



# Epicutaneously sensitized food-induced anaphylaxis is ameliorated with “oral tolerance” to antigen

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## Abstract

Food allergy is an antigen-specific immunological adverse reaction after exposure to a given food. Multiple clinical studies showed that oral immunotherapy (OIT) is effective for the prevention and treatment for food allergy that is developed in infants and children. However, the effectiveness of OIT for epicutaneously sensitized food allergy remains unclear. Previously, we established a mouse model of epicutaneous-sensitized food allergy. In this model, systemic allergic reaction including intestinal and skin symptoms, such as anaphylaxis, was observed. We treated this model with OIT in two ways (OIT before sensitization or OIT during the sensitization phase) and evaluated the preventive effect of both methods. OIT before sensitization significantly ameliorated mast cell degranulation in sensitized skin, but there was no decrease in rectal temperatures or in mast cell degranulation in the jejunum. However, OIT administered during the sensitization phase significantly ameliorated the decrease in rectal temperature and mast cell degranulation in the skin and jejunum. OIT before sensitization increased the regulatory T cells in mesenteric lymph node (MLN), but not in the spleen, and it reduced antigen-specific IgG, but not IgE, production compared with the non-OIT control. However, OIT during sensitization caused a greater increase in regulatory T cells in both the MLN and spleen and reduced antigen-specific IgE and IgG generation compared with the non-OIT control group. Thus, OIT during the sensitization phase was effective for the prevention of epicutaneous-sensitized food allergy.

## KEYWORDS

basophil, food allergy, mast cells, OIT, regulatory T cells

## 1 | INTRODUCTION

Food allergy is a common allergic disease that is defined as an antigen-specific immunological adverse reaction after exposure to a given food.<sup>1</sup> It affects 5% to 10% of infants and children<sup>1,2</sup> and 10%

of adults.<sup>3</sup> Skin, especially inflamed skin, was shown to be one of the most important organs for sensitization to food allergens.<sup>4</sup> Genetic and environmental factors cause skin barrier dysfunction and skin inflammation, which promotes allergen penetration and sensitization to the food allergen in certain cases of food allergy.<sup>5</sup> A case-controlled study

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showed that a loss-of-function mutation in the filaggrin gene, which is an important component of the corneum that is associated with skin barrier function, is associated with development of peanut allergy.<sup>6</sup> Multiple cohort studies demonstrate that the percentages of food sensitization are much higher in children with atopic dermatitis compared with healthy controls.<sup>7</sup> The severity of atopic dermatitis was associated with the sensitization to food such as cow's milk and peanut in infants.<sup>8</sup> In adulthood, the use of soaps containing hydrolysed wheat protein induces epicutaneous sensitization to the protein and caused the development of wheat allergy in 2011 Japanese patients.<sup>9</sup> An association between occupation (eg cooks, food-processing workers) and food allergy also has been reported.<sup>10</sup> Therefore, epicutaneous sensitization may be the resulting mechanism that contributes to the development of certain types of food allergy in childhood and adulthood.

Oral administration of food allergens induces immune tolerance and inhibits allergic reaction.<sup>4</sup> Early exposure to food allergens decreases the risk of developing a food allergy in infants and children.<sup>11</sup> A randomized trial of peanut consumption in infants with severe eczema, egg allergy or both showed that the prevalence of peanut allergy at 5 years of age was 13.7% in the avoidance group and 1.9% in the consumption group.<sup>12</sup> Oral immunotherapy (OIT) is also effective for desensitizing individuals with food allergy.<sup>11,13,14</sup> In a double-blind, placebo-controlled study of egg allergy, 75% of children who received OIT with egg white powder, but not those in the placebo group, were desensitized.<sup>13</sup> OIT combined with omalizumab achieved desensitization in children with cow's milk allergy.<sup>14</sup> However, the efficacy of OIT in preventing and treating food allergy that developed in adults remains unclear. Additionally, it is unclear if OIT can prevent and treat epicutaneously sensitized food allergy.

Previously, we established a mouse model using adult mice.<sup>15</sup> In this model, mice exhibit systemic allergic reactions such as intestinal and skin symptoms after antigen challenge, which mimics anaphylaxis. In the present study, we examined the preventive efficacy of OIT on epicutaneous-sensitized food allergy using this model. We treated the epicutaneous-sensitized food allergy model mice with OIT in the following two ways: OIT before sensitization or OIT during the sensitization phase. Then, we compared these mice with non-OIT-treated control mice or each other and evaluated the preventive effect in each OIT group. OIT before sensitization significantly ameliorated skin symptoms, but not intestinal symptoms. However, OIT during sensitization significantly ameliorated skin and intestinal symptoms. Thus, OIT effectively prevents and alleviates epicutaneous-sensitized food allergy symptoms above a certain level. This may enable the avoidance of severe and life-threatening allergic reactions such as anaphylaxis.

## 2 | METHODS

### 2.1 | Mice

BALB/c mice (7 weeks old) were purchased from Sankyo Labo Service. All experiments were conducted in accordance with the

Guidelines for Animal Use and Experimentation of Tokyo Medical and Dental University (A2019-022C2).

### 2.2 | Epicutaneous-sensitized food-induced anaphylaxis

Epicutaneous-sensitized food-induced anaphylaxis was induced as described previously.<sup>15</sup> Briefly, dorsal hair was removed with an electric shaver and depilatory cream followed by tape stripping. A patch with 50  $\mu$ l of a 20-mg/ml ovalbumin (OVA) solution was placed onto the tape-stripped back skin for 1 week. The same procedure was repeated twice at 2-week intervals. On day 50, mice were orally challenged with 200  $\mu$ l of 0.25-g/ml OVA solution (50 mg of OVA). Changes in the rectal temperature were measured for 60 min. Mice were sacrificed 2 h after oral challenge. Then, whole blood, skin (ear lobes, sensitized lesions), jejunum, mesenteric lymph node (MLN), and spleen were collected for analysis.

### 2.3 | Oral immunotherapy

OIT was induced by offering to the mice, ad libitum, a 1% OVA solution dissolved in drinking water, as described previously.<sup>16</sup> The dose was calculated as 50 mg/day based on a drinking volume of 5 ml/day. The timing of OIT administration occurred in two ways. One way was oral administration of 1% OVA water for 1 week before starting the first sensitization to evaluate the preventive effect of OIT before sensitization (OIT [presensitization]). The other way was oral administration of 1% OVA water from the start of the first sensitization to the end of the final sensitization to evaluate the preventive effect of OIT during the sensitization phase (OIT [on sensitization]). The procedure, oral administration of 1% OVA water for 5 consecutive days and normal drinking water for the following 2 days, was repeated seven times. In the control group, mice were not orally administered OVA until the oral challenge (non-OIT).

