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Letter to the Editor

Epicutaneous allergen administration without antigen delivery device induces local T cell response and alleviates food allergic enteropathy

Dear Editor,

Epicutaneous immunotherapy (EPIT) in which immune responses are modulated by epicutaneous (EC) antigen administration has been reported^{1–5} and anticipated as a novel allergen immunotherapy (AIT).^{6,7} EC immunization has also been shown to inhibit immune response in animal models of contact sensitivity,⁸ multiple sclerosis,⁹ rheumatoid arthritis,¹⁰ hapten-induced colitis¹¹ and in multiple sclerosis patients.¹² However, most reports of EPIT employ a particular device to deliver antigen on skin without scratching or tape stripping, or apply allergen with TLR ligands.¹³ In order to investigate its mechanism and efficacy more simply, we aimed to establish a new EPIT model without any antigen delivery device in a food allergy model.

To examine whether EC OVA administration to intact skin without any device can deliver OVA to skin draining lymph nodes (DLNs) of sensitized mice, Alexa-fluor 647 conjugated OVA in PBS was applied to dorsal skin of both ears following pre-wetting with PBS. We examined the frequency of Alexa 647⁺ cells in CD11c cells of three groups of mice; the OVA-Alexa group, a group treated with unconjugated Alexa N-hydroxysuccinimide ester and one treated with PBS (Supplementary Fig. 1; for more information concerning methods of this study, see the Supplementary Methods section). There was a significant increase in Alexa⁺ cells when OVA-Alexa was applied, while no significant increase was observed in the case of unconjugated Alexa. These results suggested that applied OVA are taken up in a healthy intact skin, delivered to secondary lymphoid tissues by CD11c⁺ cells. Similar delivery by dendritic cells have been observed for myelin peptides applied transdermally to multiple sclerosis patients in human studies.¹⁴

Next, we examined whether EPIT can suppress allergic symptoms elicited by allergen food challenge (Fig. 1A). Sensitized mice were epicutaneously administrated OVA in PBS (EPIT group) or PBS only (control group) repeatedly for 3 weeks (EPIT course). Following to EPIT course, both group of mice were fed with egg white (EW)-diet to induce allergic symptoms. In this food allergy model, allergic symptoms such as weight loss, T cell-mediated enteropathy, IgE production, and Th2 response are elicited by EW-diet feeding.¹⁵ To evaluate efficacy of EPIT for suppressing food allergy symptoms, the rate of body weight change relative to the start of feeding (day 22) was calculated. Body weight of the control group mice decreased, while that of the EPIT group was significantly alleviated (Fig. 1B). There was no significant difference between the

two groups during the EPIT course (Supplementary Fig. 2). Rectal temperature was measured before EW-diet feeding (day 22) and after antigen challenge (day 27), respectively. There was no significant difference in the two groups before EW-diet feeding, while after allergen challenge with EW-diet feeding, rectal temperature drop was significantly rescued in the EPIT group compared to the control group with dropped rectal temperature (Fig. 1C). Intestinal inflammation observed in the jejunal tissue was shown in Figure 1D. Typical morphological signs of inflammation, such as villous branching, goblet cell hyperplasia and infiltration into the mucosal epithelium in the control group were severer compared to the EPIT group. The ratio of villous height to crypt depth as parameters for monitoring morphology^{16,17} decreased in the control group by EW-feeding than that of the EPIT group, showing the jejunal enteropathy significantly relieved by EPIT (Fig. 1E). These results clearly show that EPIT without any device is effective to suppress systemic allergic symptoms, including T cell-mediated food allergic enteropathy.

Next, we analyzed the parameters related to suppression of allergic symptoms in this EPIT model. We measured OVA-specific IgE antibody levels in sera by ELISA. There was no significant difference between the two groups after immunization (day 0) and EPIT course (day 22). On day 27, robust OVA-specific IgE production elicited by EW-feeding was observed in the control group, which was significantly suppressed in the EPIT group (Fig. 1F). Foxp3⁺ Tregs are known for their suppressive function in antigen specific manner. After EW-diet feeding, we obtained spleen (SPL), mesenteric lymph nodes (MLNs), DLNs and analyzed Foxp3 expression for individual mice. In SPL and MLN, the frequency of Foxp3 expression was significantly higher in the EPIT group than that of control group (Fig. 1G). On the other hand, in the skin DLN, such significant difference was not observed.

