

Overcoming food allergy through acquired tolerance conferred by transfer of Tregs in a murine model

H. Yamashita, K. Takahashi, H. Tanaka, H. Nagai & N. Inagaki

Laboratory of Pharmacology, Department of Bioactive Molecules, Gifu Pharmaceutical University, Daigakunishi, Gifu, Japan

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Correspondence

Hirotaka Yamashita, Laboratory of Pharmacology, Department of Bioactive Molecules, Gifu Pharmaceutical University, Daigakunishi 1-25-4, Gifu 501-1196, Japan.
Tel.: +81-(0)58-237-8100
Fax: +81-(0)58-237-8105
E-mail: hyamashita@gifu-pu.ac.jp

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Abstract

Background: The number of food allergy patients is increasing. Some children outgrow their food allergies through tolerance, whereas others remain susceptible throughout their lives. We aimed to contribute to food allergy therapeutics by understanding induction of oral tolerance in a murine food allergy model.

Methods: We modified an existing murine food allergy model by using ovalbumin (OVA) to induce oral tolerance, either by pretreating mice with OVA or by transferring mesenteric lymph node (MLN) cells or T cells derived from mice treated with OVA.

Results: Pretreatment with OVA prevented food allergy, with complete suppression of OVA-specific immunoglobulin (Ig)E and IgA antibody production and interleukin (IL)-4, IL-10, and IL-9 mRNA expression. The proportion of regulatory T cells (Tregs) in MLN cells and expression of transforming growth factor- β mRNA increased. In the transfer model, anaphylaxis secondary to OVA intake was suppressed by transfer of whole MLN cells and Tregs from OVA-treated mice. However, OVA-specific IgE and IgA expressions were partially attenuated by transfer of antigen-specific and nonspecific Tregs, but not by whole MLN cells from OVA-treated mice. In the Treg transfer model, IL-4 and IL-10 mRNA expression decreased, but IL-9 mRNA expression increased.

Conclusion: We concluded that oral tolerance for food antigens is induced in two ways: (i) by initial exposure to antigen, or inherent tolerance, and (ii) by transfer of Tregs, or acquired tolerance. Because food allergies occur when inherent tolerance is absent, understanding of acquired tolerance is important for the development of therapies for food allergy.

The number of food allergy patients is increasing. The prevalence of food allergies in children in Japan is 3.6–17%; atopic dermatitis (13–18%) and asthma (2.9–14.3%) have similar incidences in children (1). Although prior studies indicated that most food allergies resolved spontaneously by 3 years of age, recent studies show that about 20% of children did not experience relief from food allergies by 3 years of age (2).

Allergies to eggs (3), milk (4), nuts (5), and wheat can result in adverse immunologic phenomena such as eczema,

diarrhea, and hypothermia; though, antigen-specific suppression of the immune system generally occurs after prior oral antigen intake. Because food allergies result from an absence of inherent mucosal tolerance to food proteins, an understanding of the mechanisms of oral tolerance could aid in the development of novel therapies for food allergies.

In this study, we modified an existing murine food allergy model (6) to improve the timing and degree of anaphylaxis and allergic diarrhea induced by antigen intake. We accomplished this goal by altering the frequency and timing of ovalbumin (OVA) administration. We confirmed that oral tolerance is induced by pretreatment with OVA in our model. Furthermore, we attempted to identify the immune cells involved in oral tolerance to food antigens by transferring lymphocytes derived from mice that were pretreated with OVA.

Abbreviations:

OVA, ovalbumin; PBS, phosphate-buffered saline; Alum, aluminum hydroxide gel; Ig, immunoglobulin; Tregs, regulatory T cells; MLN, mesenteric lymph node; HBSS, Hank's balanced salt solution; BSA, bovine serum albumin; RT-PCR, reverse transcriptase-polymerase chain reaction.

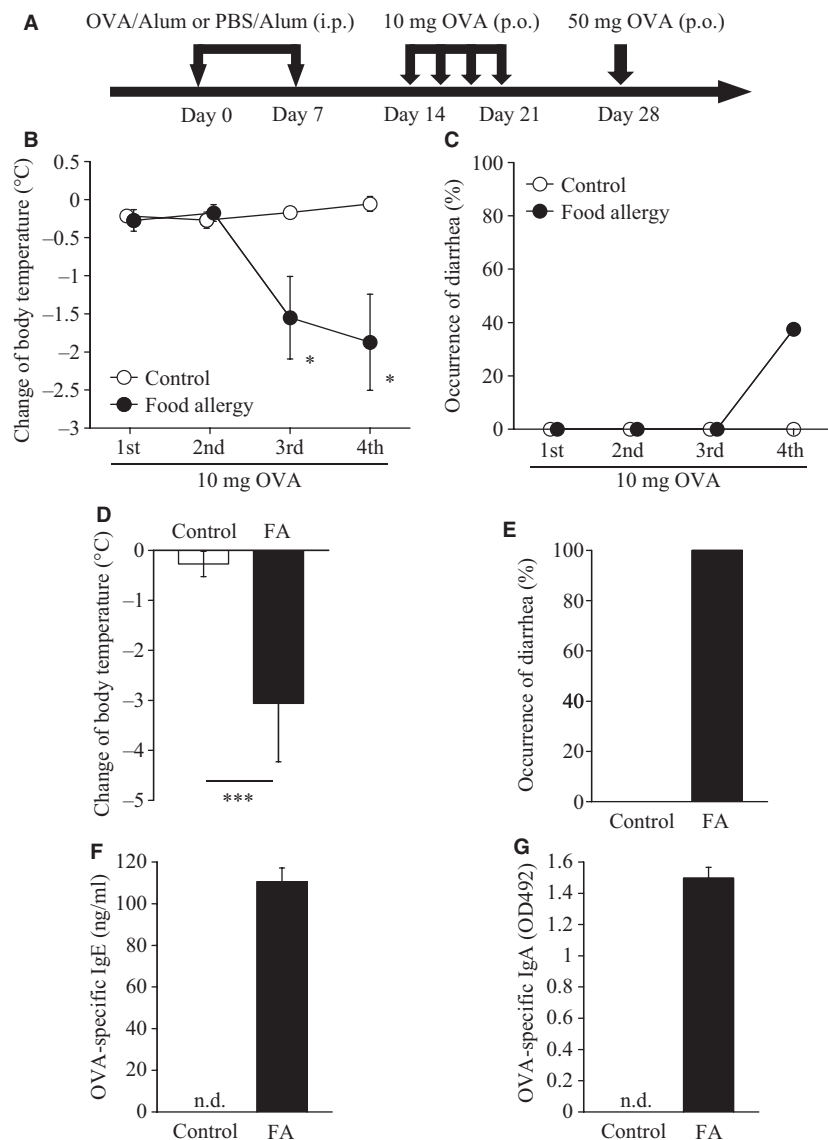


Figure 1 Characterization of food allergy model. Food allergy was induced by four oral administrations of 10 mg OVA followed by 50 mg OVA (A). Food allergy was estimated by changes in rectal temperature (B,D) and diarrhea score (C,E) in food allergy induction

by 4 × 10 mg OVA; elicitation phases: single dose of 50 mg OVA. Ovalbumin-specific IgE (F) and IgA (G) antibodies were measured by ELISA. Each value represents the mean ± SEM of seven or eight mice (* P < 0.05, *** P < 0.001 vs control; n.d., not detected).