### 2.4 | Measurement of mouse mast cell protease-1 and OVA-specific antibodies

Mouse mast cell protease-1 (mMCP-1) levels in serum were measured using an ELISA kit (eBioscience). OVA-specific IgE, IgG, IgG1, IgG2a and IgG2b levels were also measured using ELISA kits (Chondrex).

### 2.5 | Histology

Tissues were fixed with 10% buffered formalin, embedded in paraffin and cut with a microtome into 3- $\mu$ m-thick sections. For detection of mast cells in the skin and jejunum, tissue sections were stained with May-Grunwald-Giemsa and toluidine blue (pH 4.1), respectively. Mast cells, around which granules were observed or which were

loosely packed with granules, were called degranulated mast cells. To detect eosinophils in the jejunum, tissue sections were stained with Luna. For immunohistochemical staining of basophils, tissue sections were treated with microwaves, followed by incubation with methanol containing 0.3% H<sub>2</sub>O<sub>2</sub> to inhibit endogenous peroxidase and with a protein-blocking solution containing 0.25% casein (Dako) to prevent the non-specific antibody binding. The tissue sections were incubated with anti-mouse mast cell protease (mMCP)-8 mAb, which was established by the corresponding author,<sup>17</sup> followed by HRP-conjugated anti-rat IgG (Santa Cruz Biotechnology,). They were incubated subsequently in 3'-diaminobenzidine tetrahydrochloride solution (DAB) (Dako) and counterstained with haematoxylin.

## 2.6 | Real-time PCR analysis

After preparing total cell RNA, first-strand cDNA was synthesized using reverse transcription with oligo-dT primers. Gene expression was measured using Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies) with a real-time PCR system and was normalized to *Gapdh*. The following primers (forward and reverse, respectively) were used for both PCR reactions: for *Gapdh*, 5'-TTCACCACCATGGAGAAGGCCG-3' and 5'-GGCATGGACTGTGGTCATGA-3'; for *mFoxp3*, 5'-CCCAGGAAAGACAGCAACCTT-3' and 5'-TTCTCACAACCGGCCACTTG-3'; for *mGata3*, 5'-TTATCAAGCCCAAGCGAAG-3' and 5'-TGGTGGTGGTCTGACAGTTC-3'; and for *mIl-4*, 5'-GGTCTCAACCCAGCTAGT-3' and 5'-GCCGATGATCTCTCTCAAGTGAT-3'.

## 2.7 | Statistical analyses

The statistical significance of differences between data was determined by ANOVA and Tukey's honestly significant difference test. A *p* value of less than 0.05 was considered to indicate a statistically significant difference.

# 3 | RESULTS

## 3.1 | Intestinal symptoms were significantly suppressed in mice with OIT during the sensitization phase

In our previous study, systemic allergic reactions such as intestinal and skin symptoms were observed in mice that were sensitized with OVA proteins on tape-stripped back skin after oral OVA challenge.<sup>15</sup> We considered this model to be an epicutaneous-sensitized food allergy model. To evaluate the preventive effect before oral exposure to antigen on epicutaneous-sensitized food allergy, we treated this model with OIT in two ways: OIT before sensitization (OIT [presensitization]) or OIT during the sensitization phase (OIT [on sensitization]). First, we compared the decrease in rectal temperature

between these two groups and the non-OIT control group. We found that a decrease in rectal temperature was significantly inhibited in the OIT (on sensitization) group but not in the OIT (presensitization) group compared with the non-OIT control group (Figure 1A). The decrease in rectal temperature was impaired significantly in the OIT (on sensitization) group compared with that in the OIT (presensitization) group (Figure 1A). We further examined the serum mMCP-1 levels in each group, and we found that serum mMCP-1 was significantly lower in the OIT (on sensitization) group but not in the OIT (presensitization) group compared with the non-OIT control group (Figure 1B). The serum level of mMCP-1 was significantly lower in the OIT (on sensitization) group than that in the OIT (presensitization) group (Figure 1B). Histologically, in the non-OIT control group, 57.9% of mast cells in the jejunum were degranulated. The percentage of mast cell degranulation was significantly decreased in the OIT (on sensitization) group (32.1%, *p* = 0.011) but not the OIT (presensitization) group (49.4%) compared with the non-OIT control group (Figure 1C–E).

## 3.2 | Mast cell degranulation in skin was inhibited in both OIT treatment groups with previous exposure to the antigen

In this model, mast cell degranulation was observed in skin such as the ear lobes and sensitization sites on the back, as well as in the intestine. In the non-OIT control group, 32.7% of mast cells were degranulated at the sensitization skin sites. The percentage of degranulated mast cells at the sites was significantly decreased in the OIT (presensitization) (17.3%) and OIT (on sensitization) groups (7.3%) compared with the non-OIT control group (Figure 2A, C–D). For ear lobes, 31.8% of mast cells were degranulated in the non-OIT control group. The percentage of degranulated mast cells in the OIT (on sensitization) group was significantly lower (7.9%) compared with the non-OIT control group. The percentage of degranulated mast cells in the OIT (presensitization) group was likely to be lower compared with the non-OIT control group, although the difference was not statistically significant (15.2%, Figure 2B, E–F).

## 3.3 | Antigen-specific antibody levels were lower in mice with previous exposure to the antigen

We next examined the status of the sensitization that was determined by antigen-specific antibody levels. OVA-specific IgE levels were significantly lower in the OIT (on sensitization) group compared with those in the non-OIT control group and OIT (presensitization) group (Figure 3A). The OVA-specific IgE levels in the OIT (presensitization) group seemed to be lower compared with the non-OIT control group, but the difference was not statistically significant (Figure 3A). In addition, OVA-specific IgG levels were significantly lower in the OIT (on sensitization) and OIT (presensitization) groups compared with the non-OIT control group