To further analyze the mechanism of EPIT, we examined the effect of EPIT before EW-feeding (Fig. 2A). We considered that immune responses may be induced in the skin DLN during the EPIT course. CD4⁺ T cells from DLN after EPIT course were cultured under OVA stimulation. In CD4⁺ T cells from DLN of EPIT group, Th2 response to OVA stimulation, such as IL-4 production (Fig. 2B), and cell proliferation (Fig. 2C) were induced OVA-dose-dependently. Effects on other cytokines such as IFN- γ and IL-10, and on SPL and MLN-derived CD4⁺ T cells by EPIT were not stable (data not shown). To identify OVA-specific Tregs, we tracked the cells proliferated in response to OVA stimulation using 5,6-calboxyfluorescein diacetate (CFSE) and analyzed Foxp3 expression by CD4⁺ T cells (Supplementary Fig. 3). In DLN of the EPIT group, the proportion of OVA-specific Foxp3⁺ cells in CD4⁺ T cells was

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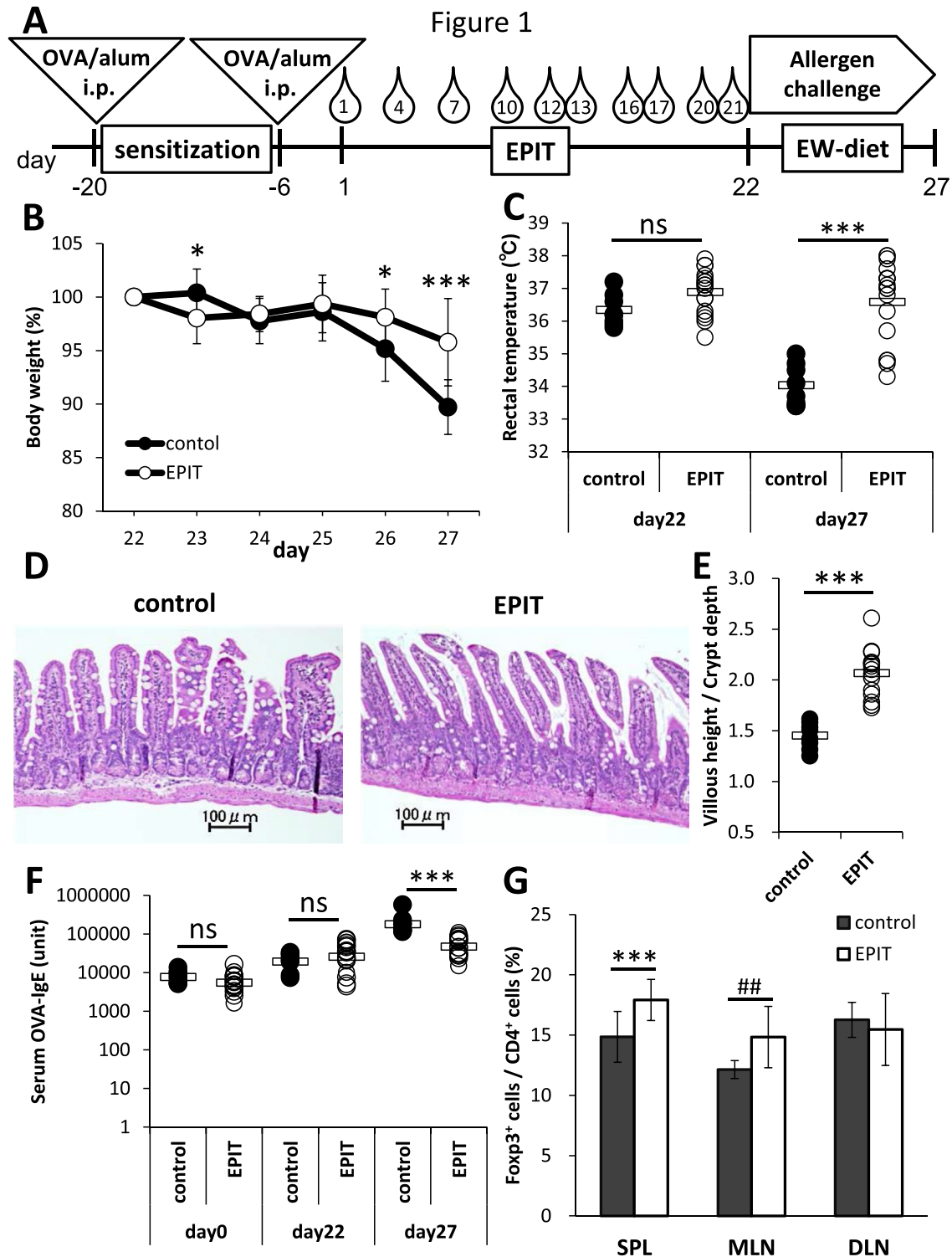


Fig. 1. EPIT suppressed T cell-mediated food allergic enteropathy as well as systemic allergic symptoms. **(A)** Experimental schedules for immunization, EPIT, and allergen challenge in a food allergy model. OVA application was performed for 3 weeks, on day 1, 4, 7, 10, 12, 13, 16, 17, 20, and day 21. After EPIT course, from day 22–27, both groups of mice were challenged with EW-diet. **(B)** Body weight during EW-diet feeding was measured. The rate of body weight change relative to the start of feeding (day 22) was calculated. **(C)** Rectal temperature was measured before (day 22) and after (day 27) EW-diet feeding. **(D)** Histological analysis of the jejunum was performed by hematoxylin eosin staining. **(E)** Morphological changes were quantitatively evaluated. We measured and calculated the ratio of villous height to crypt depth. **(F)** OVA-specific IgE antibody levels were measured by ELISA. **(G)** Fc γ R3 expression by CD4⁺ T cells analyzed for individual mice after EW-diet feeding (day 27). The percentage of cells expressing Fc γ R3 in CD4⁺ T cells is shown. These data represent 2–3 independent experiments. Control n = 8, EPIT n = 16–17. Results are shown as Mean $\pm$ SD, or as individual values. Statistical differences are shown as *p < 0.05, ***p < 0.001 (Student's t-test), ##p < 0.01 (Welch's t-test), ns; not significant.

