215)

Keywords: allergy, lipopolysaccharide, ovalbumin, tolerance

Introduction

Food allergy is defined as an aberrant T helper (Th) 2-mediated immune response to a dietary antigen[1, 2]. Cytokines produced by Th2 cells, such as interleukin (IL)-4, IL-5 and IL-13, participate not only in dietary antigen-specific IgE production but also in mucus secretion, muscle contraction, and eosinophil/mast cell proliferation in intestinal tissues[1, 3]. Mast cells express FcεRI, which binds antigen-specific IgE. Upon reexposure to the dietary antigen, cross-linking of the surface IgE stimulates mast cells to release a variety of chemical mediators, which can induce food allergic symptoms, such as diarrhea and anaphylactic reactions[4].

Components of environmental microbes are potent activators of the immune system with the capacity to shift active immune responses towards priming of Th1 or Th2[5]. Lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, has the capacity to induce Th1-polarized adaptive immune responses via toll-like receptor (TLR) 4 signaling[6, 7] and exerts crucial effects on the development of asthma, which is a Th2-mediated allergic disease[8–11]. It has also been shown that LPS exposure drives the development of Th2 airway hypersensitivity through TLR4 signaling in murine models of pulmonary inflammation[12–15]. It is therefore likely that the condition of LPS determines the subsequent immune responses. In the digestive tract, to maintain tolerance to food antigens, the immune system is strictly controlled by several regulatory mechanisms, such as the enzymatic digestion of immunogenic epitopes in food, the constitutive stimulation of gut-associated lymphoid tissue by commensal flora and the action of unique cell populations with regulatory function. The stimulation of gut-associated lymphoid tissue with noninvasive commensal microbiota has a profound impact on the responsiveness of lymphocytes and the generation of Th1-biased memory effector cells[16–18]. It also induces tolerance to orally administered antigens[19]. Since LPS is contained in commensal flora as a basic component with biological activity, it may function as a crucial regulator for food allergy and produce tolerance to dietary antigens.

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The involvement of regulatory T cells (Treg) in food allergy has been reported[20, 21]. $CD4^+$ $CD25^+$ $Foxp3^+$ Treg participate in the induction of oral tolerance to cow's milk and peanut extract allergy[22, 23]. Treg producing IL-10, Tr1, inhibit Th2-mediated allergic diarrhea and eosinophilic infiltration into the small intestine of Peyer's patch-lacking mice[24, 25]. Treg producing transforming growth factor (TGF)-β, Th3, are crucial for intestinal homeostasis. Food allergy and anaphylaxis caused by impaired production of TGF-β can be prevented by administration of TGF-β[26–28]. Recently, it has been shown that systemic stimulation with LPS enhances the production of IL-10 and TGF-β in mice, suggesting that LPS regulates immune responses to antigens through induction of Treg producing IL-10 or TGF-β[29, 30].

In this study, to assess the role of LPS in food allergy, we sensitized BALB/c mice with crude ovalbumin (OVA) (hereafter called cOVA) or with cOVA and LPS by intraperitoneal (i.p.) injection. Th2-mediated allergy was observed in the former case, and tolerance was observed in the latter case. The abrogation of the allergic reaction in tolerant mice was not due to induction of anergy and apoptosis in primed T cells or to the shift from Th2 to Th1. On the other hand, IL-10 expression was upregulated in spleen $CD4^+$ T cells of tolerant mice. These spleen $CD4^+$ T cells suppressed the growth of $CD4^+$ T cells expressing an OVA-specific T cell receptor (TCR) in co-culture. These results indicate that systemic exposure to LPS during OVA sensitization activates a population of $CD4^+$ T cells producing IL-10, resulting in suppression of Th2-mediated allergic reactions.

Materials and Methods

Mice

Female BALB/c ByJ and BALB/c Cr mice were purchased from Clea Japan Inc. (Tokyo, Japan) and Japan SLC (Hamamatsu, Japan), respectively. $OVA_{323–339}$-specific TCR transgenic mice (DO11.10) on a BALB/c background were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed under specific pathogen-free conditions and used for experiments at 6–8 weeks of age.

Endotoxin activity in OVA

To evaluate the contamination of LPS in cOVA (Grade V, A-5503; Sigma-Aldrich, St. Louis, Mo, USA) and purified OVA (hereafter called pOVA) (Profos AG, Regensburg, Germany), endotoxin activities of cOVA and pOVA were examined by the *Limulus* amebocyte lysate assay (Seikagaku Biobusiness Corp., Tokyo, Japan). LPS derived from *Escherichia coli* 0013:H10 was used as a standard for endotoxin activity. Endotoxin activity of cOVA was assayed after it was diluted so that the activity fell within the linear range of the standard. pOVA was assayed for endotoxin activity without dilution. The endotoxin activity of cOVA (5 μg/ml) and that of pOVA (5 μg/ml) were estimated to be 52 EU/mg and < 1 EU/mg, respectively, based on the value of 545 nm absorbance. For further experiments, cOVA was used as an antigen for *in vivo* sensitization, and pOVA was used as that for *ex vivo* stimulation.

Sensitization and challenge protocol

Based on a previous study about oral allergen-induced diarrhea, we generated the following protocol for the food allergic and tolerant models[31]. Mice (n=3–10) were sensitized with 100 μg of cOVA by i.p. injection with or without various doses of LPS derived from *Escherichia coli* 055:B5 (LIST Biological Laboratories Inc., Campbell, CA, USA) in 200 μl phosphate-buffered saline (PBS) on days 0 and 14. Mice (n=3–5) sham sensitized with PBS on days 0 and 14 were used as the control. One week after the second sensitization, mice were orally challenged with 100 mg of cOVA in 250 μl of sterile saline at 2- or 3-day intervals. Before each intragastric challenge, mice were deprived of food for 3–4 h to minimize degradation of the antigen in the stomach. Mice were carefully monitored for 1 h after each challenge, and those defecating profusely liquid stool were marked as acute diarrhea positive. Diarrhea occurrence was assessed for 15–60 min after each challenge with cOVA. Mice were sacrificed within 1 h after the last challenge. Blood was taken from the femoral artery, and sera were prepared and stored at −20˚C until use. Duodenums (approximately 10 cm distal to the stomach) and colons (distal part of the cecum) were promptly removed. A part of each of these tissues was embedded in OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), and the remaining tissues were quickly frozen and stored at −80˚C until use. To characterize spleen or mesenteric lymph node (MLN) cells, mice were sensitized with 100 μg of cOVA by i.p. injection with or without 100 μg of LPS in 200 μl PBS on days 0 and 7. Mice sham sensitized with PBS on days 0 and 7 were used as the control. Four days or 1 week after the second sensitization, spleens and MLNs were removed. All experimental procedures were approved by the Animal Care Committee of Hyogo College of Medicine and performed in accordance with the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences.