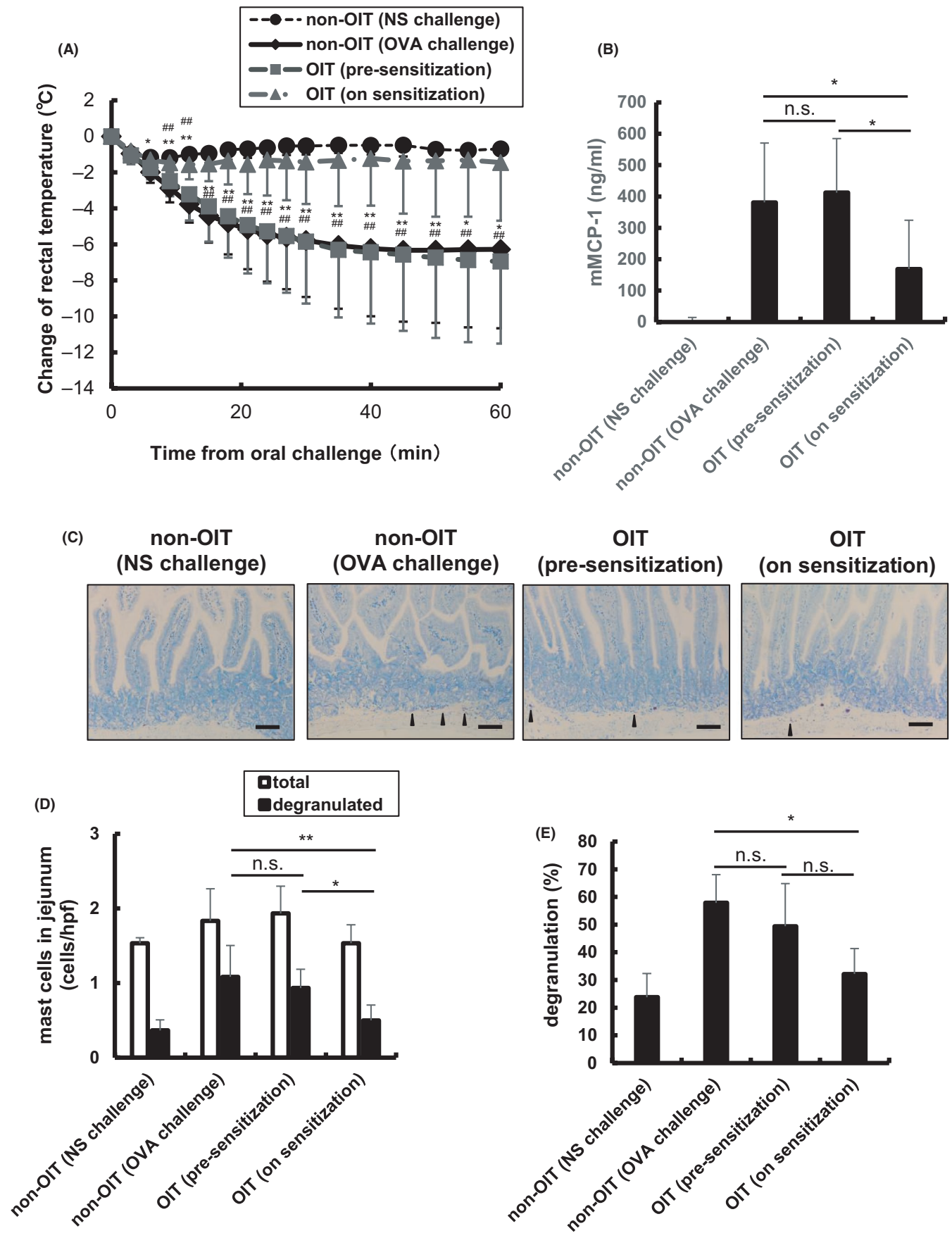


**FIGURE 1** Decrease in rectal temperature and mast cell degranulation in the jejunum. A, Changes in the rectal temperature were measured for 60 min. B, mMCP-1 concentrations in the serum were determined by ELISA. Data are the mean  $\pm$  standard deviation; n.s., not significant;  $^{*}/\#$   $p < 0.05$ ;  $^{**}/\#\#$   $p < 0.01$ . In (A), stars indicate the comparison between the OIT (on sensitization) group and non-OIT control group (OVA challenge); hashes indicate the comparison between the OIT (presensitization) group and OIT (on sensitization) group. Data sets are representative of three experiments. Data sets shown are experiments that included seven to ten mice in each group. C, Degranulated mast cells (arrowheads) in the lamina propria in the jejunum (Toluidine blue,  $\times 200$ ). Scale bar, 50  $\mu$ m. D, The number of total (closed bars) and degranulated (open bars) mast cells per high-power field in the jejunum. E, The percentage of degranulated mast cells in the jejunum. Data are presented as the mean  $\pm$  standard deviation; n.s., not significant;  $^{*}p < 0.05$ ;  $^{**}p < 0.01$ . Data sets are representative of three experiments. Data sets shown are experiments that used five mice in each group. NS challenge, normal saline challenge

(Figure 3B). OVA-specific IgG levels in the OIT (on sensitization) were significantly lower than those in the OIT (presensitization) group (Figure 3B). We further examined the OVA-specific IgG isotype. OVA-specific IgG1 levels were significantly lower in the OIT (on sensitization) group compared with those in the non-OIT control group (Figure 3C). OVA-specific IgG1 levels in the OIT (presensitization) group were also lower compared with those in the non-OIT control group, but the difference was not significant (Figure 3C). However, OVA-specific IgG2a levels in both the OIT (on sensitization) and OIT (presensitization) groups were comparable to the levels in the non-OIT group (Figure 3D). OVA-specific IgG2b levels in the OIT (on sensitization) group were also comparable with the levels in the non-OIT group (Figure 3E). OVA-specific IgG2b levels in the OIT (presensitization) group were lower compared with the levels in the non-OIT group (Figure 3E) but these differences were not significant (Figure 3E).

### 3.4 | Induction of regulatory T cells in lymph organs in mice with OIT (on sensitization) and OIT (presensitization) was systemic and local, respectively

Regulatory T cells have been reported to be associated with acquisition of both immune tolerance and desensitization to oral antigens.<sup>11,18</sup> To evaluate the systemic and local induction of regulatory T cells and type 2 helper T cells in lymph organs, we examined the forkhead box P3 (Foxp3), Gata binding protein-3 (Gata3) and interleukin (IL)-4 transcription levels in the spleen and MLN, respectively. In the MLN, Foxp3 transcription levels were significantly higher in the OIT (on sensitization) and OIT (presensitization) groups compared with the non-OIT control group (Figure 4A). Gata3 transcription levels were significantly lower in the OIT (on sensitization) compared with the non-OIT control group. However, Gata3 gene expression levels in the OIT (presensitization) group were not changed (Figure 4B). In addition, the IL-4 gene level was significantly lower in the OIT (on sensitization) group compared with the non-OIT control group. The IL-4 gene level in OIT (presensitization) tended to be low, but the difference was not statistically significant (Figure 4C). In the spleen, Foxp3 transcription levels were significantly higher in the OIT (on sensitization) group but not in the OIT (presensitization) group compared with the non-OIT control group (Figure 4D). The Gata3 transcription levels in the OIT (on sensitization) and OIT (presensitization) groups were equal to those in the non-OIT control group (Figure 4E). However, IL-4 gene expression levels were

significantly lower in both OIT (on sensitization) and OIT (presensitization) groups compared with the non-OIT control group (Figure 4F).