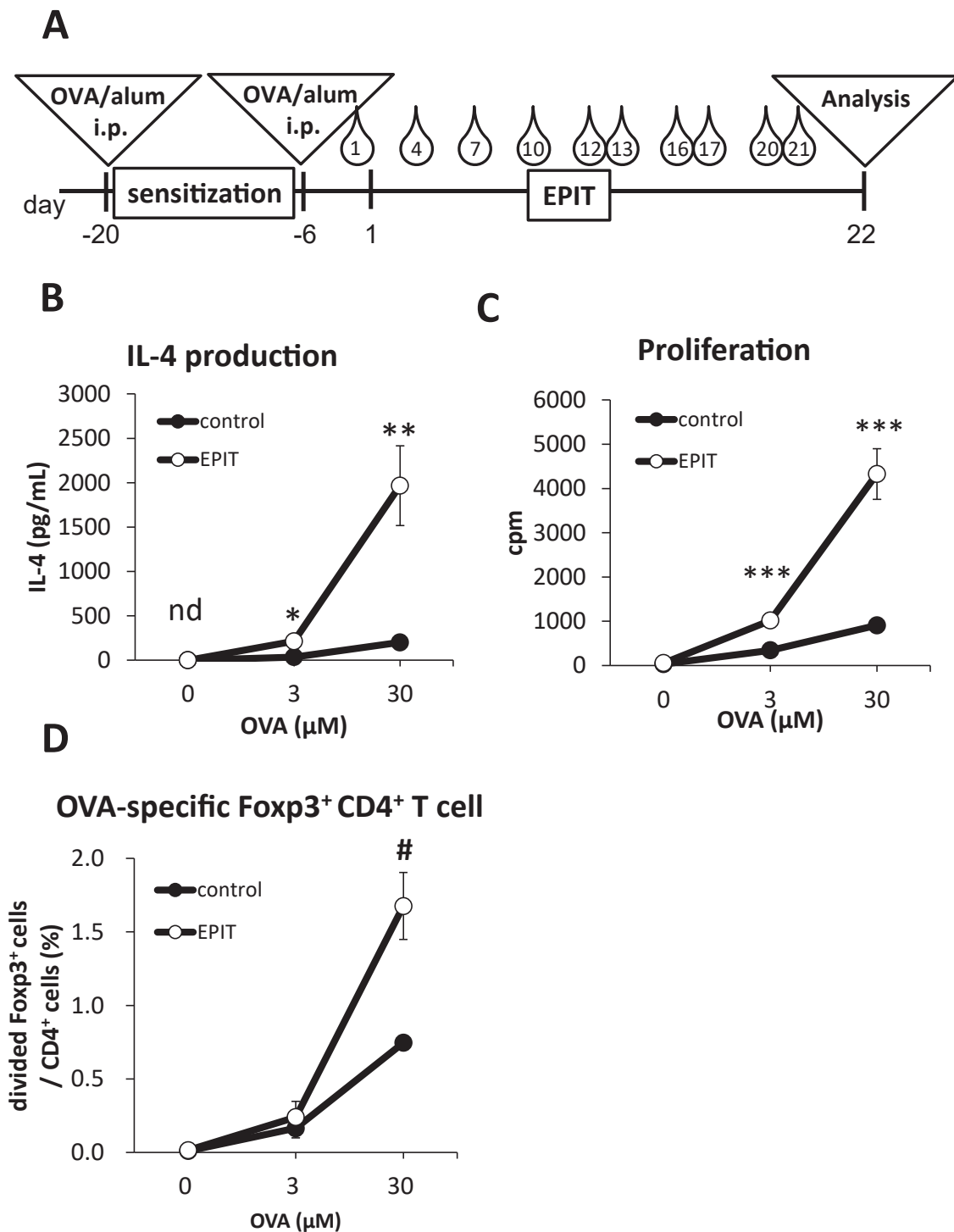


Fig. 2. Effect of EPIT on CD4⁺ T cells from DLNs. **(A)** Experimental schedules for immunization, EPIT in a food allergy model. **(B)** IL-4 production in supernatants was measured by ELISA. **(C)** Cell proliferation in response to OVA stimulation evaluated by incorporation of [³H]-thymidine into the cells. **(D)** OVA-specific CD4⁺ cells that proliferated in response to OVA stimulation were detected using CFSE, and Foxp3 expression by the CD4⁺ T cells was analyzed. The percentage of Foxp3⁺CD4⁺ divided cells in CD4⁺ T cells is shown. These data represent 2 independent experiments. **(B)** **(C)** control n = 2–3, EPIT n = 2–3 **(D)** control n = 6 EPIT n = 7. Results are shown as Mean $\pm$ SD of cultured 3 wells. Statistical differences are shown as *p < 0.05, **p < 0.01, ***p < 0.001 (Student's t-test), #p < 0.05 (Welch's t-test). nd; not detected.