Cells and culture

Spleens and MLNs were removed from sensitized and control (PBS-sensitized) BALB/c ByJ mice at the indicated days after the second sensitization and dispersed mechanically. Single-cell suspensions from spleens and MLNs were prepared from the dispersed tissues by passing through nylon mesh cell strainers (BD Biosciences, Bedford, MA, USA) and excluding RBCs by lysis. $CD4^+$ T cells were negatively selected from single-cell suspensions by magnetic separation with a $CD4^+$ T cell Isolation Kit (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions (> 95% $CD4^+$ T cells as determined by FACS analysis). Spleen $CD4^+$ T cells from naive DO11.10 mice were isolated in a similar manner.

For preparation of antigen-presenting cells (APCs), the spleens removed from naive BALB/c ByJ and DO11.10 mice were cut into small pieces, treated with 400 Mandl units/ml collagenase D (Roche, Mannheim, Germany) and 10 μg/ml

DNase I (Roche) with continuous stirring at 37˚C for 35 min and incubated at 37˚C for 5 min after adding EDTA to adjust the final concentration to 10 mM. Single cells were prepared from the spleen by vigorously pipetting the suspension of small tissue pieces and passing the suspension through nylon mesh cell strainers. After lysing of RBCs, the $CD4^+$ T cell-depleted fraction was isolated from the cell suspensions with a $CD4^+$ T cell Isolation Kit (Miltenyi Biotec). The isolated cells were irradiated at 30 Gray and used as APCs.

All cells used in this study were cultured in RPMI 1640 medium containing 10% FCS at 37ºC in a 5% CO_2 humidified atmosphere.

OVA-specific proliferation assay

Co-culture of 3.5×10^5 MLN $CD4^+$ T cells from control (PBS-sensitized) and sensitized BALB/c ByJ mice was performed with 1×10^5 APCs from BALB/c ByJ mice in the presence of the indicated concentration of pOVA in 96-well flat bottom microplates for 72 h. MLN $CD4^+$ T cells in each well were labeled with 0.25 µCi [^{3}H]-thymidine (PerkinElmer, Waltham, MA, USA) for the last 12 h of incubation, and the incorporated radioactivity in MLN $CD4^+$ T cells was measured by a liquid scintillation counter (TopCount, Packard BioScience Company, Meriden, CT, USA).

Ex vivo co-culture assay

Co-culture of 5×10^5 spleen $CD4^+$ T cells from naive DO11.10 mice was performed with 5×10^5 nonirradiated or irradiated spleen $CD4^+$ T cells from sensitized BALB/c ByJ mice in the presence of pOVA (50 µg/ml) and 1×10^5 APCs from DO11.10 mice in 96-well round bottom microplates for 72 h. Cells in each well were labeled with 0.25 µCi [^{3}H]-thymidine (PerkinElmer) for the last 12 h of incubation, and the incorporated radioactivity was measured by a liquid scintillation counter (TopCount).

Enzyme-linked immunosorbent assay (ELISA) for OVA-specific IgE, IgG and IgG2a

The level of serum OVA-specific IgE was measured by ELISA (Dainippon Sumitomo Pharma, Tokyo, Japan). For measurement of serum OVA-specific IgG1 and IgG2a, sera were diluted 1:1,000 for IgG1, 1:10 for IgG2a and serially diluted 1:4. Immunoplates (Nalge Nunc International, Naperville, IL, USA) were coated with 50 µg/ml cOVA in borate saline containing 1% (W/V) bovine serum albumin (BSA) and incubated overnight at 4˚C. After blocking with 1% BSA in PBS for 1 h at room temperature, serially diluted serum samples were added into the immunoplates, and the immunoplates were incubated for 2 h at room temperature. After washing with borate saline containing 1% BSA and 0.05% Tween-20, horseradish peroxidase-conjugated anti-mouse IgG1 diluted 1:1,000 (X56; BD Biosciences Pharmingen, Franklin Lakes, NJ, USA) or horseradish peroxidase-conjugated anti-mouse IgG2a diluted in 1:1,000 (R19–15; BD Biosciences Pharmingen) was added the immunoplates, and the immunoplates were incubated for 1 h at room temperature. The color reaction was developed with 3,3',5,5'-tetramethylbenzidine blue (DAKO, Glostrup, Denmark) for 30 min and stopped by the addition with 6N HCl. Optical density (OD) was measured at 405 nm within 30 min. Data represent OD values at a serum dilution of 1:125,000 for IgG1 and 1:50 for IgG2a, which were in the middle of the linear part of the OD curve, respectively.

Immunohistochemistry

Frozen sections (6 µm) were prepared from OCT-embedded tissues and fixed with 10% formalin. Sections were immunostained with rat anti-mouse c-kit mAb (ACK2), a gift from Dr. Shin-Ichi Nishikawa (Biohistory Research Hall, Osaka, Japan) and then with Histofine Simple Stain mouse MAX-PO (Rat) (Nichirei Corporation, Tokyo, Japan). Cells immunoreactive for c-kit were visualized with Simple Stain DAB Solution (Nichirei). The sections were lightly counterstained with hematoxylin. The average number of c-kit-positive cells in a high-power field (HPF) was calculated from the total number of c-kit-positive cells in 10 HPFs of 5 mice.