## 4 | DISCUSSION

It remains unknown whether OIT is effective for epicutaneous-sensitized food allergy. Here, we examined whether OIT has a preventive effect on epicutaneous-sensitized food allergy using the mouse model that we established previously.<sup>15</sup> We treated this model with OIT in the following two ways: OIT before sensitization or OIT during the sensitization phase. We showed that the systemic allergic reaction observed after an antigen challenge was relieved by OIT during the sensitization phase more effectively than that observed by OIT before sensitization.

Intestinal symptoms were significantly inhibited in the OIT (on sensitization) group compared with the non-treated control group. Inhibition in the OIT (presensitization) group was not statistically significant. However, skin symptoms were significantly suppressed in both OIT treatment groups compared with non-treated control group. Therefore, the preventive effect in the OIT (on sensitization) group was considered to be general, whereas the preventive effect in the OIT (presensitization) group was a partial effect.

Immune tolerance to a food allergen results when the antigen presentation in gut-draining lymph nodes leads to the development of regulatory T cells that inhibit Th2 cells, and these Th2 cells consequently induce B cells to switch to IgE-producing cells.<sup>11,18</sup> In this study, the induction of regulatory T cells in lymph organs was observed in MLNs in the OIT (presensitization) group, and this was also observed in systemic lymph organs, such as the spleen, in addition to MLN in the OIT (on sensitization) group. Therefore, we speculated that the difference in the preventive effects in both OIT treatment groups might depend on the degree of regulatory T cell development in lymph organs. In OIT (on sensitization), Gata3 expression levels, which indicate Th2 cell induction, were decreased in the MLN but not the spleen. This finding indicates that regulatory T cells that are induced OIT during sensitization and locally, but not systemically, ameliorated Th2 cell differentiation. In OIT (presensitization), inhibition of Th2 differentiation was not observed locally or systemically. However, IL-4 expression levels were ameliorated in the spleen and in the MLN in OIT (on sensitization). This was also observed in the spleen in OIT (presensitization). In food-allergic mice, a large amount of follicular helper T cells that generate elevated IL-4 and IL-21 levels were detected in lymph nodes, and they promoted antigen-specific

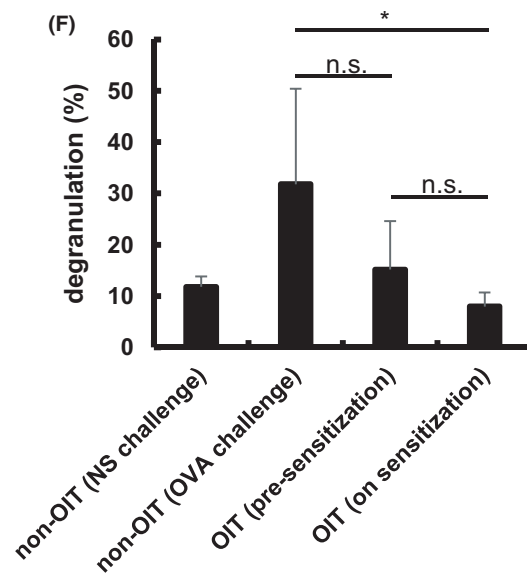
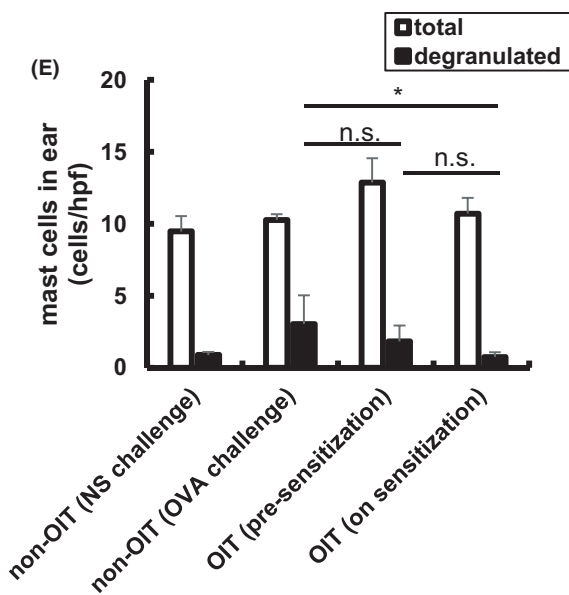
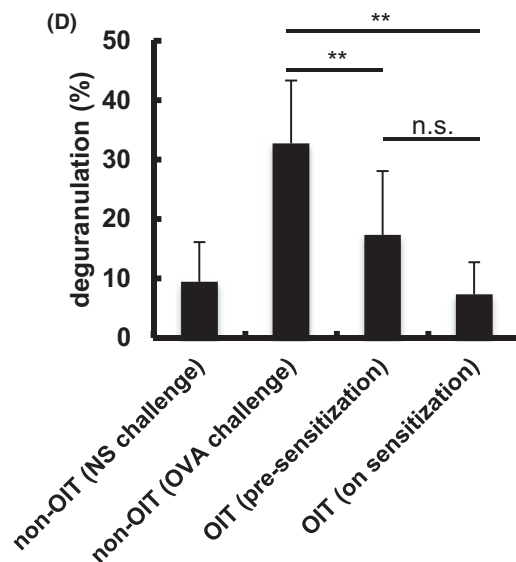
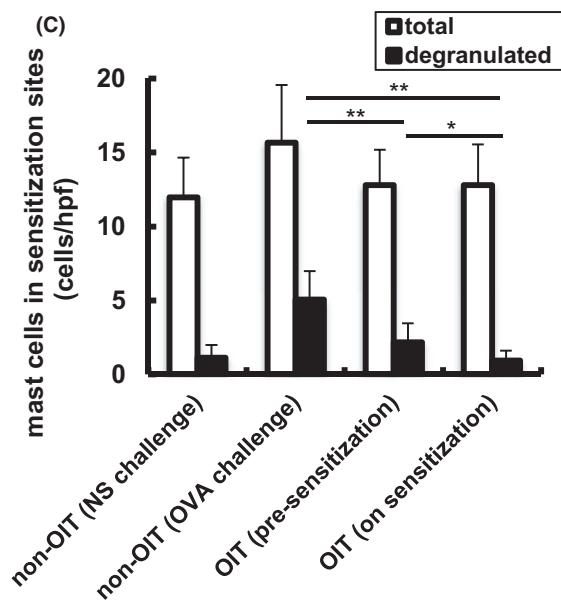
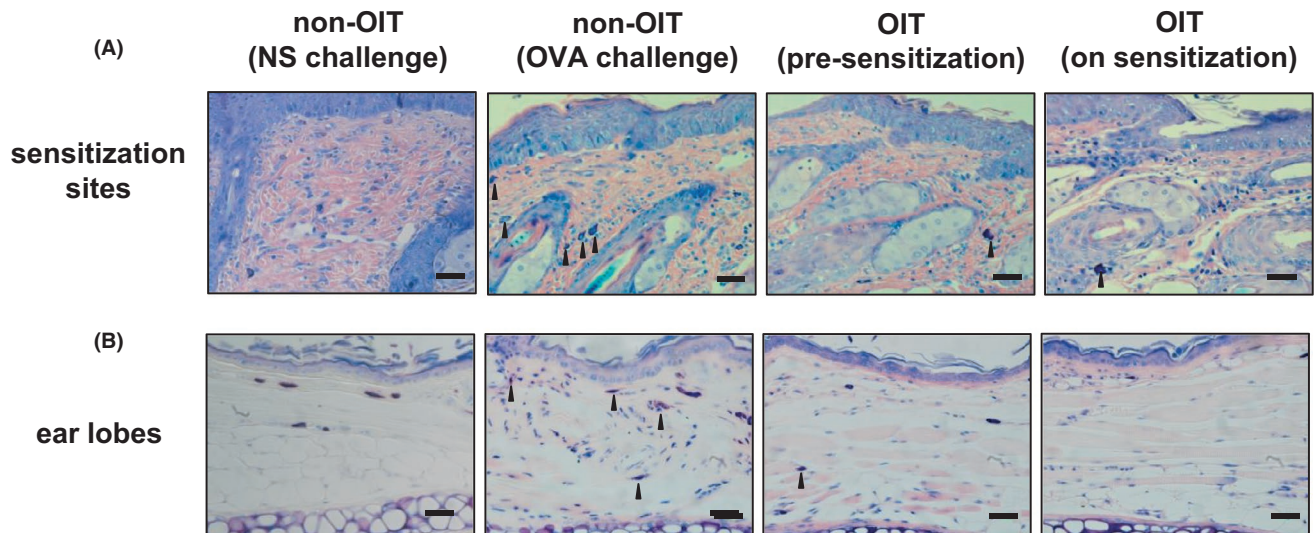