We found that oral tolerance to food allergens is possible under two circumstances: inherent tolerance and acquired tolerance. Moreover, oral tolerance in an allergen pretreatment model differed from that in a cell transfer model. Analysis of types of tolerance may lead to the development of novel therapies for food allergies.

Methods

Murine food allergy model

Female BALB/c mice (Japan SLC Inc., Hamamatsu, Japan) were sensitized with two intraperitoneal (i.p.) injections of

1 µg OVA (Sigma-Aldrich Co., St. Louis, MO, USA) mixed with aluminum hydroxide gel (alum) each over a span of 1 week. All the mice were orally administered OVA at a dose of 10 mg/mouse dissolved in phosphate-buffered saline (PBS, p.o.) four times a week to induce food allergy (Fig. 1A). One week after the last administration, allergic responses were elicited in all of the mice through oral treatment with OVA at a dose of 50 mg/mouse dissolved in PBS.

Food allergy was evaluated by changes in rectal temperature for 1 h using a thermometer (KN-91; Natume, Tokyo, Japan) and by allergic diarrhea. Allergic diarrhea was assessed by a severity score of fecal form from 0 to 3: score 0, solid state; score 1, funicular form; score 2, slurry; score 3,

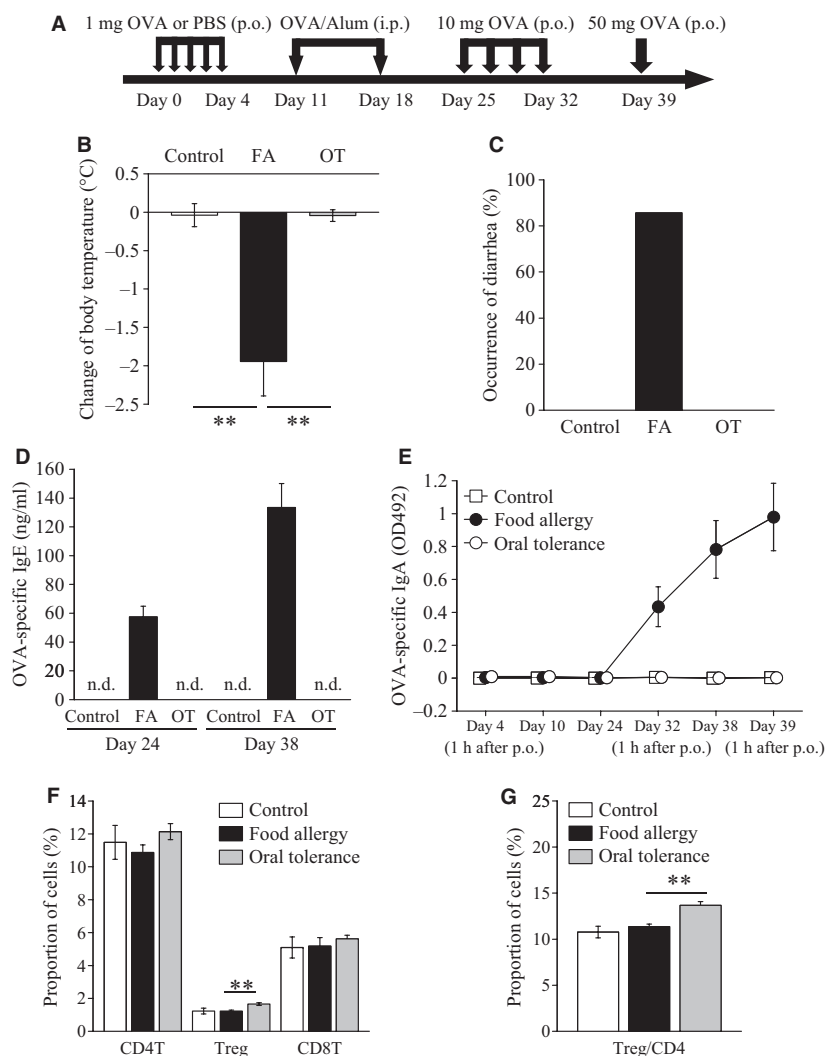


Figure 2 Evaluation of oral tolerance induced by antigen pretreatment. Oral tolerance was induced with five administrations of 1 mg ovalbumin (OVA) for 5 days before the induction of food allergy (A). Food allergy was estimated by changes in body temperature (B), diarrhea score (C), and levels of OVA-specific IgE (D) and IgA (E) antibody

ies. Proportions of CD4⁺ CD25⁺ T cells (CD4T), CD4⁺ CD25⁺ T cells (Tregs), and CD4⁺ CD8⁺ T cells (CD8T) to lymphocytes (F) or of Tregs to CD4T (G) in mesenteric lymph nodes were measured by FACS. Each value represents the mean $\pm$ S.E.M. of 5–7 mice (** P < 0.01 vs food allergy; n.d., not detected).

watery state. Criterion for scoring is shown in Fig. S1. Data were expressed as the percentage of mice with diarrhea according to the method of Brandt et al. (6). Fecal conditions with scores of 2 and 3 were defined as diarrhea.

Hematocrit value was measured as an indicator of anaphylactic shock (7). Blood was collected in heparinized capillary tubes and centrifuged for 5 min at 15 000 g (Hematocrit KH-1200S; Kubota, Tokyo, Japan). Hematocrit value was calculated as the length of packed red blood cells divided by the total length of the serum and red cell layer in the capillary tube.

Model of inherent oral tolerance in food allergy

To evaluate inherent oral tolerance, mice were pretreated with oral OVA at 1 mg/mouse every day for 5 days, before

sensitization with OVA/alum. The mice were then orally treated with OVA to evoke food allergy (Fig. 2A).