significantly higher than that of the control group (Fig. 2D). No significant effect of EPIT was observed in SPL, MLN-derived CD4⁺ T cells (data not shown).

For assessment of EPIT in clinical application, various factors should be considered and investigations with multiple models are needed. In our EPIT model, we employed no device. The skin does not receive shaving or hair removal, and irritation to the skin can be less than other EPIT model with device. We performed histological analysis after the EPIT course, and there was no loss of stratum corneum or any other lesions in the EPIT group. Some cell infiltration was observed for both EPIT and control groups but this was not restricted to the side of application (data not shown). This study has significance in showing that antigen can be taken up without device and affect immune responses and that not only Th2 response, but also suppressive reaction can be induced.

In other reports using Viaskin, systemic induction of Tregs and decrease of splenic Th2 response *ex vivo* and specific IgE after the EPIT course has been reported.^{2–5} Our EPIT model is characteristic in that there is no change in systemic specific IgE before the allergen challenge. There was no change of specific IgG, IgG1 or IgG2a titers either (Supplementary Fig. 4). EPIT induced Th2 and Treg response which was confined to the local response in the DLNs of the skin. The allergic responses were suppressed systemically at the time of massive allergen invasion accompanying systemic induction of Tregs. Since the Tregs migrated out of the DLN, no significant difference in Foxp3⁺CD4⁺ T cell frequency was observed between the two groups in DLN after allergen challenge, although specific IgE antibody titer in the EPIT group was significantly lower compared to the control group. Our results suggest that modification of T cell response without altering the antibody response during the EPIT course may be sufficient to alleviate response after allergen challenge. However, it is possible that induction of specific IgG antibody will further increase efficacy of EPIT.

In this model, the effects of EPIT on the immune system stay local, which suggests that the risk of systemic adverse events might be low. Our model should be useful in elucidating mechanisms to increase biological safety and immunological efficacy of EPIT.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2020.04.010>.

Conflict of interest

SH received a grant from Torii Pharmaceutical. KOD is an employee of Torii Pharmaceutical. The rest of the authors have no conflict of interest.

Mamiko Morinaga^{a,b}, Haruyo Nakajima-Adachi^{a,b}, Erika Hiraide^{a,b}, Noriko Kitamura^c, Osamu Kaminuma^{c,d}, Takachika Hiroi^c, Katsuyo Ohashi-Doi^e, Satoshi Hachimura^{a,b,*}

^a Research Center for Food Safety, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

^b Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

^c Department of Genome Medicine, Allergy and Immunology Project, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

^d Department of Disease Model, Research Institute of Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan

^e Research Laboratories, Torii Pharmaceutical Co., Ltd., Tokyo, Japan

* Corresponding author. Research Center for Food Safety Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan.

E-mail address: ahachi@mail.ecc.u-tokyo.ac.jp (S. Hachimura).

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Supplementary Methods

Animals

Female BALB/c mice were purchased from Clea Japan Inc (Tokyo, Japan). All experiments were performed in accordance with guidelines for the care and use of laboratory animals of the University of Tokyo, and were approved by the Animal Use Committee of the Faculty of Agriculture, the University of Tokyo (approval no. P13-847, P17-098 and P18-029).

Epicutaneous administration of Alexa-fluor 647 conjugated OVA

BALB/c mice (6-9 weeks-old) were sensitized with 50 μ g OVA (Sigma-Aldrich, St. Louis, MO, USA) with 1 mg of alum (Thermo Fisher Scientific, Waltham, MA, USA) in 100 μ L PBS (Nissui Pharmaceutical, Tokyo, Japan) by intraperitoneal injection on day -20 and day -6. We applied 25 μ g Alexa-fluor 647 conjugated OVA (Lifetechnologies, Carlsbad, CA, USA) in 5 μ L PBS (OVA-Alexa group n=5), 5 μ g Alexa-fluor 647 N-hydroxysuccinimide ester in 5 μ L (Alexa group n=5) or PBS only (PBS group; n=3) to the skin of both ears following pre-wetting with 5 μ L PBS. The solution was applied with a micropipette under anesthesia with isoflurane and was spread using the side of the tip carefully not to damage the skin.