**FIGURE 2** The degranulation of mast cells in the skin of the sensitization sites and ear lobes. A, B, Degranulated mast cells (arrowheads) in the skin at the sensitization sites (A) and ear lobes (B) (May-Grunwald-Giemsa,  $\times 400$ ). Scale bar, 20  $\mu\text{m}$ . C, The cell number of total (closed bars) and degranulated (open bars) mast cells per high-power field at the sensitization sites. D, The percentage of degranulated mast cells at the sensitization sites. E, The number of total (closed bars) and degranulated (open bars) mast cells per high-power field in the ear lobes. F, The percentage of degranulated mast cells in the ear lobes. Data are presented as the mean  $\pm$  standard deviation; n.s., not significant; \* $p < 0.05$ , \*\* $p < 0.01$ . Data sets are representative of three experiments. Data sets shown are experiments that used five mice in each group. NS challenge, normal saline challenge

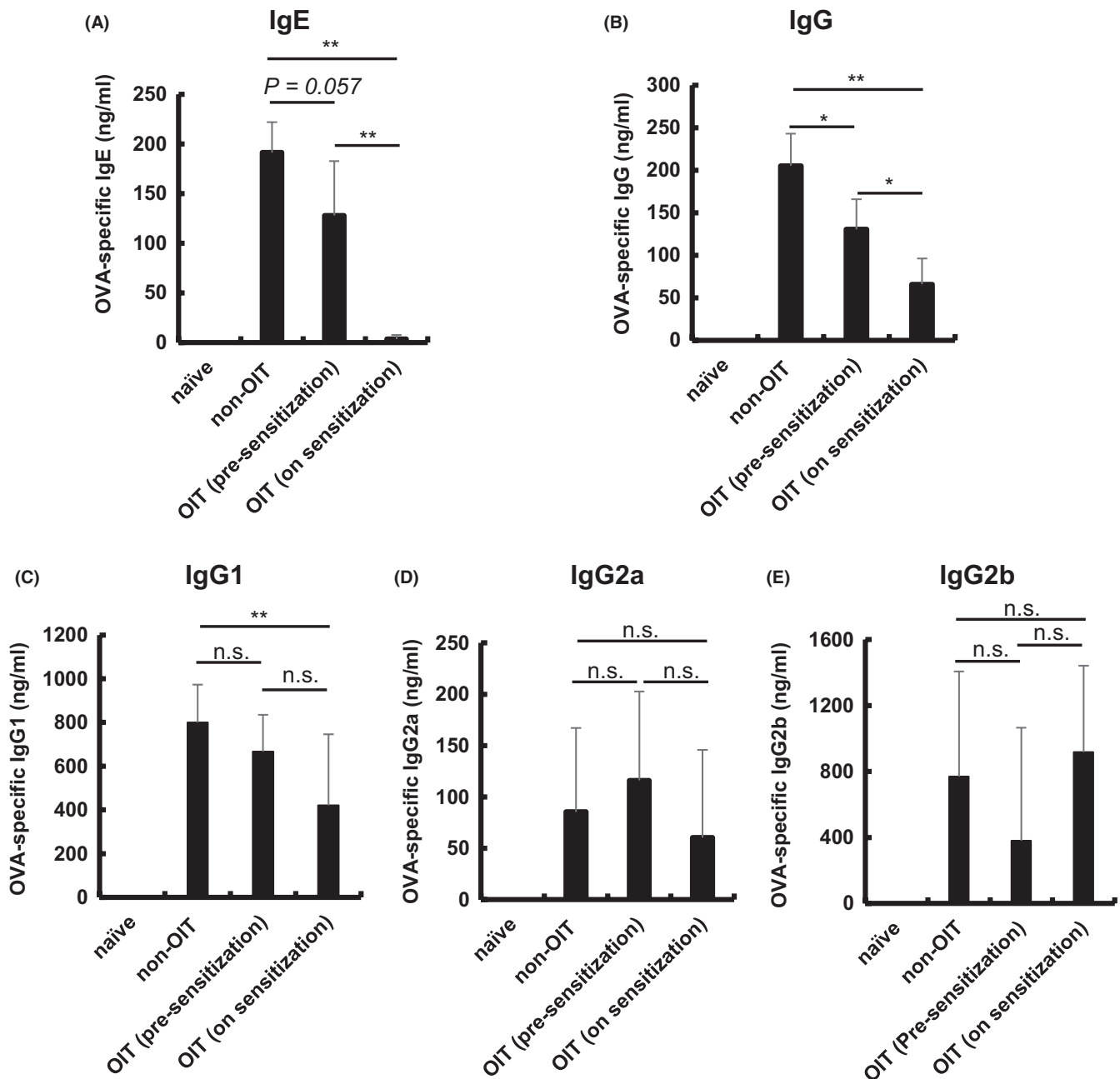


**FIGURE 3** The serum OVA-specific IgE and IgG levels. OVA-specific IgE (A), IgG (B), IgG1 (C), IgG2a (D) and IgG2b (E) concentrations in serum were determined by ELISA. Data are presented as the mean  $\pm$  standard deviation; n.s., not significant; \* $p < 0.05$ , \*\* $p < 0.01$ . Data sets are representative of three experiments. Data sets shown are experiments that used 7–10 mice in each group

IgE secretion.<sup>19</sup> Basophils were recruited into lymph organs and that sensitized the skin site after epicutaneous sensitization, and they contributed to the development of food allergy.<sup>20</sup> Basophils together

with dendritic cells promote Th2 differentiation via IL-4 in vitro.<sup>21</sup> These findings suggested that regulatory T cells that are induced in the spleen might suppress IL-4 production via follicular helper T cells

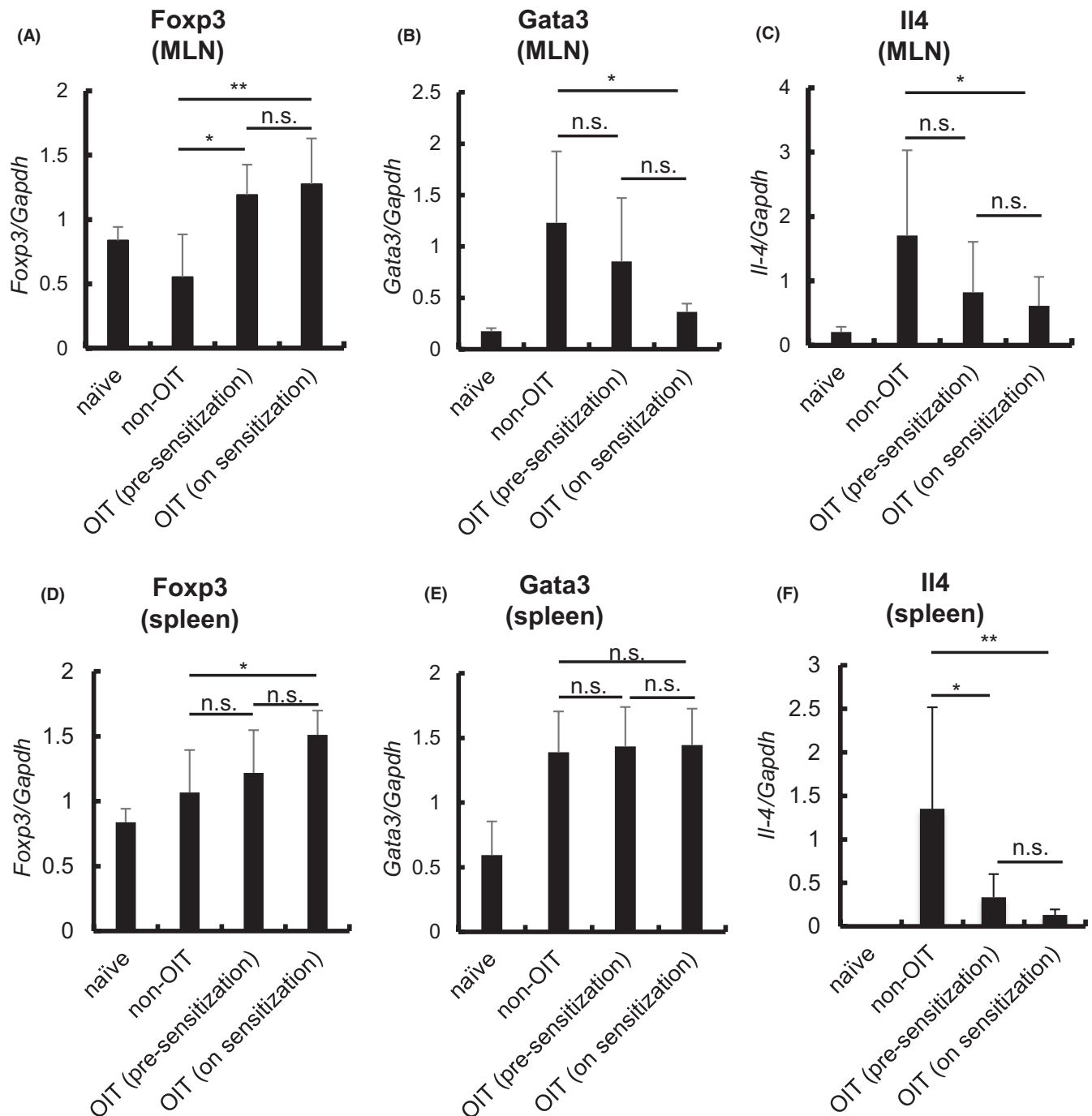