Adoptive transfer for acquired tolerance

For induction of acquired tolerance, lymphocytes isolated from mesenteric lymph node (MLN) were intravenously injected into recipient mice. Donor mice were administered OVA at a dose of 1 mg/mouse or PBS every day for 5 days. Mesenteric lymph node cells (1×10^7 cells) from donor mice, or CD4⁺ CD25⁺ T cells (regulatory T cells, Tregs, 1.7×10^5 cells), CD4⁺ CD25⁺ (CD4T, 5.6×10^5 cells) or CD4⁺ CD8⁺ cells (CD8T, 5.6×10^5 cells) sorted from 1×10^7 MLN cells by FACSaria II (BD Biosciences, Franklin Lakes, NJ, USA), were intravenously injected into recipient mice. We

then attempted to induce food allergy in recipient mice (Fig. 4A).

ELISA measurements

Ovalbumin-specific immunoglobulin (Ig)E was measured by ELISA according to previously described protocols (8, 9). Ovalbumin-specific IgA in sera and feces was measured after IgE detection with the following modifications: for measurement of specific IgA, anti-mouse IgA (STAR137; Serotec Ltd., Oxford, UK) was used as a capture antibody instead of anti-mouse IgE (MCP419; Serotec). Fecal IgA was measured from supernatant of feces suspended in PBS containing 0.1% sodium azide. IgA levels were measured by absorbance at 492 nm, with blank absorbance subtracted.

Interleukin (IL)-9 protein levels in sera were measured using an ELISA MAX™ Set Deluxe kit (BioLegend, San Diego, CA, USA).

Amounts of mouse mast cell protease-1 (MMCP-1) as an indicator of mucosal mast cell degranulation (6) were measured in sera collected after challenge with 50 mg OVA by ELISA READY-SET-GO! kit (eBioscience Inc., San Diego, CA, USA) according to manufacturer's instruction.

Flow cytometric analyses

Mesenteric lymph node cells were suspended in Hank's balanced salt solution (HBSS) containing 1% bovine serum albumin. Mesenteric lymph node cells were stained with FITC-labeled CD3 (clone 145-2C11) and PE-labeled CD25 (clone 7D4) from Beckman Coulter Inc. (Fullerton, CA, USA), APC-Cy7-labeled CD4 (clone L3T4) from BD Biosciences, and PE-Cy5-labeled CD8 α (clone 53-6.7) from eBioscience Inc. CD4⁺ CD25⁺ cells (Tregs), CD4⁺ CD25⁻ cells, and CD4⁻ CD8⁺ cells were sorted from the stained MLN cells and analyzed by FACSaria II, with purities more than 95%.

mRNA expression

mRNA levels in jejunum and MLNs were measured by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Cytokine expression was analyzed by Thermal Cycler Dice (Takara Bio Inc., Ohtsu, Japan), using glyceraldehyde-3-phosphate dehydrogenase (GADPH) expression as the standard.

Statistical analyses

The statistical significance of the difference between values was evaluated using Student's or Welch's *t*-tests. When the *P* value was <0.05, the difference was considered significant.

Results

Timing of induced food allergy is consistent in the modified model

Mice injected with OVA/alum (food allergy, FA) or PBS/alum (control) was orally administered OVA at a dose of 10 mg/

mouse four times (Fig. 1A). A gradual decrease in body temperature and an increase in diarrhea were induced in OVA-sensitized mice (Fig. 1B,C). One week after the last administration of 10 mg OVA, the mice were administered a single dose of 50 mg OVA. Compared to the control group, decreased body temperature and watery diarrhea were induced in the FA group (Fig. 1D,E). Ovalbumin-specific IgE and IgA antibodies were produced in the FA mice (Fig. 1F,G).

The rectum temperatures at multiple points (0, 15, 30, 45, 60, 75, 90, 105, 120, 135, and 180 min after the 50-mg OVA challenge) in FA mice and oral tolerance mice (dosed according to the protocol for Fig. 2A) are shown in Fig. S2. In our model, the peak of hypothermia was between 45 and 60 min after challenge.

Oral pretreatment with OVA prevents anaphylaxis

Mice were given 1 mg OVA serially for 5 days for induction of oral tolerance (Fig. 2A). Decrease in body temperature and induction of diarrhea following 50-mg OVA intake were not observed, and OVA-specific IgE and IgA were not elevated in this group (the oral tolerance group, OT group) as compared to the food allergy group (FA group; Fig. 2B–D).

Mesenteric lymph node cells collected after challenge were homogenized in HBSS and analyzed by flow cytometry. The proportions of CD4⁺ CD25⁻ (CD4T) and CD8⁺ T cells were not affected by induction of food allergy and oral tolerance, but the proportion of CD4⁺ CD25⁺ T cells (Tregs) of total cells or of CD3⁺ CD4⁺ cells increased in cases of induced oral tolerance (Fig. 2F,G). Flow cytometry diagrams are shown in Fig. S3.

Expression of interferon- γ (IFN- γ), IL-4, IL-5, IL-9, and IL-10 increased, while expression of transforming growth factor- β (TGF- β) decreased in the jejunums of mice in which food allergy was induced, as compared to control mice (Fig. 3). In mice with induced oral tolerance, expressions of IL-4, IL-9, and IL-10 were suppressed by pretreatment with OVA. There was no difference in IFN- γ expression between food allergy and tolerant mice, and TGF- β expression increased with oral pretreatment with antigen (Fig. 3).

Hematocrit was calculated as an indicator of anaphylactic shock. MMCP-1 levels in sera collected 1 h after challenge with 50 mg OVA were measured as indicators of mucosal mast cell degranulation (6). In the FA group, MMCP-1 levels and hematocrit values were elevated compared with control and OT groups (Fig. S4A,B).

Food allergy is suppressed by transfer of lymphocytes from OVA-treated mice

To analyze oral tolerance in food allergy, MLN cells derived from donor mice that were orally administered 1 mg OVA or vehicle (PBS) serially for 5 days were injected into recipient mice (Fig. 4A). In the recipient mice to which lymphocytes from OVA-treated or PBS-treated mice were transferred, anaphylaxis after OVA intake was suppressed (Fig. 4B,C). There was no difference in the levels of OVA-specific IgE and IgA in sera from mice injected with MLN cells obtained from