Epicutaneous immunotherapy (EPIT) schedule

BALB/c mice (6-weeks-old) were sensitized with 50 μ g OVA with 1 mg of alum in 100 μ L PBS by intraperitoneal injection on day -20 and day -6. As EPIT, we

applied 5 μ L of 30 mg/mL OVA/PBS (EPIT group; n=16-17) or PBS only (control group; n=8) to the skin of both ears (300 μ g OVA /head for EPIT group) following pre-wetting with 5 μ L PBS as described above. OVA application was performed for three weeks, on day 1, 4, 7, 10, 12, 13, 16, 17, 20, and day 21. After EPIT course, from day 22 to day 27, both groups of mice were fed with egg white (EW)-diet containing egg white protein as protein source to induce food allergic inflammation. It is estimated that a mouse ingests approximately 200 mg of OVA per day. On day 22 or 27, mice were euthanized for analysis.

Histological analysis

Intestinal tissues were harvested from the jejunum, and fixed with 10% neutral formalin as described previously.¹ Each section was embedded in paraffin, sliced down into 4 μ m thick. Intestinal inflammation of jejunal tissue was observed by hematoxylin · eosin staining. To quantify the morphological changes (crypt elongation, villous atrophy, and goblet cell hyperplasia) of the jejunum, we measured villous height and crypt depth as a typical parameter for monitoring morphology.² To evaluate the inflammatory changes of the jejunum, multiple randomly selected villi per mouse were measured, and the ratio of villous height to crypt depth was calculated.

Rectal Temperature

Rectal temperature was measured before EW-diet feeding (day 22) and after allergen challenge (day 27), respectively.

Serum OVA-specific IgE, IgG, IgG1 and IgG2a

OVA-specific antibody levels in sera were measured by ELISA. For

measurement of IgE, immunoplates (Thermo Fisher scientific) were coated with rat anti-mouse IgE antibody (R35-92, BD Biosciences, San Jose, CA, USA). After blocking with bovine serum albumin, sera diluent and standards were applied. Specific antibody was detected by biotinylated OVA, and streptavidin-alkaline phosphatase conjugate (BD Pharmingen) and enzyme substrate (p-nitrophenylphosphate; Tokyo Chemical Industry Co., Tokyo, Japan) were subsequently added. For measurements of IgG, IgG1 and IgG2a, immunoplates were coated with OVA by adding 100 µg/mL OVA solution. After blocking, sera diluent and standards were applied. Then, alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Abcam), alkaline phosphatase-conjugated goat anti-mouse IgG1 antibody (Abcam) or alkaline phosphatase-conjugated goat anti-mouse IgG2a antibody (Abcam) was added, followed by enzyme substrate.

Flowcytometry

For flowcytometric analysis, single-cell suspensions prepared from spleen, mesenteric lymph nodes, superficial cervical lymph nodes as draining lymph nodes (DLNs) or ear skin harvested from each mouse or cells collected from each well of cultured plates. Single-cell suspension of lymphocytes from DLNs was prepared after incubation in 10%FCS-RPMI containing 0.6 mg/mL collagenase (Wako, Osaka, Japan) and 10 µg/mL DNaseI (Roche, Basel, Switzerland). Each ear was peeled into two pieces and chopped finely. These were treated with the collagenase solution and then passed through a 70 µm cell strainer to prepare a cell suspension. In experiments of Alexa-fluor 647 conjugated OVA application, CD11c⁺ cells were separated by MACS cell isolation

system (Miltenyi Biotech, Bergisch Gladbach, Germany). CD11c or CD4 molecules on cell surface were stained with APC/Cy7-conjugated anti-mouse CD11c (N418, BioLegend, San Diego, CA), FITC or APC-conjugated anti-mouse CD4 mAb (GK1.5, BioLegend) after Fc receptor blocking with purified anti-mouse CD16/32 Ab (93, BioLegend). Intracellular staining of Foxp3 was performed with Foxp3 staining buffer set (eBioscience, San Diego, CA) and PE-conjugated anti-mouse Foxp3 mAb (FJK-16s, eBioscience). Analysis was performed using FACSVerse (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

Cell culture

For antigen stimulation in *ex vivo*, CD4⁺ T cells were cultured with antigen-presenting cells (APCs) in the presence of OVA protein. DLNs were obtained from each mouse, pooled in each group, and single-cell suspension was obtained. CD4⁺ T cells are isolated using MACS cell isolation system (Miltenyi Biotech). Splenocytes from naïve BALB/c mice were treated with 50 µg/mL mitomycin C (Sigma-Aldrich) and co-cultured as antigen presenting cells (APCs). For analysis of Foxp3 expression in OVA-specific CD4⁺ T cells, CD4⁺ T cells were labeled with 5,6-Carboxyfluorescein diacetate, succinimidyl ester (CFSE; eBiosciences) to detect cells that proliferated in response to OVA stimulation. In this experiment, CD4⁺ T cells were stained with CFSE as follows. CD4⁺ T cells isolated by MACS were suspended at a concentration of 1.0×10^7 cells/mL in PBS. CFSE solution in dimethyl sulfoxide (Sigma-Aldrich) was added to cell suspension at 3 µM. Cells were incubated 7-8 min at room temperature and washed with 5%FCS-RPMI 2 times, and suspended in 10 % FCS-RPMI.