**FIGURE 4** Foxp3, Gata3 and IL-4 gene expression levels in lymph organs. A–C, Foxp3 (A), Gata3 (B) and IL-4 (C) transcript levels in MLN were measured using real-time PCR and normalized to Gapdh. (D–F) Foxp3 (D), Gata3 (E) and IL-4 (F) transcript levels in the spleen were measured using real-time PCR and normalized to Gapdh. Data are presented as the mean  $\pm$  standard deviation; n.s., not significant; \* $p < 0.05$ , \*\* $p < 0.01$ . Data sets are representative of three experiments. Data sets shown are experiments that used seven to ten mice in each group

and basophils, which promote antigen-specific IgE generation and Th2 differentiation.

In OIT (on sensitization), the systemic induction of regulatory T cells in lymph organs might contribute to effective suppression of the antigen-specific IgE generation and impairment of epicutaneous sensitization establishment, resulting in better symptom relief. However, in OIT (presensitization), regulatory T cell induction

was limited only in MLN, which induced a mild reduction of antigen-specific immunoglobulins, while antigen-specific IgG was significantly reduced. In addition, antigen-specific IgE tended to be suppressed, although the degree of suppression was not significant. Under such conditions, symptoms in the gut that was directly exposed to a large amount of antigen might not be suppressed, although the symptoms in the skin that was exposed to a

relatively small amount of antigen, which is spread via the blood, might be suppressed. In addition, suppression of skin symptoms in OIT (presensitization) may have been caused by infiltration of regulatory T cells into skin sites. Further studies (eg infiltration of regulatory T cells into peripheral tissues) are needed to clarify the discrepancy between skin symptoms and gut symptoms in OIT (presensitization).