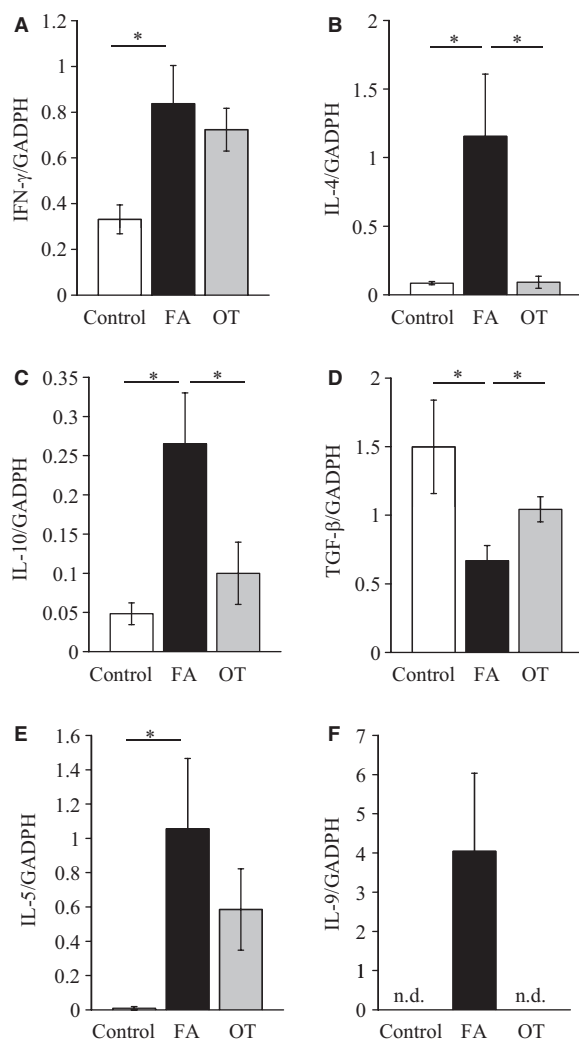


Figure 3 Expression of cytokines in the induction of oral tolerance by antigen pretreatment. Interferon- γ (A), IL-4 (B), IL-10 (C), TGF- β (D), IL-5 (E), and IL-9 (F) mRNA expressions in the jejunum were measured by real-time RT-PCR. Results were normalized to GAPDH mRNA expression. Each value represents the mean $\pm$ SEM of 5–7 mice (* P < 0.05 vs food allergy; n.d., means not detected).

OVA-treated mice (OVA) or vehicle-treated mice (PBS; Fig. 4D,E).

Tolerance by transfer is dependent on Tregs

Mesenteric lymph node cells were sorted into CD4⁺ CD25⁺ T cells (Tregs), CD4⁺ CD25[−] T cells (CD4T), and CD8⁺ CD4[−] T cells (CD8T) to elucidate the cells associated with oral tolerance. Cells of each type (including whole MLN cells) were transferred to recipient mice. A decrease in body temperature was suppressed by transfer of Tregs, but not by transfer of CD4⁺ or CD8⁺ T cells from the OVA- or PBS-treated mice (Fig. 5A). Diarrhea was also inhibited by injection of Tregs derived from OVA-treated mice (Fig. 5B).

Ovalbumin-specific IgE and IgA levels were attenuated by injection of Tregs, but IgE and IgA levels did not significantly differ between the mice in which whole MLN cells were transferred from OVA- and PBS-treated mice (Fig. 5 C,D). Transfer of cells did not affect mRNA expression of IFN- γ in the jejunum (Fig. 6A). IL-4 and IL-10 expressions were downregulated by injection of whole cells derived from OVA-treated mice, compared to PBS-treated mice, and significantly downregulated by injection of Tregs derived from the PBS- or OVA-treated mice (Fig. 6B,C). Transforming growth factor- β expression tended to be upregulated by transferring whole cells derived from OVA-treated mice and was significantly increased after transfer of Tregs derived from the OVA-treated mice (Fig. 6D). Levels of IL-9 mRNA and protein were decreased when whole cells were transferred, but significantly elevated when Tregs were transferred from OVA-treated mice (Fig. 6E,F).

Hematocrits were decreased after transfer of whole MLN cells from OVA-treated mice, and each transfer of Tregs (Fig. S4C). In contrast, MMCP-1 levels were elevated after each transfer of Tregs (Fig. S4D).

Discussion

We modified the frequency and timing of administration of food antigen in an existing murine food allergy model (6), because anaphylaxis was not consistently induced in all mice. We consistently induced allergic diarrhea and decreased rectal temperatures in our model through challenge with 50 mg OVA in mice. Moreover, OVA-specific IgE and IgA levels increased, and both Th1 and Th2 inflammatory cytokine expressions increased after induction of food allergy.

Food allergy results from an absence of oral tolerance, which is a lack of immune response of T cells and B cells to dietary antigens introduced through the oral route (10–13). We thus evaluated the induction of oral tolerance in our model of food allergy. We confirmed that pretreatment with oral OVA effectively prevented the decreases in body temperature, allergic diarrhea, and hematocrit value elevation associated with anaphylactic response in food allergy. Moreover, production of OVA-specific IgE antibodies was completely inhibited. We defined oral tolerance resulting from the pretreatment of food antigen as inherent tolerance.

In the adoptive transfer model, although body temperature decrease and allergic diarrhea were inhibited by transfer of MLN cells isolated from mice orally pretreated with OVA, IgE and IgA levels in the sera remained high, consistent with transfer of cells from the PBS-treated mice. Furthermore, transferring Tregs isolated from MLN in both OVA- and PBS-treated mice suppressed anaphylactic responses. We defined oral tolerance resulting from the transfer of Tregs as acquired tolerance.

It is known that immune suppression through oral tolerance in allergies depends on IL-10 or TGF- β , which are produced by CD4⁺ CD25⁺ T cells (14, 15). Transforming growth factor- β is one of the primary molecules that induces