CFSE-labeled CD4⁺ T cells and CD4-depleted APCs, prepared with MACS cell separation system (Miltenyi Biotech), were cultured in presence of OVA, and subsequently subjected to flowcytometric analysis.

Cells were cultured as follows; for analysis of cell proliferation, CD4⁺ T cells; 2.0×10^5 cells/well, APCs; 8.0×10^5 cells/well, for measurement of cytokine production, CD4⁺ T cells; 4.0×10^5 cells/well, APCs; 8.0×10^5 cells/well, and for analysis of proliferation of OVA-specific CD4⁺ T cells with CFSE, CFSE stained CD4⁺ cells; 2.0×10^5 cells/well and CD4-depleted APCs; 8.0×10^5 cells/well. Each well for co-culture were prepared in total 200 μ L 10 % FCS-RPMI in 96 well flat-bottom plates, and incubated under 37 °C, 5 % CO₂ for 3 days.

Cytokine production

IL-4 level in the supernatants was measured by sandwich ELISA. Immunoplates (ThermoFisher) were coated with rat anti-mouse IL-4 antibody (11B11, BD Pharmingen). After blockage with BSA, supernatant diluent and standards (rIL-4; PeproTech, Rocky Hill, NJ, USA) were applied. IL-4 was detected by biotinylated rat anti-mouse IL-4 antibody (BVD6-24G2, BD Pharmingen), and streptavidin-alkaline phosphatase conjugate and enzyme substrate (p-nitrophenylphosphate) were subsequently added. Absorbance at 405 nm (ref. 490 nm) was measured with Microplate Reader Model 680 (Bio-Rad, Hercules, CA, USA), and data analyzed by Microplate Manager III (Bio-Rad).

Cell proliferation

To assess T cell proliferation, [³H]-thymidine (18.5 kBq/well; Moravek

Biochemicals, Brea, CA, USA) was added to the wells after 3 days culture. After additional 24 hours incubation, cells were harvested and [³H]-thymidine incorporation was counted. T cell proliferation was evaluated as the number of counts per minute (cpm). In this assay, T cells which recognizes OVA as antigen (both Tregs and helper T cells) should all proliferate.

Statistical analysis

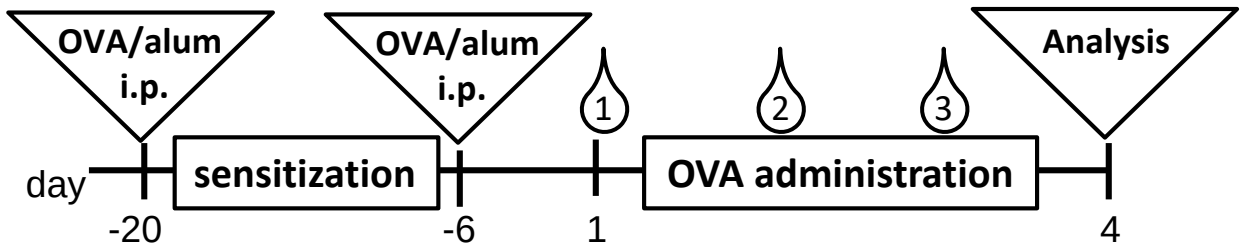
Results are shown as Mean $\pm$ SD, or as individual values. For statistical analysis, Student's *t*-test, Welch's *t*-test or Tukey's HSD test was used.