The preventive effect of OIT is transient if the treatment is not continued long enough.<sup>22,23</sup> Muto et al used another mouse model of food allergy that was cutaneously sensitized with OVA in the presence of sodium dodecyl sulphate, and they showed that intra-gastric pretreatment of antigen before sensitization three times per week for 2 weeks successfully ameliorated rectal changes after the antigen challenge and antigen-specific IgE production.<sup>20</sup> Leonard et al used an orally OVA-sensitized mouse model, and they showed amelioration of the decrease in the rectal temperature in mice that received 2 weeks of OIT after sensitization. However, this amelioration was reversed 2 weeks after OIT was discontinued. In the OIT (presensitization) group in our study, the period of antigen pretreatment was only 1 week, and the period from the discontinuation of OIT to antigen challenge was 7 weeks. These findings suggest that pretreatment with antigen for a much longer time might yield more effective alleviation of symptoms even before sensitization. Additionally, more effective alleviation of symptoms might be observed if the antigen challenge was performed earlier after discontinuation of OIT.

Smaldini et al demonstrated that OIT-induced infiltration of regulatory T cells into the jejunum was associated with decreasing food-allergic reactions using a mouse model that was orally sensitized using cow's milk proteins.<sup>24</sup> They showed that, in the OIT-treated jejunum, the Foxp3 transcription levels were upregulated and Gata3 expression levels were downregulated.<sup>24</sup> In our study, in the jejunum, Foxp3 expression levels in both OIT (presensitization) and OIT (on sensitization) groups were not changed. However, Gata3 expression levels in both OIT groups were significantly lower than those of the non-OIT group (Figure S1), which might be associated with an induction of regulatory T cells in MLN. To clarify whether regulatory T cells had infiltrated into the jejunum in the OIT groups, further detailed studies using a flow cytometer are necessary.

In this study, OIT also suppressed basophil recruitment into the skin at the sensitization site in both ways (before and during sensitization) to about half the level of the non-OIT control groups (Figure S2). Thymic stromal lymphopoietin (TSLP) is essential for food antigen sensitization via the skin.<sup>20</sup> TSLP-elicited basophils in epicutaneous-sensitized skin promote an antigen-specific Th2 cytokine response when draining the lymph nodes, promoting intestinal food allergy.<sup>25</sup> These findings suggested that OIT might suppress basophil recruitment into the sensitization site, which results in hampering the formation of epicutaneous sensitization to food allergens. Further studies are necessary to reveal the mechanisms by which OIT inhibits the recruitment of basophils to the sensitization sites.

OIT for egg allergy is used in regular clinical practice. The most frequent adverse reactions (ARs) were gastrointestinal ARs. Non-IgE-mediated eosinophilic esophagitis is an AR to OIT for egg.<sup>26</sup> The

incidence of eosinophilic esophagitis during OIT for egg was relatively high (2.7%).<sup>26</sup> In our study, eosinophil infiltration into the jejunum in both OIT (presensitization) and OIT (on sensitization) was equal to those in the non-OIT control group (Figure S3). Daily or frequent exposure to the causative food sometimes leads to chronic food protein-induced enterocolitis syndrome.<sup>27</sup> Most cases occurred in infancy. The symptoms were characterized by intermittent vomiting, abdominal distension, chronic diarrhoea and poor weight gain.<sup>27</sup> In our study, we used adult mice. In addition, gastrointestinal manifestations and weight loss were not observed in OIT (presensitization) or in OIT (on sensitization).

Food allergy sometimes causes severe and potentially lethal allergic reactions such as anaphylaxis. Over 30% of child and adult patients reported severe reactions such as anaphylaxis or at least one food allergy-related emergency department visit.<sup>2,3</sup> In patients with epicutaneously sensitized hydrolysed wheat allergy, 25% of patients exhibited anaphylactic shock.<sup>9</sup> In the present study, OIT had a preventive effect on epicutaneously sensitized food allergy above a certain level in two ways (before or during the sensitization phase). OIT during the sensitization phase demonstrated better relief of both intestinal and skin symptoms. Thus, OIT has the potential to prevent a severe reaction or life-threatening anaphylaxis.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## AUTHOR CONTRIBUTIONS

YM, TU, KO, YH, NU and HI performed experiments. TU, KI and HY designed experiments and analysed data. TU and HY wrote the manuscript.

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## SUPPORTING INFORMATION

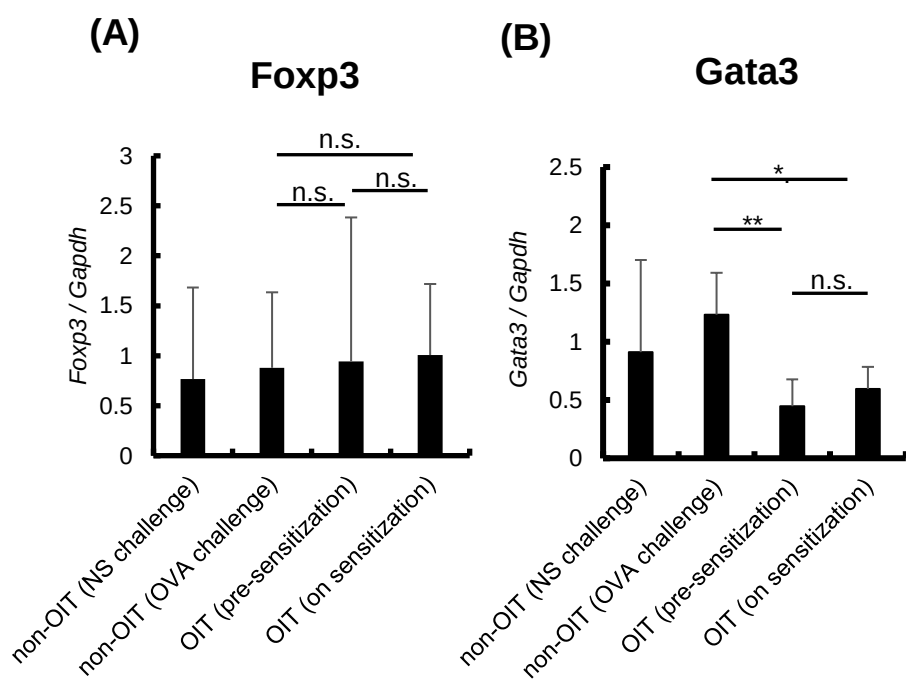
Additional supporting information may be found online in the Supporting Information section.