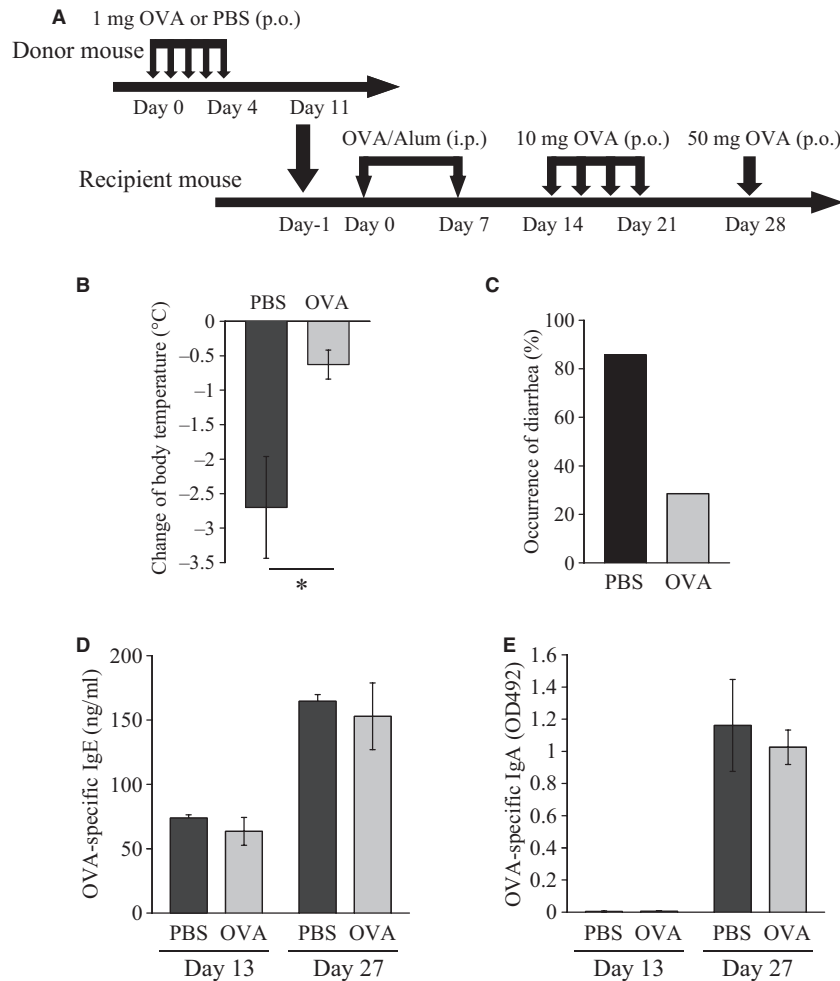


Figure 4 Analysis of oral tolerance by transfer of mesenteric lymph node (MLN) cells isolated from ovalbumin (OVA)-treated mice. Mesenteric lymph node cells from donor mice were transferred to recipient mice to induce food allergy (A). Food allergy was evaluated by changes in body temperature (B) and diarrhea

score (C) in the recipient mice. Ovalbumin-specific IgE (D) and IgA (E) antibodies were measured by ELISA. Each value represents the mean $\pm$ SEM of six or seven mice (* P < 0.05 vs the group transferred with MLN cells derived from OVA-treated mice).

and maintains Tregs (16, 17). Secreted and cell surface-associated forms of TGF- β suppress activation of effector T cells in intestinal inflammation (18, 19). Because mRNA expression of IL-10 decreased and TGF- β increased both in the model of oral tolerance by pretreatment with food antigen and with transferred Tregs, TGF- β producing Tregs or Tregs induced and maintained by TGF- β might play a role in oral tolerance and food allergy.

IgA is an important regulatory factor in the mucosal immune system. IgA is secreted by plasma cells and reacts with antigens upon exposure of mucosal surfaces in the intestine to pathogens and food antigens (20). In our model, OVA-specific IgA was produced in the sera after oral administration of OVA, but not after i.p. injection of OVA/alum alone (Fig. 2E, from day 24–32). Additionally, specific IgA injection systematically inhibited anaphylactic responses induced by ingested food antigen (21). Therefore, IgA against food antigens is produced for protection against food allergy.

However, serum IgA expression in a model of inherent tolerance was completely suppressed, whereas expression was partially suppressed by transfer of specific and nonspecific Tregs in a model of acquired tolerance. We assume that food proteins are not recognized as antigens in inherent tolerance and that immunoglobulins against the proteins are not elevated by oral administration or intraperitoneal injection. Therefore, suppressions of IgE and IgG, rather than IgA, contribute to inherent tolerance. In acquired tolerance, the recipient mice recognized food proteins as antigens, so immunoglobulins for these proteins were induced and then partially suppressed by inhibitory Tregs. Both suppression of IgE and induction of IgA might therefore be important to acquired tolerance.

Local IgA in feces of food allergy mice is lower than that of immunotolerant mice, while systematic IgA in sera is greater (22). In contrast, IgA expression in feces as well as in sera was significantly lower in tolerant mice sensitized by OVA with cholera toxin (23). Previously, we found a

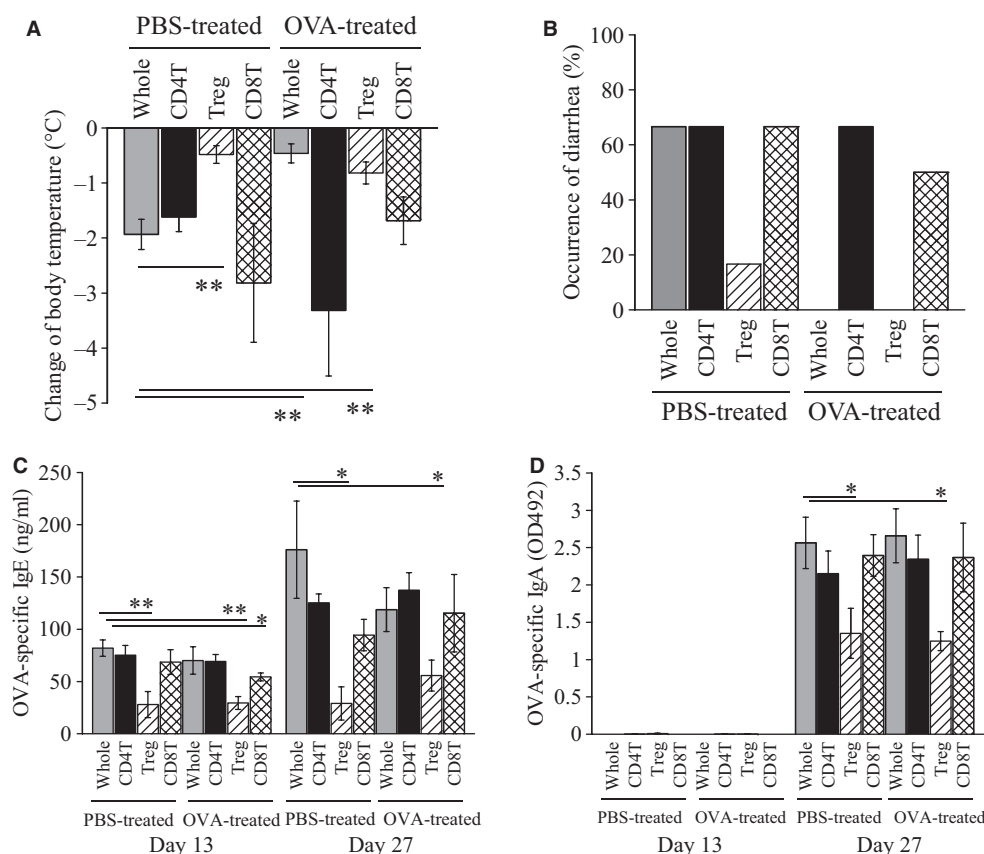


Figure 5 Search for the pivotal cells involved in oral tolerance acquired by transfer of sorted T cells. CD4⁺ CD25⁺ T cells (CD4T), CD4⁺ CD8⁺ T cells (CD8T), and CD4⁺ CD25⁺ T cells (Treg) were sorted from mesenteric lymph node (MLN) cells in donor mice and transferred to recipient mice in an attempt to induce food allergy. Food allergy was evaluated by changes in body temperature (A),

diarrhea score (B), and ovalbumin (OVA)-specific IgE (C) and IgA (D) antibodies in the recipient mice. Each value represents the mean $\pm$ SEM of six mice (phosphate-buffered saline [PBS]- or OVA-treated refers to mice transferred with MLN cells from PBS- or OVA-treated mice, respectively; * P < 0.05, ** P < 0.01 vs whole cells from PBS-treated mice).