Supplementary References

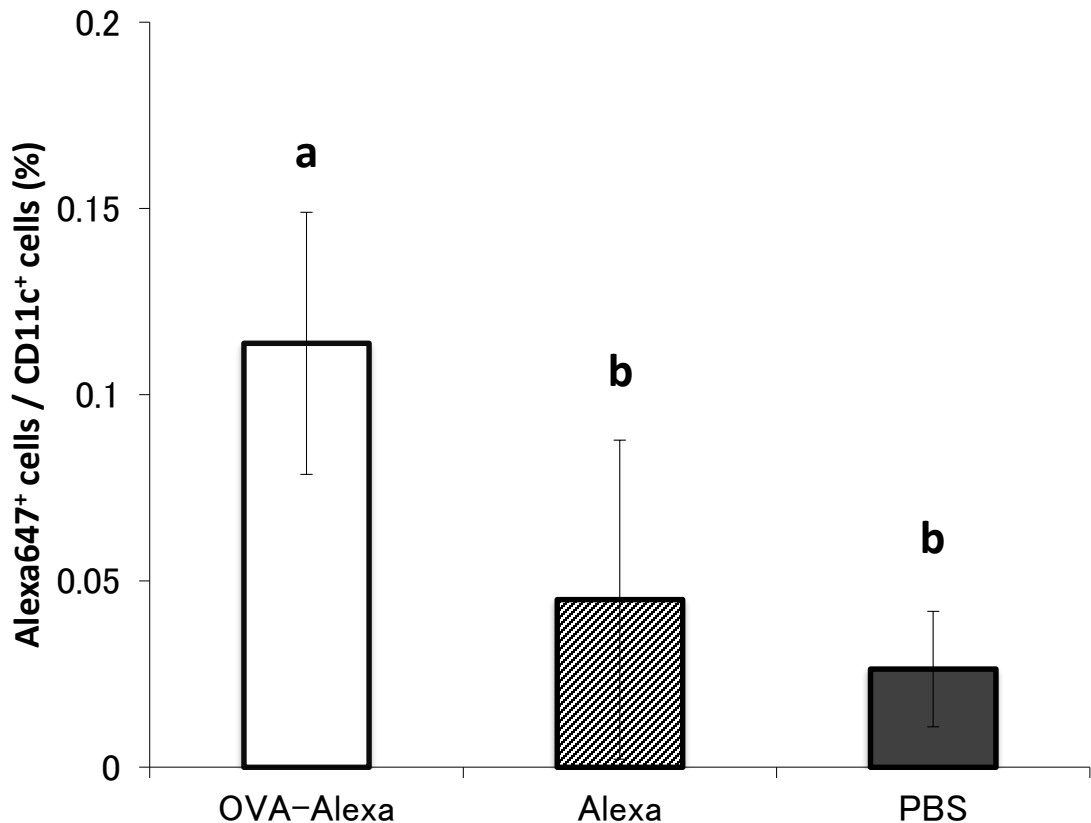
1. Burggraf M, Nakajima-Adachi H, Hachimura S, Ilchmann A, Pemberton AD, Kiyono H, et al. Oral tolerance induction does not resolve gastrointestinal inflammation in a mouse model of food allergy. *Mol Nutr Food Res* 2011;**55**:1475-83.
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Supplementary Figure 1