Quantitative Real-time RT-PCR

Frozen tissues were immersed in RNA later (Ambion, Austin, TX, USA) at 4˚C overnight. Total RNA was extracted from the frozen tissues, whole spleen cells and $CD4^+$ T cells by using ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan) and reverse transcribed to produce cDNA with a PrimeScript RT kit (Takara Bio Inc., Otsu, Japan). The expression of Th1-, Th2- and Treg-type cytokine mRNA was examined by SYBR Green-based quantitative real-time RT-PCR with 40 cycles of 5 seconds of denaturation at 95˚C, 30 seconds of annealing at 64˚C and 15 seconds of dissociation at 95˚C with a TP800 Thermal Cycler (Takara Bio Inc.). The amount of PCR products was analyzed by the standard curve method and calculated by software (Multiplate RQ) with β-actin cDNA as an internal control. The following oligonucleotides were used as primers for PCR: 5'-ACC-GTT-TAA-CCA-GAA-CGT-TGA-ATT-G-3' (sense) and 5'-TCC-ACG-AAT-TTG-GAC-AGG-TTT-ACT-C-3' (antisense) for the *IL-3* gene, 5'-GGA-TGT-GCC-AAA-CGT-CCT-CAC-AGC-3'(sense) and 5'-GCT-TAT-CGA-TGA-ATC-CAG-GC-ATC-G-3' (antisense) for the *IL-4* gene, 5'-AAT-TCC-TGT-AGC-GCA-GGC-TGG-3' (sense) and 5'-TAC-TTG-AGA-CCC-TGA-TGC-AAC-G-3' (antisense) for the *IL-5* gene, 5'-CTG-AAG-ACC-CTC-AGG-ATG-CGG-CT-3' (sense) and 5'-TCT-AGG-TCC-TGG-AGT-CCA-GCA-GAC-3' (antisense) for the *IL-10* gene, 5'-GCT-CAT-GGC-TGG-TGC-AAA-GA-3' (sense) and 5'-GAG-ACG-CCA-TTC-CAC-ATG-TCA-3' (antisense) for the *IL-12β* gene, 5'-CGG-CAC-AGT-CAT-TGA-AAG-CCT-A-3' (sense) and 5'-GTT-GCT-GAT-GGC-CTG-ATT-GTC-3' (antisense) for the *interferone* (*IFN)-γ* gene, 5'-CAA-TTC-CTG-GCG-TTA-CCT-TGG-3' (sense) and 5'-TGG-AGT-TTG-TTA-TCT-TTG-CTG-TCA-C-3' (antisense) for the *TGF-β* gene and 5'-CAT-CCG-TAA-AGA-CCT-CTA-TGC-CAA-C-3' (sense) and 5'-ATG-GAG-CCA-CCG-ATC-CAC-A-3' (antisense) for the *β-actin*

gene. The PCR products were confirmed by electrophoresis in 0.7% agarose gel and staining with ethidium bromide.

Statistical analysis

Data are summarized as means ± standard deviations (SDs). Comparisons between two groups and among three groups were made with the independent *t*-test and one-way analysis of variance, followed by the post hoc pairwise independent *t*-test, respectively. The multiplicity in pairwise comparisons was corrected by Bonferroni's procedure. All probability values are 2-sided, and values of $P<0.05$ were considered to indicate statistical significance. Statistical analysis was performed using the SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA).

Results

Establishment of an allergic model

BALB/c ByJ or BALB/c Cr mice were sensitized twice by i.p. injection of cOVA (100 µg) prior to repeated intragastric challenges with a high dose of cOVA (100 mg) (Fig. 1A). Acute diarrhea occurred in both strains, but BALB/c ByJ mice were found to be less sensitive to lethal anaphylactic shock than BALB/c Cr mice (data not shown). BALB/c ByJ mice were chosen for the following experiments.

BALB/c ByJ mice sensitized with cOVA developed acute diarrhea after the 4th or 5th challenge with cOVA (Fig. 1B). The diarrhea usually occurred within 30 min after each challenge and lasted for at least 1 h (Fig. 1C). Mice also showed systemic manifestations, such as hypothermia, less movement and hair ruffles. The ceca and colons removed from these mice were severely edematous and hyperemic and contained watery soft feces (Fig. 1C). In contrast, no control mice sham sensitized with PBS and challenged with cOVA developed diarrhea and systemic manifestations during the experimental period (Fig. 1B). The ceca and colons removed from control mice showed no edematous and hyperemic changes and contained solid feces (Fig. 1C). These results indicate that acute diarrhea resulted from immune reactions to cOVA sensitization but not from an osmotic load and/or nonspecific irritation in the digestive tract. In addition, the allergic reaction was induced, without an additional exogenous adjuvant, in mice sensitized with cOVA and challenged with cOVA. Mice sensitized with 100 µg cOVA, hereafter called allergic mice, are simple to produce and useful for studying food allergic diseases.