correlation between IgA in sera and feces (unpublished data) and confirmed that the fecal IgA level in inherently tolerant mice did not increase (data not shown). We therefore propose that the serum IgA level reflects a local IgA levels.

It is known that IL-9 is an important cytokine involved in the development of oral antigen-induced intestinal anaphylaxis through mast cell differentiation and degranulation, and allergic diarrhea is attenuated in IL-9 knockout mice (24, 25). Inhibition of IL-9 production and MMCP-1 release was thereby strongly involved in the suppression of anaphylaxis through antigen ingestion in inherent tolerance.

In contrast, mRNA expression of IL-9 in the jejunum and MLNs (data not shown) and protein levels in sera were elevated after transfer of Tregs. Because IL-9 produced by CD4⁺ CD25⁺ Tregs has immunosuppressive effects on allograft tolerance owing to migration of mast cells (26), high levels of IL-9 released from Tregs might lead to the inhibitory effects in cases of acquired tolerance in food allergy. MMCP-1 levels in the sera also elevated by transfer of Tregs. However, parenchymal responses, such

as hypothermia, allergic diarrhea, and hematocrit value, were suppressed. Therefore, we assume that Tregs associated with IL-9 production indirectly affect acquired tolerance through differentiation and degranulation of particular mast cells.

The results of transferring whole cells did not correspond perfectly to transferring Tregs. In the transfer of whole MLN cells, IL-4 producing effector cells may have stimulated the production of immunoglobulins. Furthermore, because transfer of naïve whole MLN cells containing Tregs did not suppress food allergy, there are several regulatory systems in acquired tolerance, namely antigen-dependent and antigen-independent systems. Transfer of Tregs from naïve mice may provide antigen-independent regulatory systems in oral tolerance. In antigen-dependent system, it is also possible that other regulatory cells (except CD4⁺ CD25⁺ T cells) may have been induced to MLN cells, or antigen-presenting cells or memory cells in MLN may have been affected because of oral tolerance by antigen loading. It is necessary to conduct more experiments.

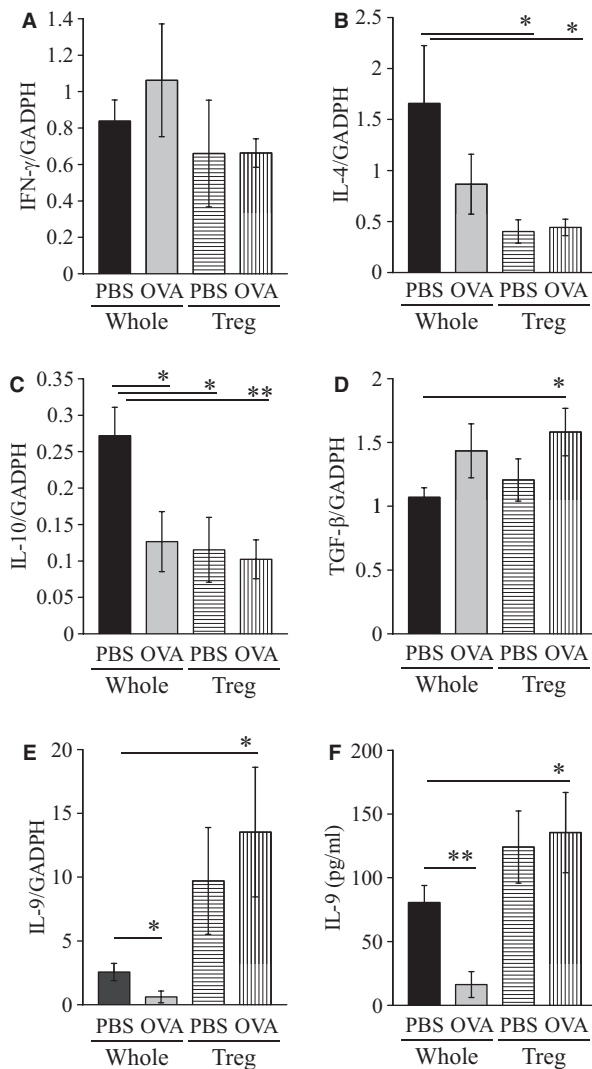


Figure 6 Expression of cytokines in the induction of oral tolerance by transfer of isolated cells. Interferon- γ (A), IL-4 (B), IL-10 (C), TGF- β (D), and IL-9 (E) mRNA expressions in the jejunum were measured by real-time RT-PCR. IL-9 levels in sera were measured by ELISA (F). Each value represents the mean $\pm$ SEM of six mice (phosphate-buffered saline [PBS] or ovalbumin [OVA] refers to mice transferred with mesenteric lymph node cells from PBS- or OVA-treated mice, respectively; * P < 0.05, ** P < 0.01 vs whole cells from PBS-treated mice).

We believe that the transfer model accurately recapitulates outgrowth of food allergies owing to the attenuation of allergic responses induced by acquired immune factors, because

IgE levels are not completely suppressed in patients (27, 28) who outgrow their allergies. Additionally, it was reported that Tregs from outgrowing patients proliferated compared to food allergy patients after stimulation of the food allergen. Additionally, Tregs in the tolerant patients did not produce IL-10 (29). Our data suggest that the induction of IL-9 associated with Treg, and not IL-10, is important for acquired tolerance. We speculate that environments rich in IL-9 and poor in IL-4 suppress immunoglobulin production and induce atypical mast cells and regulatory responses. A more detailed analysis is necessary to elucidate the mechanism by which IL-9 affects inflammatory cells.