A

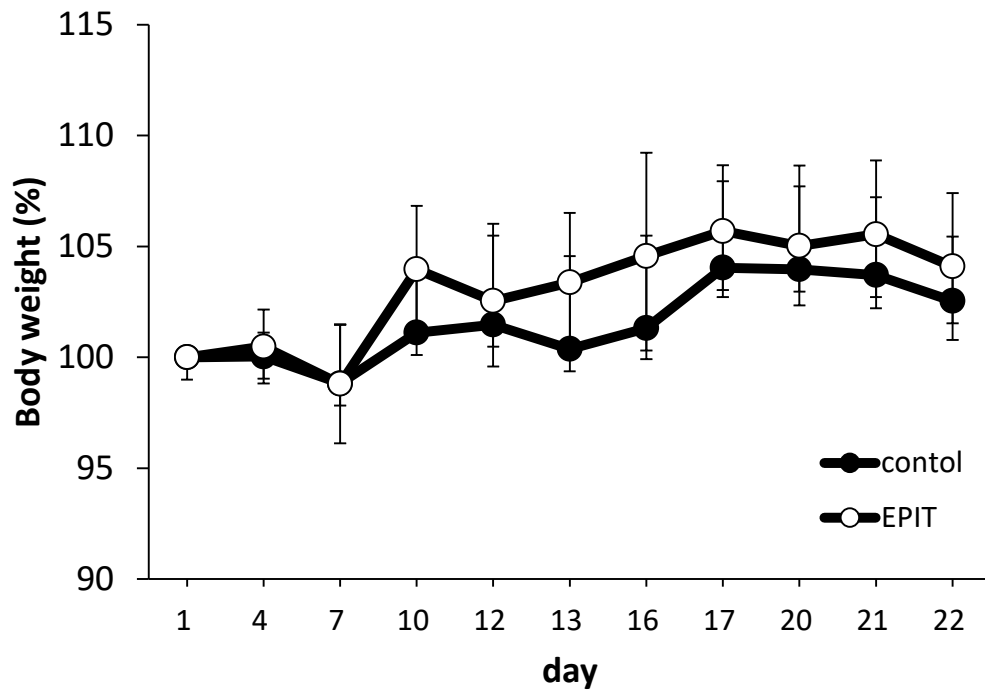


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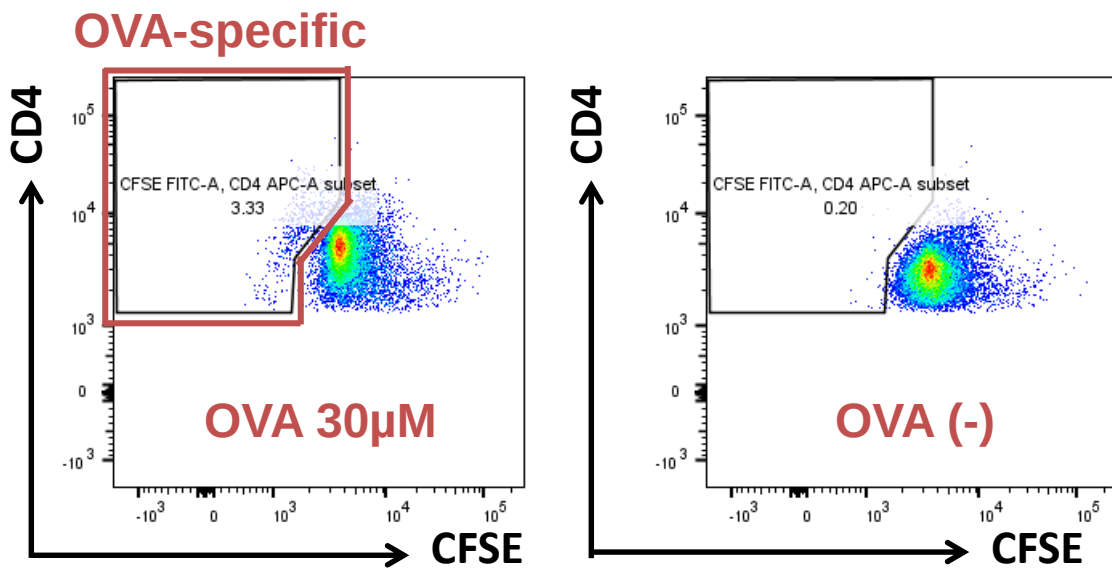
OVA administration on intact skin. (A) Experimental schedules for sensitization and administration of OVA (B) Intake of OVA to CD11c⁺ cells in skin-draining lymph nodes were analyzed using flowcytometry. OVA-Alexa, Alexa NHS ester, or PBS was applied, and the frequency of Alexa 647⁺ cells in CD11c cells were examined for individual mice. These data represent 3 independent experiments. OVA-Alexa n=5, Alexa n=5, PBS n=3. Values not sharing a common letter are significantly different (Tukey's HSD test, $p < 0.05$).

Supplementary Figure 2



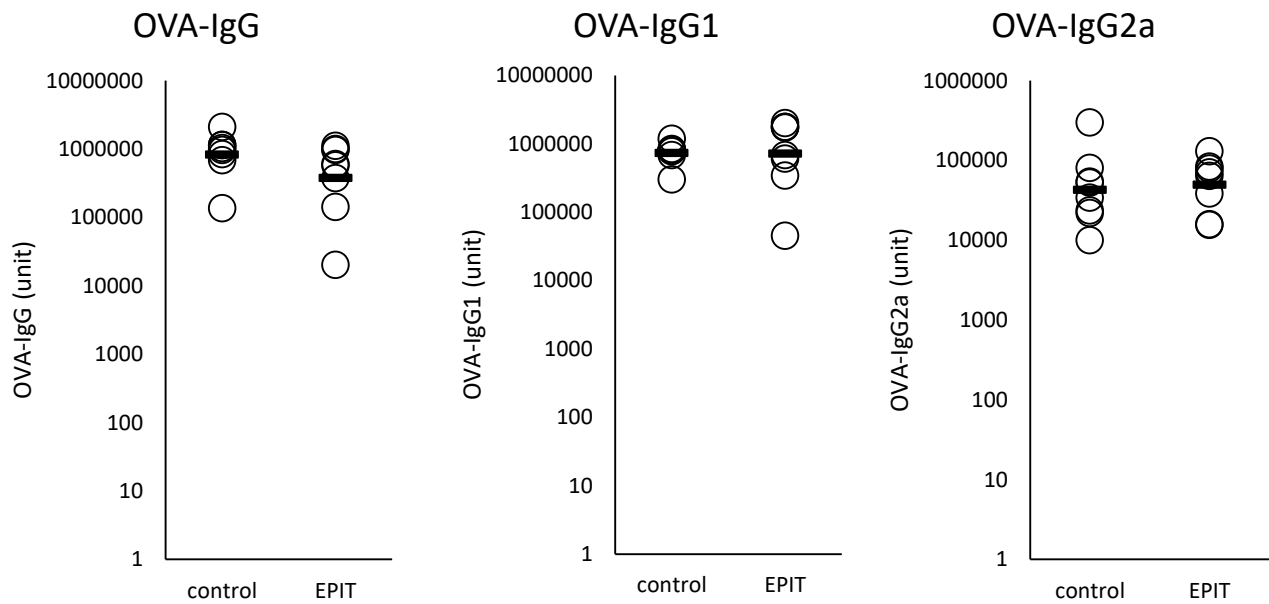
Body weight during the EPIT course was measured. The rate of body weight change relative to the start of EPIT course (day 1) was calculated.

Supplementary Figure 3



Identification of OVA-specific CD4⁺ T cells. Divided cells were identified by comparing the fluorescence intensities of OVA-stimulated and non-stimulated samples.

Supplementary Figure 4



OVA-specific IgG, IgG1 and IgG2a antibody levels at day22 after the EPIT course were measured by ELISA.