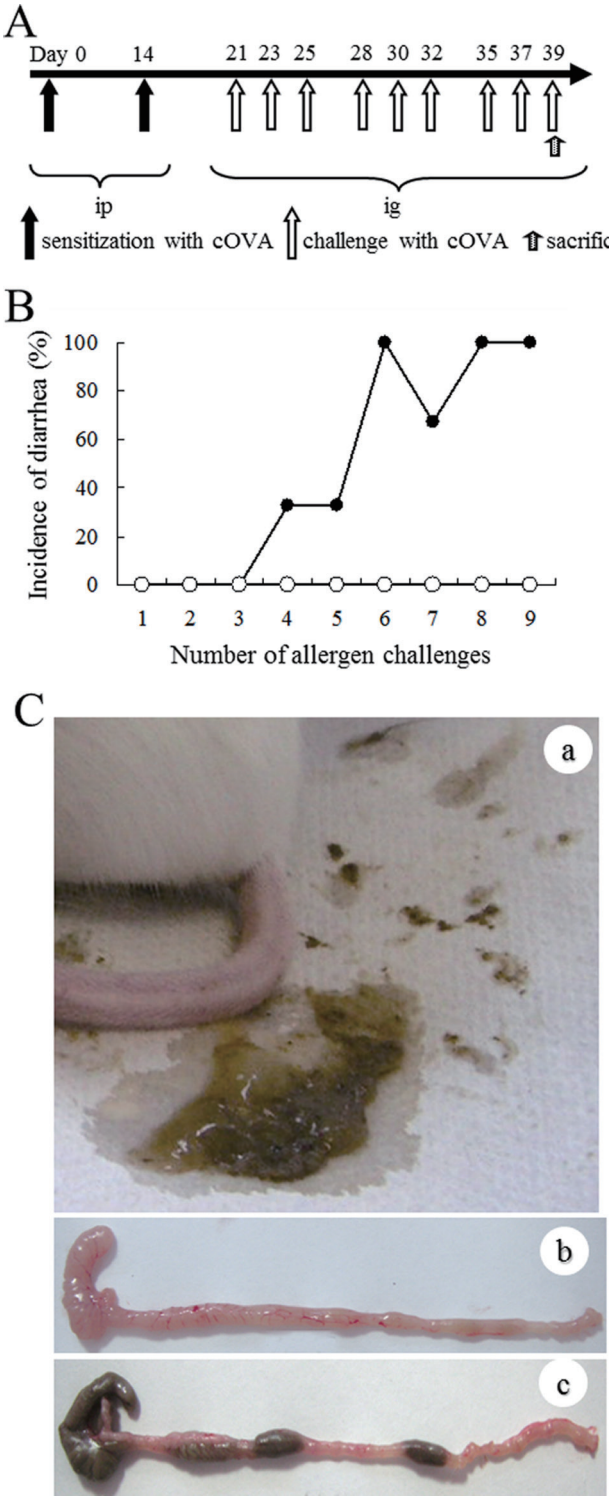

Fig. 1. Establishment of an allergic model. (A) The experimental protocol for induction of allergy in mice. BALB/c ByJ or BALB/c Cr mice were sensitized twice by i.p. injection of cOVA (100 µg) at a 2-week interval (black arrows) and then challenged by intragastric administration (ig) of cOVA (100 mg) on the indicated days (white arrows). Mice were sacrificed within 1 h after the last challenge (dotted arrow). Mice sensitized with PBS were used as a control. (B) Incidence of diarrhea. cOVA-sensitized (closed circle) (n=8) and PBS control BALB/c ByJ mice (open circle) (n=3) were monitored for 1 h, and diarrhea occurrence was assessed for 15 - 60 min after each challenge with cOVA. Similar results were obtained in 5 experiments carried out in this study, and the representative results are shown. (C) Allergic reactions of the alimentary tract. (a) Mice sensitized with cOVA showed severe mucous diarrhea within 30 min after challenge with cOVA. (b) The ceca and colons removed from cOVA-sensitized, cOVA-challenged mice showed severe edematous and hyperemic changes and contained watery soft feces after repeated oral challenges with cOVA, and (c) those from control mice showed no edematous and hyperemic changes and contained solid feces.

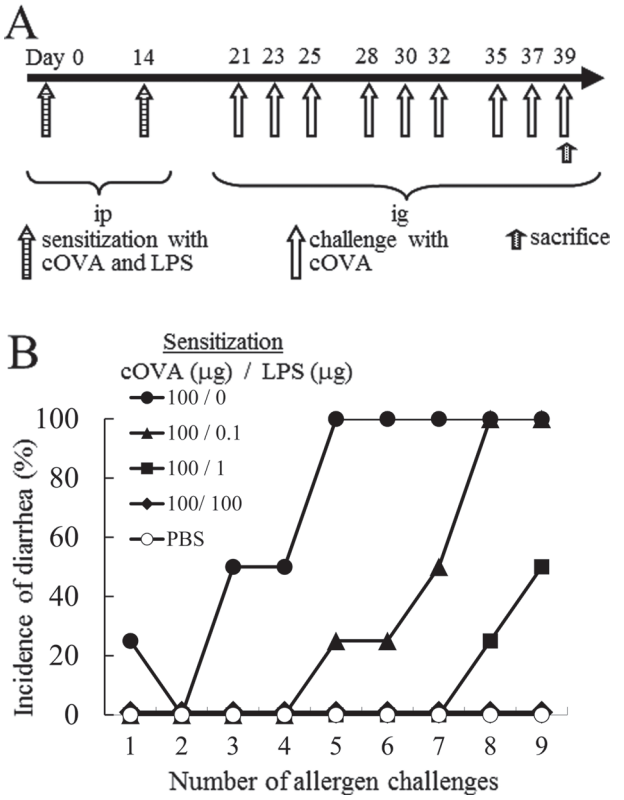

Fig. 2. Prevention of diarrhea by simultaneous sensitization with cOVA and LPS. (A) The experimental protocol for induction of LPS-mediated tolerance in allergic mice. Mice were sensitized by simultaneous i.p. injection of cOVA (100 μg) and various doses of LPS (striped arrows) twice and repeatedly challenged by the intragastric administration (ig) of cOVA (100 mg) on the indicated days (white arrows). Mice were sacrificed within 1 h after the last challenge (dotted arrow). Allergic mice, sensitized with cOVA alone, were used as a positive control, and PBS-sensitized mice were used as a negative control. (B) Prevention of diarrhea. The incidence of diarrhea decreased with increasing doses of LPS (▲ 0.1 μg (n=4) ■ 1 μg (n=4) and ♦ 100 μg (n=4)). The presence of LPS (100 μg) completely abrogated diarrhea. Mice sensitized with cOVA alone and those sensitized with PBS are indicated by closed (● (n=4)) and open (○ (n=3)) circles, respectively. Similar results were obtained in 5 experiments carried out in this study, and representative results are shown.

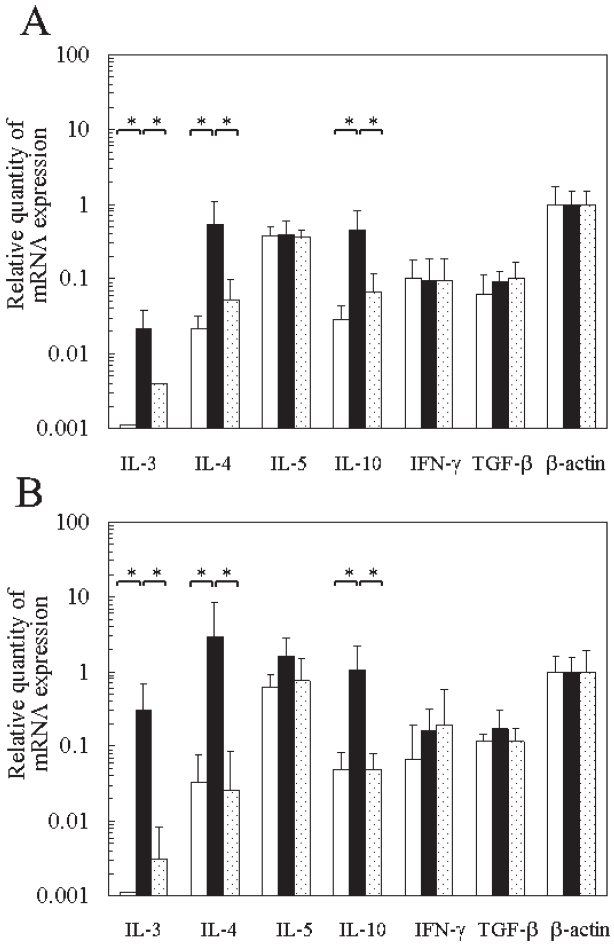

Fig. 3. The expression of Th1-, Th2- and Treg-type cytokine mRNAs in duodenal and colonic tissues. Th1-, Th2- and Treg-type cytokine mRNAs were measured by quantitative real-time RT-PCR in duodenal (A) and colonic (B) tissues removed from control mice (white columns), allergic mice (black columns) and tolerant mice (dotted columns). Each value of mRNA expression was calibrated to that of β-actin expression. Each bar represents the mean ± SD of 5 control mice, 5 tolerant mice and 6 allergic mice. Significant differences (*P < 0.05) were found between columns by the independent *t*-test with Bonferroni's correction for pairwise comparisons. In control mice, IL-3 mRNA was below the level of detection. Similar results were obtained in 3 experiments, and representative results are shown.