Our data reveal two types of food allergy suppression in mice. The first involves oral tolerance through preingestion of antigen, and the second involves tolerance acquired through transfer of Tregs. We believe that the former type is related to inherent tolerance and the latter type is an acquired tolerance for food antigens. Because food allergies are induced when inherent oral tolerance is absent, induction of acquired tolerance via Tregs is important for the development of new therapies for food allergy. Moreover, because our data showed that transfer of non-antigen-loaded Tregs also suppressed symptoms of food allergy, an understanding of the detailed mechanisms of tolerance induction by Tregs associated with IL-9 is important for understanding outgrowing and treatment of food allergy.

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Conflict of interest

The authors have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article found at: <http://www.wiley-online-library.com>

Figure S1. Criterion for diarrhea.

Figure S2. Change in rectum temperature over 180 min.

Figure S3. Diagrams of flow cytometric analyses.

Figure S4. Hematocrit and MMCP-I levels in sera.

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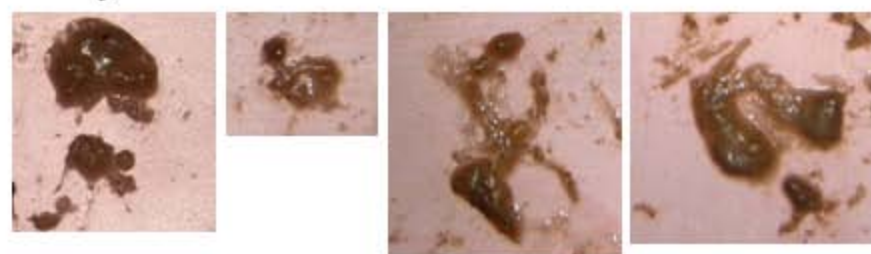
Solid state