**Figure S1** Foxp3 and Gata3 gene expression levels in the jejunum. Foxp3 (A) and Gata3 (B) transcript levels in the jejunum were measured using real-time PCR and normalized to Gapdh. Data are presented as the mean  $\pm$  standard deviation; n.s., not significant; \* $p < 0.05$ ; \*\* $p < 0.01$ . Datasets are representative of two experiments. Datasets shown are experiments that used seven to ten mice in each group. NS challenge, normal saline challenge

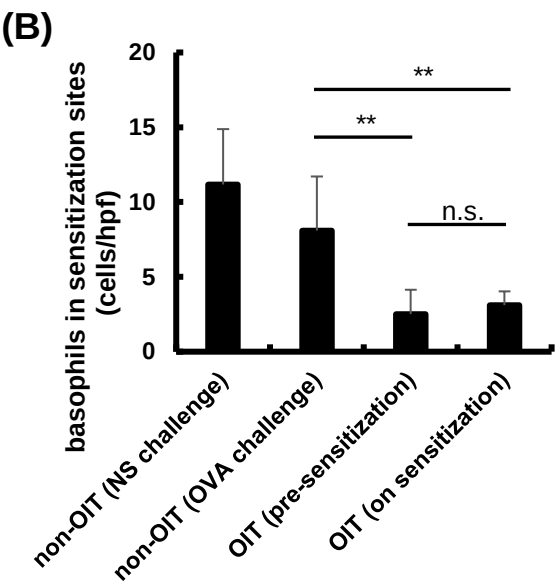
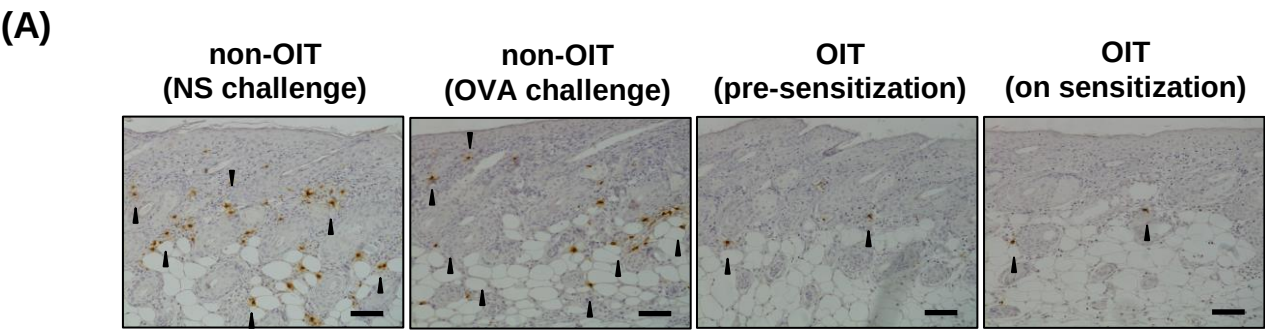
**Figure S2** Basophil infiltration into sensitized lesions was reduced. (A) Brown-stained basophils (arrowheads) in the sensitized lesions (reaction products visualized using DAB,  $\times 400$ ). Scale bar, 20  $\mu$ m. (B) The number of basophils per high-power field in the sensitized lesions. Data are presented as the mean  $\pm$  standard deviation; n.s., not significant; \*\* $p < 0.01$ . Datasets are representative of three experiments. Datasets shown are experiments that used five mice in each group. NS challenge, normal saline challenge

**Figure S3** Eosinophil infiltration into jejunum. (A) Red-stained eosinophils (arrowheads) in the jejunum (Luna stain,  $\times 200$ ). Scale bar, 50  $\mu$ m. (B) Number of eosinophils per high-power field in the sensitized lesions. Data are presented as the mean  $\pm$  standard deviation; n.s., not significant. Datasets shown are experiments that used five mice in each group. NS challenge, normal saline challenge

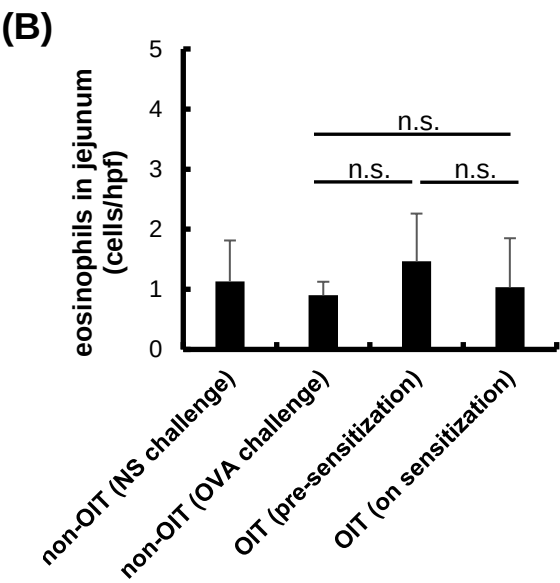
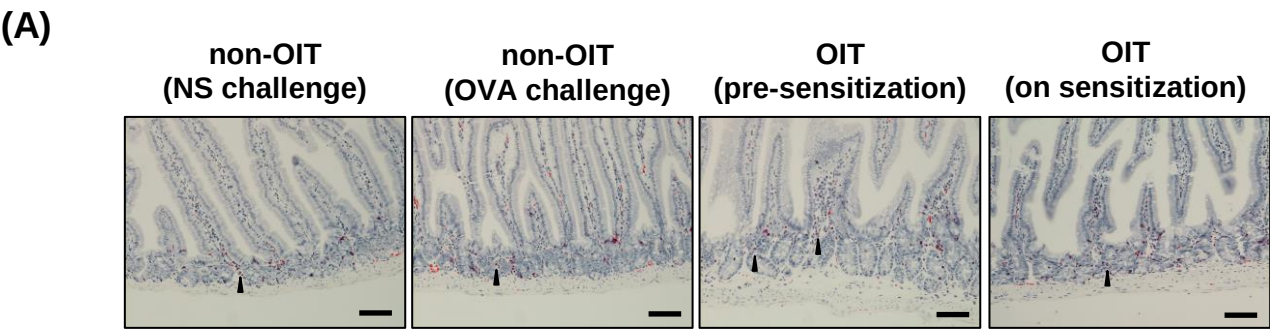
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Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3