Establishment of a tolerant model

Mice were simultaneously sensitized with a constant dose of cOVA (100 μg) and various doses of LPS prior to cOVA challenges (Fig. 2A). The incidence of diarrhea decreased with increasing doses of LPS, and the presence of 100 μg LPS completely abrogated diarrhea (Fig. 2B). These results indicate that the presence of a high dose of LPS during cOVA sensitization converted the allergic reactions to tolerant ones against a dietary antigen even if mice were repeatedly challenged with cOVA subsequently. The mice sensitized with 100 μg cOVA and 100 μg LPS, hereafter called the tolerant mice/model, are useful for studying the mechanism by which tolerance to a food allergy is induced in the digestive tract.

Cytokine profiles in the intestinal tissues

To ascertain the involvement of the Th2 immune response in the allergic model, we examined the mRNA expression of Th1-, Th2- and Treg-type cytokines in duodenal and colonic tissues of control, allergic and tolerant mice by quantitative real-time RT-PCR (Fig. 3A and B). The relative expression of IL-3, IL-4 and IL-10 in allergic mice was higher than that in control mice. On the other hand, the levels of IL-4 and IL-10 were almost the same in control and tolerant mice. The IL-5, IFN-γ and TGF-β expression levels were almost equal among these three types of mice. These

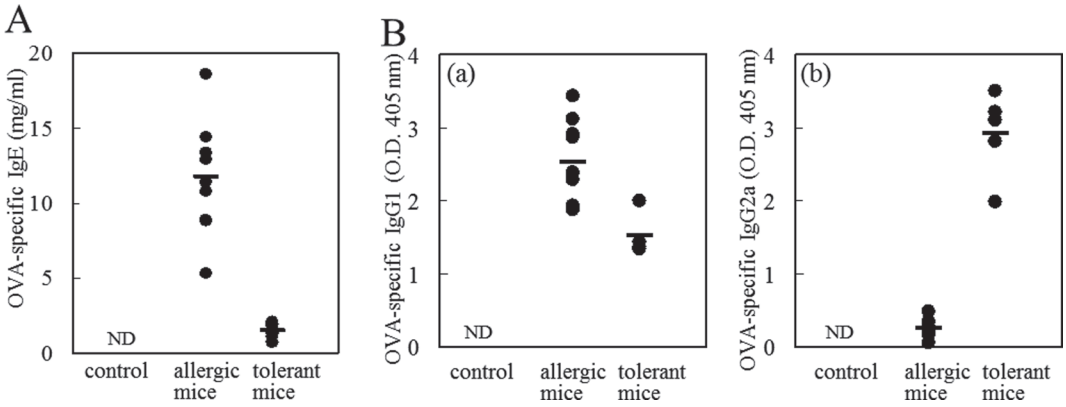

Fig. 4. Production of OVA-specific antibodies in allergic mice. Blood samples were obtained from PBS-sensitized control, allergic and tolerant mice within 1 h after the last challenge, and their sera were prepared. (A) Increases in OVA-specific IgE levels in allergic mice. Serum levels of OVA-specific IgE were determined by ELISA. Each dot indicates the OVA-specific IgE concentration in one mouse. We used control (n=5), allergic (n=10) and tolerant (n=8) mice. (B) Increases in OVA-specific IgG1, but not IgG2a, levels in allergic mice. The titers of OVA-specific IgG1 (a) and IgG2a (b) in sera were determined by ELISA. Each dot indicates the values of OVA-specific IgG1 and IgG2a in one mouse. We used control (n=5), allergic (n=10) and tolerant (n=5) mice. Lines depict mean values. ND, not detectable.

results indicate that enhanced expression of Th2 cytokines, IL-3 and IL-4 and IL-10, contributes to the induction of acute diarrhea in allergic mice and that downregulation of these cytokines prevents acute diarrhea in tolerant mice. In addition, it appears that IL-5, IFN-γ and TGF-β are not involved in the induction of diarrhea in allergic mice and the prevention of diarrhea in tolerant mice.

OVA-specific antibodies in cOVA-challenged allergic and tolerant mice

We examined serum levels of OVA-specific IgE and serum titers of OVA-specific IgG1 and IgG2a in control, allergic and tolerant mice by ELISA. The concentrations of OVA-specific IgE were significantly higher in allergic mice (11.7 mg/ml) than in tolerant mice (1.5 mg/ml) (Fig. 4A). The titers of OVA-specific IgG1 were undetectable, 2.53 at an OD of 405 nm and 1.51 at an OD of 405 nm in control, allergic and tolerant mice, respectively (Fig 4B(a)). The titers of OVA-specific IgG2a were undetectable, 0.25 at an OD of 405 nm and 2.92 at an OD of 405 nm in control, allergic and tolerant mice, respectively (Fig. 4B(b)). Thus, the levels of OVA-specific IgG1 were significantly higher in allergic mice than in control and tolerant mice, and the levels of OVA-specific IgG2a were markedly reduced in allergic mice as compared with tolerant mice. Additionally, the level of OVA-specific IgG1 was significantly higher than that of OVA-specific IgG2a, suggesting that OVA-specific

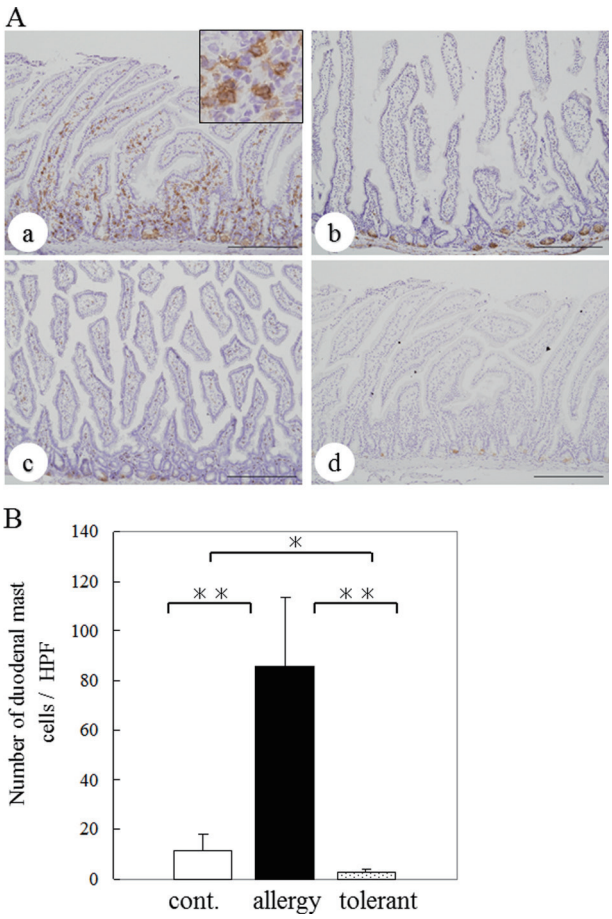

Fig. 5.