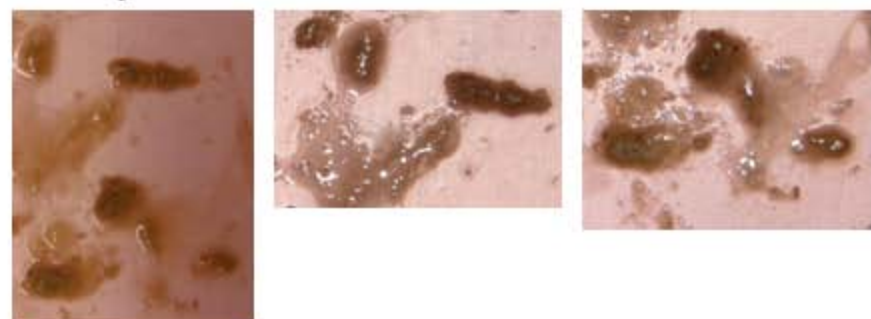
Funicular form

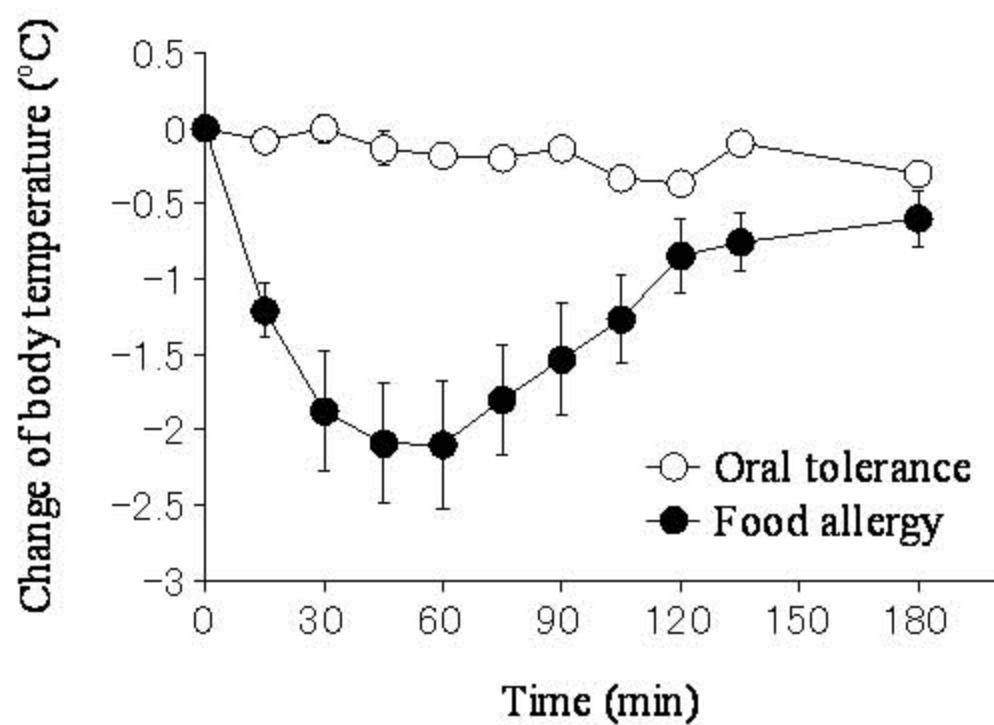


Slurry



Watery state





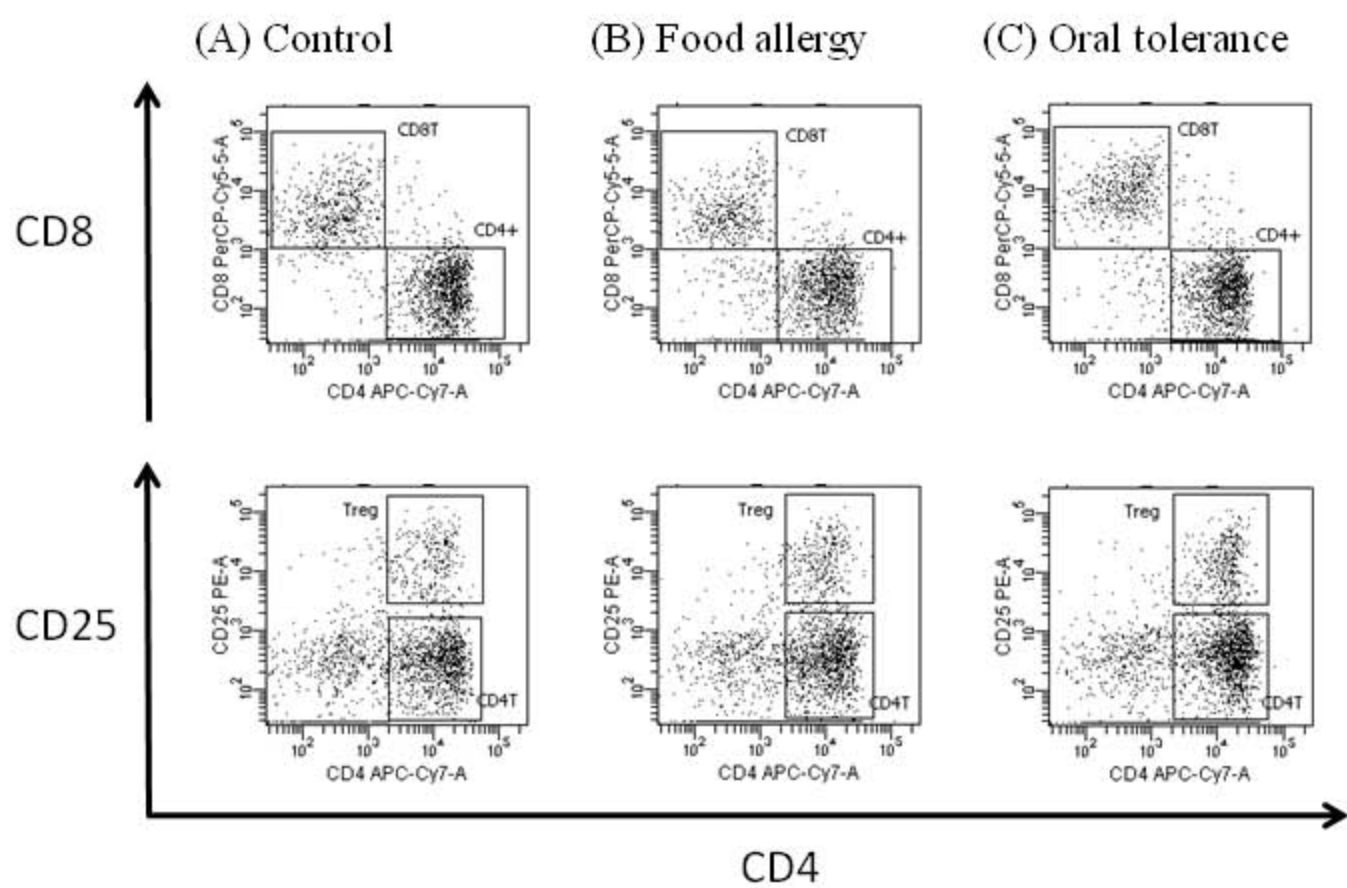
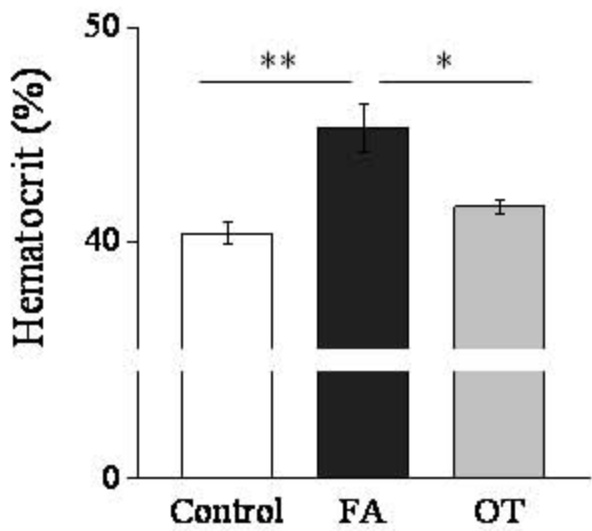
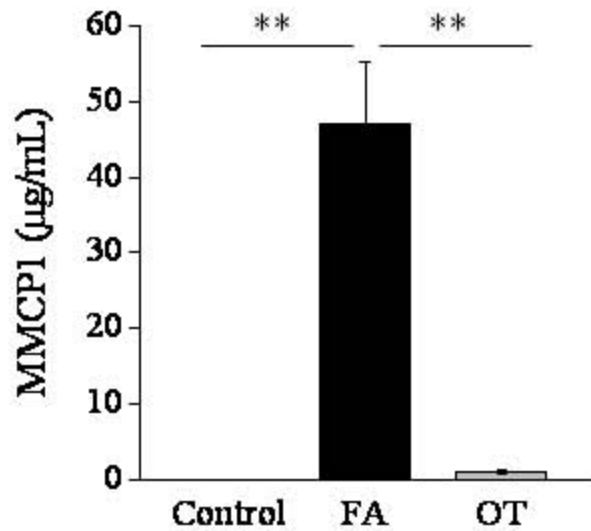


Figure E3

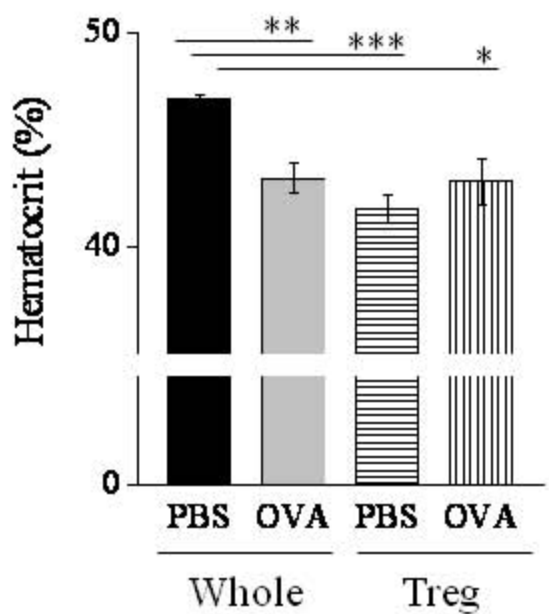
(A)



(B)



(C)



(D)

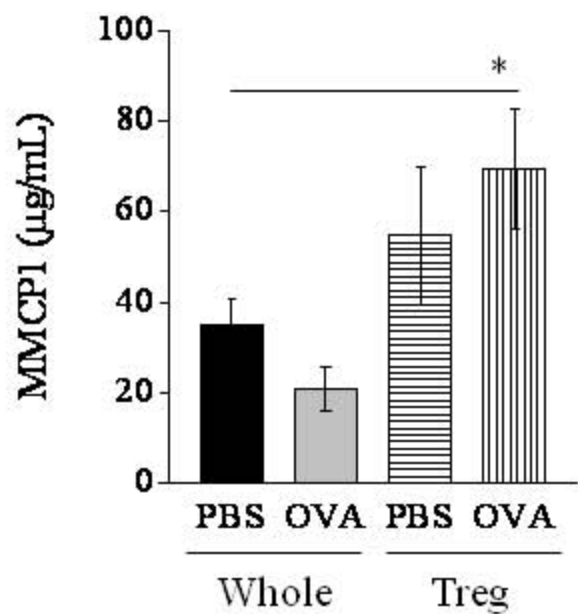


Figure E4