Fig. 5. Involvement of mast cells in allergic and tolerant mice. (A) Duodenal sections of allergic mice, tolerant mice and control mice were immunostained with anti-c-*kit* antibody (ACK2). Numerous mast cells infiltrated into the duodenal mucosa of allergic mice (a), but mast cells were hardly detectable in the duodenal mucosa of tolerant mice (b) and control mice (c). The secondary antibody does not show a nonspecific reaction (d). Scale bars represent 200 μm. (B) Increase of mast cells in allergic mice. The numbers of mast cells in the duodenal mucosa of allergic (n=5), tolerant (n=5) and control mice (n=5) were counted under a light microscope. Each bar indicates the mean ± SD of 5 mice. Significant differences (*P < 0.01; **P < 0.005) were found between columns by the independent *t*-test with Bonferroni's correction for pairwise comparisons.

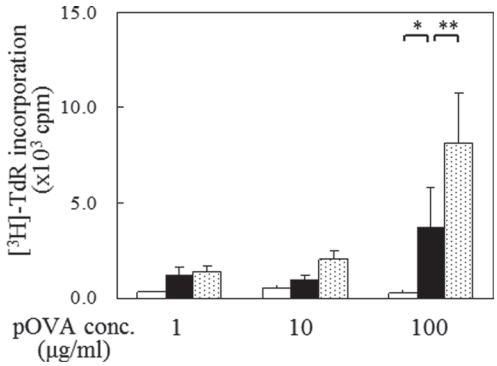

Fig. 6. Proliferative ability for OVA stimulation of MLN CD4$^+$ T cells from allergic and tolerant mice. MLNs were removed from control mice (n=5) (white columns), allergic mice (n=5) (black columns) and tolerant mice (n=5) (dotted columns) 1 week after the second sensitization. MLN CD4$^+$ T cells were purified by magnetic separation and co-cultured with APCs from BALB/c ByJ mice in the presence of the indicated concentration of pOVA. Proliferative ability of MLN CD4$^+$ T cells was examined with a [^{3}H]-thymidine incorporation assay (cpm). Each bar represents the mean ± SD of 5 mice. Significant differences (*P < 0.05; **P < 0.01) were found between columns by the independent *t*-test with Bonferroni's correction for pairwise comparisons. Similar results were obtained in 3 experiments, and representative results are shown.

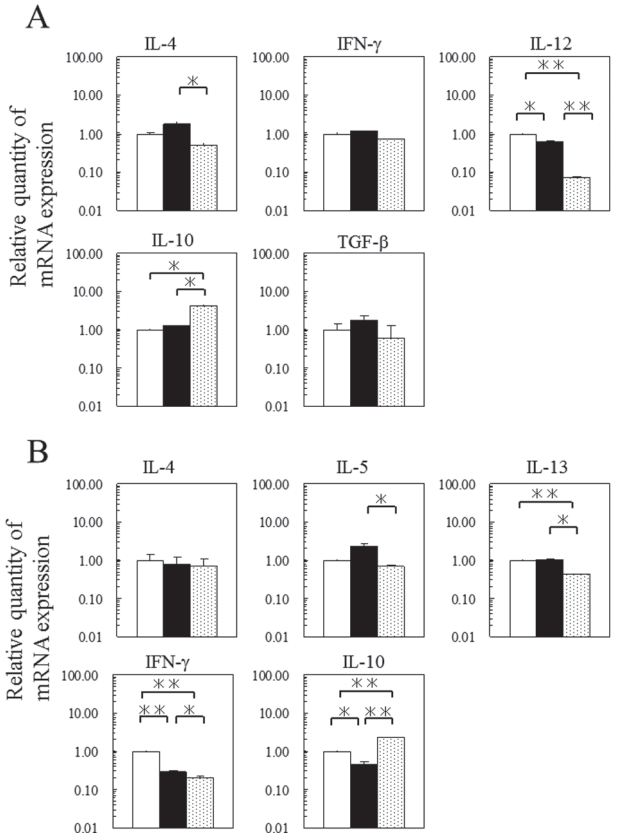

Fig. 7. The expression of Th2-, Th1- and Treg-type cytokine mRNA in whole spleen cells and spleen CD4$^+$ T cells. Spleens were removed from control mice (white columns), allergic mice (black columns) and tolerant mice (dotted columns) 4 days after the second sensitization. Total RNA was extracted from whole spleen cells (A) and purified CD4$^+$ T cells (B) and converted to cDNA. Expression of cytokines at the mRNA level was examined by quantitative real-time RT-PCR. Each value of mRNA expression was calibrated to that of β-actin expression. Each bar represents the mean ± SD of 3 mice. Significant differences (*P < 0.05; **P < 0.01) were found between columns by the independent *t*-test with Bonferroni's correction for pairwise comparisons. Similar results were obtained in 3 experiments, and representative results are shown.

IgG1 was predominantly produced in allergic mice. These data indicate that IgE-mediated Th2 immune responses participate in the induction of food allergy in mice sensitized with cOVA.

Mast cells in cOVA-challenged allergic and tolerant mice

Mast cells are known to be effectors for allergic reactions. Immunostaining with an antibody to c-kit, a marker of mast cells, showed that numerous c-kit-positive mast cells infiltrated into the duodenal mucosa of allergic mice (Fig. 5A). In contrast, c-kit-positive mast cells were hardly detectable in the duodenal mucosa of tolerant and control mice (Fig. 5A). The secondary antibody did not show a nonspecific reaction. The number of mast cells in the duodenal mucosa was significantly increased in allergic mice (Fig. 5B). Proliferation of mast cells in allergic mice was also observed in the colonic mucosa (data not shown). These results indicate that the acute diarrhea observed in allergic mice results from extensive proliferation of intestinal mast cells and that tolerance is induced by suppressing mast cell proliferation.

Antigen-specific responses of MLN CD4$^+$ T cells in the allergic and tolerant models

To examine whether the tolerant reaction against a dietary antigen is caused by anergy and/or apoptosis of primed T cells in tolerant mice, we compared the proliferative ability of MLN CD4$^+$ T cells in response to pOVA stimulation between allergic and tolerant mice. The level of [^{3}H]-thymidine incorporation of CD4$^+$ T cells from tolerant mice was similar to that from allergic mice at a low dose of pOVA, albeit higher than that from allergic mice at a high dose of pOVA (Fig. 6). In addition, there was no apparent difference in the number of MLN lymphoid cells and their viability between allergic and tolerant mice (data not shown). These results indicate that abrogation of the allergic reaction in the tolerant mice is not due to the unresponsiveness of CD4$^+$ T cells to OVA restimulation and the decrease in MLN cells caused by the apoptotic mechanism.

Cytokine profiles in the spleen

Whole spleen cells were prepared from control, allergic and tolerant mice, and the expression of Th1-, Th2- and

Treg-type cytokines was examined by quantitative real-time RT-PCR (Fig. 7A). IL-4 expression in tolerant mice was similar to that in control mice and lower than that in allergic mice. The levels of IFN-γ and TGF-β expression were comparable among control, allergic and tolerant mice. IL-12 expression was markedly lower in tolerant mice than in control and allergic mice. On the other hand, IL-10 expression was upregulated in tolerant mice as compared with control and allergic mice. Next, $CD4^+$ T cells were isolated from spleen cells of control, allergic and tolerant mice, and the expression of Th1-, Th2- and Treg-type cytokines was examined by quantitative real-time RT-PCR (Fig. 7B). IL-4 expression was comparable among control, allergic and tolerant mice. IL-5 expression in tolerant mice was similar to that in control mice and lower than that in allergic mice. IL-13 expression was lower in tolerant mice than in control and allergic mice. IFN-γ expression was markedly lower in tolerant mice than in control mice and slightly lower in tolerant mice than in allergic mice. IL-10 expression was markedly upregulated in tolerant mice as compared with control and allergic mice. In addition, there was no apparent difference in TGF-β expression among control, allergic and tolerant mice (data not shown). These results showed that the cytokine profile in tolerant mice corresponded to neither a Th1- or Th2-mediated immune response, indicating that the unresponsiveness to cOVA challenge in tolerant mice is not caused by the shift from Th2 to Th1.

Effect of $CD4^+$ T cells from tolerant mice on the proliferation of OVA-specific $CD4^+$ T cells from DO11.10 mice

To examine whether $CD4^+$ T cells from tolerant mice have the capability to suppress proliferation of OVA-specific $CD4^+$ T cells, spleen $CD4^+$ T cells from naive DO11.10 mice, which contained a transgene that expressed OVA-specific TCR in T cells, were co-cultured with either spleen $CD4^+$ T cells from allergic or tolerant mice in the presence of pOVA and APCs, and a [^{3}H]-thymidine uptake assay was performed in each co-culture. [^{3}H]-thymidine incorporation in the co-culture of DO11.10 $CD4^+$ T cells with $CD4^+$ T cells from allergic mice was almost equal to that in the culture of DO11.10 $CD4^+$ T cells alone (Fig. 8A). On the other hand, [^{3}H]-thymidine incorporation was remarkably decreased when DO11.10 $CD4^+$ T cells were co-cultured with $CD4^+$ T cells from tolerant mice. In addition, the reduction of [^{3}H]-thymidine incorporation in the co-culture of DO11.10 $CD4^+$ T cells with $CD4^+$ T cells from tolerant mice was dependent on the number of $CD4^+$ T cells from tolerant mice (data not shown).

To estimate [^{3}H]-thymidine uptake by growth of only DO11.10 $CD4^+$ T cells, DO11.10 $CD4^+$ T cells were co-cultured with X ray-irradiated $CD4^+$ T cells from allergic or tolerant mice, and [^{3}H]-thymidine uptake was examined in each co-culture. The levels of [^{3}H]-thymidine incorporation in the co-culture of DO11.10 $CD4^+$ T cells with X ray-irradiated $CD4^+$ T cells from allergic and tolerant mice were lower than that in the culture of DO11.10 $CD4^+$ T cells alone, but the levels of [^{3}H]-thymidine incorporation were comparable in X ray-irradiated $CD4^+$ T cells from allergic and tolerant mice (Fig. 8B). In addition, when the levels of [^{3}H]-thymidine incorporation in co-culture were compared between nonirradiated and X ray-irradiated $CD4^+$ T cells from tolerant mice, the level was higher in the co-culture

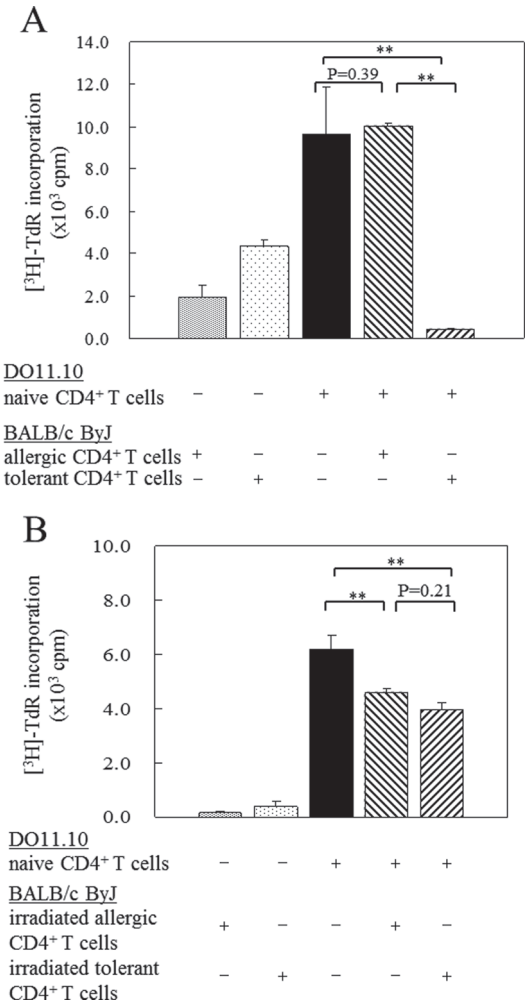

Fig. 8. Inhibition of DO11.10 $CD4^+$ T cell proliferation by $CD4^+$ T cells from tolerant mice. Naive DO11.10 spleen $CD4^+$ T cells (5×10^5) were co-cultured with nonirradiated or irradiated spleen $CD4^+$ T cells (5×10^5) from allergic or tolerant mice in the presence of pOVA (50 μg/ml) and APCs (1×10^5) from DO11.10 mice. Proliferative ability of cells was examined with a [^{3}H]-thymidine incorporation assay (cpm). (A) [^{3}H]-thymidine incorporation in the co-culture of naive DO11.10 $CD4^+$ T cells with $CD4^+$ T cells from allergic or tolerant mice. (B) [^{3}H]-thymidine incorporation in the coculture of naive DO11.10 $CD4^+$ T cells with irradiated $CD4^+$ T cells from allergic or tolerant mice. The levels of [^{3}H]-thymidine incorporation of each cell culture and co-culture as designated below the graph are shown. Each bar indicates the mean ± SD of triplicate cultures. Significant differences (*$P < 0.01$) were found between columns by the independent *t*-test with Bonferroni's correction for pairwise comparisons. Similar results were obtained in 3 experiments, and representative results are shown.

with X ray-irradiated $CD4^+$ T cells than in that with nonirradiated $CD4^+$ T cells. These results showed that viable $CD4^+$ T cells from tolerant mice are able to suppress the growth of DO11.10 $CD4^+$ T cells, suggesting that they produce a suppressor for the OVA-specific response of DO11.10 $CD4^+$ T cells.

Discussion

Food allergy is considered to be an aberrant Th2-mediated immune response against dietary antigens[1, 2]. LPS, an outer wall component of Gram-negative bacteria, is a potent activator of the immune system, and it may play a crucial role in T cell priming[5]. Recent studies have demonstrated that LPS exposure primes Th2 cells in airway allergy, but the role of LPS in food allergy has not been well investigated[12–15]. In the present study, we established two distinct mouse models: one is an IgE-mediated, Th2-type allergic model induced by cOVA sensitization alone, and the other is a tolerant model induced by sensitization with both cOVA and LPS. In the allergic model, sensitization with cOVA contaminated with a little LPS could induce Th2-mediated reactions in mice. In the tolerant model, tolerance was increased with increasing doses of LPS during sensitization, and a high dose (100 μg) of LPS completely suppressed Th2-mediated allergic reactions. These results indicate that the development of food allergy may be induced by a low dose of LPS exposure and prevented by a high dose of LPS exposure during sensitization.

It is generally accepted that Th1 priming requires TLR4 signaling. It is also thought that the priming of Th2 occurs through a default pathway at a low level of TLR signaling, but the mechanism has been poorly defined[6–11]. Regarding airway allergy, Eisenbarth *et al*. reported that a high dose of LPS exposure during sensitization induces both IL-12 production and a Th1 response, whereas a low dose of LPS exposure is not sufficient to induce a Th1 response but is required to induce Th2 inflammation[12]. In our model, an IgE-mediated, Th2-type food allergy could be induced by cOVA sensitization. Because there is a possibility that a little LPS contaminated the cOVA and influenced the induction of allergic reactions, a low dose of LPS exposure during sensitization may play a crucial role in the priming of Th2 in both airway and food allergic reactions. On the other hand, unlike airway hypersensitivity, a high dose of LPS exposure failed to induce both IL-12 mRNA expression and Th1-mediated responses in our model. The reason for this discrepancy may lie in the environmental variation between the respiratory tract, where LPS is supplied mainly from exogenous sources, and the digestive tract, where an abundance of LPS is constitutively present.

Endotoxin tolerance is a phenomenon in which cells or organisms exposed to a low dose of endotoxin enter into a transient unresponsive state and are unable to respond to further challenges with the endotoxin[32, 33]. In endotoxin tolerance, dendritic cells (DCs) and monocytes/macrophages are unresponsive to subsequent antigen stimulation by downregulating the expression of MHC class II and costimulatory molecules. It has been reported that endotoxin tolerance attenuates airway allergic inflammation through direct suppression of the T-cell stimulatory effect of DCs[34]. On the other hand, in our food allergic and tolerant models, MLN $CD4^+$ T cells from tolerant mice showed sufficient antigen responses after reexposure to the antigen. These results indicate that unresponsiveness in tolerant mice is not caused by endotoxin tolerance.

The production state of IL-12 is crucial to determine the subsequent acquired immunity[13, 30, 35]. Upregulation of IL-12 is generally related to the induction of Th1-mediated responses, and its downregulation is generally related to that of Th2-mediated responses. It is therefore thought that downregulation of IL-12 results in allergic reactions mediated by aberrant Th2 cells. However, IL-12 mRNA expression decreased markedly in spleen cells of our tolerant mice, showing that the development of Th2-mediated allergic reactions is prevented under IL-12 exhausted conditions. It is therefore likely that factors other than IL-12 participate in the suppression of Th2-mediated allergic reactions and the induction of tolerance. Our preliminary FACS analysis showed prominent decreases in $CD11_C^{high}$ DCs, which produce IL-12 and induce Th1-mediated responses, in the spleen of tolerant mice (data not shown). So, it is possible that downregulation of IL-12 mRNA expression is caused by a decrease in $CD11_C^{high}$ DCs in the spleen of tolerant mice.

Several studies have shown the crucial role of Treg in allergic diseases[20, 21, 36]. The involvement of Treg producing TGF-β, Th3, in the induction of oral tolerance and the maintenance of intestinal homeostasis has been reported[27, 28]. In our tolerant mice, upregulation of TGF-β mRNA expression was not observed. These results indicate that TGF-β does not participate in the induction of tolerance. On the other hand, the enhanced mRNA expression of IL-10, originally known as a Th2-derived cytokine synthesis inhibitory factor, was clearly identified in our tolerant mice. Groux *et al.* first reported Treg producing IL-10, Tr1, which suppress antigen-specific immune responses and actively downregulate a pathological immune response in vivo[37]. Subsequent studies have shown that Tr1 are able to inhibit the development of airway allergic inflammation in mouse models [21, 38, 39]. In a similar way, Tr1 may be induced by sensitization with cOVA and LPS and regulate the immune response to OVA in our tolerant mice. Wakkache *et al*. reported that $CD11c^{low}CD45RB^{high}$ DCs that produce IL-10 are enriched after LPS stimulation, inducing the differentiation of Tr1[40]. In addition to the decrease in $CD11_C^{high}$ DCs, our preliminary FACS analysis showed that the proportion of $CD11c^{low}CD45RB^{high}$ DCs remarkably increased in the spleen of tolerant mice (data not shown). There is a possibility that, in our tolerant mice, LPS exposure during sensitization functions as a trigger for activation of $CD11c^{low}CD45RB^{high}$ DCs, promoting Tr1 development and IL-10 production by Tr1. Our tolerant mice may be useful in study of the function of $CD11c^{low}CD45RB^{high}$ DCs and Tr1 as crucial regulators for

food allergy.

Thus, LPS is crucial for the regulation of Th2-mediated allergic reactions to dietary antigens. Understanding of the mechanisms involved in the induction of LPS-induced tolerogenic cells can potentially provide useful information for developing novel strategies to prevent food allergy or to treat allergic disease by therapeutic manipulation of the innate immune system.

Declaration of Conflicting of Interests: The authors declare that they have no conflicts of interest.

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