



## Full Paper

## Oral allergy induction through skin exposure to previously tolerated food antigens in murine models



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## ARTICLE INFO

## Article history:

Received 14 February 2023

Received in revised form

16 March 2023

Accepted 27 March 2023

Available online 30 March 2023

## Keywords:

Food allergy

Oral tolerance

Murine model

Percutaneous sensitization

## ABSTRACT

Food allergies (FAs) are caused by a failure of the immune system to regulate oral tolerance (OT). The use of soap containing hydrolyzed wheat overrides acquired OT to wheat through skin exposure. However, in mouse models, the experimental OT is robust, suggesting that acquired OT to allergens prevents the development of FAs. We aimed to analyze the mechanisms and developed a mouse model of FA that overrides acquired OT via skin exposure. Three murine FA models (intraperitoneal [IP], epicutaneous [EC], and intradermal [ID]) were compared to evaluate if allergies to ovalbumin (OVA) that had been previously tolerated orally could be induced. In the ID model, OT was overridden, and allergic reactions of severe anaphylaxis were developed. To analyze this effect in the ID model, we measured the migration of dendritic cells (DCs) into lymph nodes. The induction of OT promoted the migration of CD103<sup>+</sup> dermal DCs; moreover, repeated percutaneous doses of OVA for sensitization gradually increased the migration of CD11b<sup>+</sup> dermal DCs. The difference in the proportion of regulatory T cells between ID-sensitized groups at the first ID injection disappeared at the tenth injection. Although OT was robust in the IP model, ID sensitization was found to override OT.

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## 1. Introduction

Immune tolerance to a wide range of foodstuffs is necessary for maintaining health. Previously, it has been thought that food allergy (FA) results from the sensitization of the immature gastrointestinal tract to certain types of food during infancy. However, a recent study suggested sensitization to food primarily develops through skin exposure to the allergen. Conversely, it is thought exposure of the intestinal tract to the allergens helps develop tolerance.<sup>1</sup>

**Abbreviations:** AUC, area under the curve; BDL, below the detection limit; DC, dendritic cell; DDC, dermal dendritic cell; EC, epicutaneous; FA, food allergy; FVD520, Fixable Viability Dye eFluor520; ID, intradermal; IP, intraperitoneal; LC, Langerhans cells; OT, oral tolerance; OVA, ovalbumin; SDLN, skin-draining lymph nodes; TSLP, thymic stromal lymphopoietin.

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Peer review under responsibility of Japanese Pharmacological Society.

In our previous research, we developed mouse models of both FA and oral tolerance (OT).<sup>2</sup> Further, the mice were intraperitoneally injected with ovalbumin (OVA) and then orally administered OVA to induce anaphylaxis. We found preliminary oral treatment with OVA prevented sensitization, almost completely suppressing the elevation of OVA-specific IgE and inducing OT. In other studies, experimental OT was found to suppress the development of autoimmune disease,<sup>3</sup> contact hypersensitivity,<sup>4</sup> and asthma.<sup>5,6</sup> This finding suggests OT is a consequence of homeostasis maintenance in a robust immune system. However, the experimentally induced OT to food allergens in these studies does not account for cases of sensitization to wheat caused by skin contact with wheat protein soap.<sup>7</sup> In the case, the patients who could eat foods containing wheat developed wheat-dependent exercise-induced anaphylaxis to use hydrolyzed wheat protein containing facial soap, and the reasons why skin exposure to a food antigen could override OT remain unclear. In the present study, we used murine models to investigate how skin exposure can cause allergen sensitization after induction of OT to a previously tolerated food antigen.

<https://doi.org/10.1016/j.jphs.2023.03.003>

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## 2. Materials and methods

### 2.1. Animals

Female BALB/c mice were purchased from Japan SLC, Inc (Hamamatsu). This study was conducted in accordance with the guidelines of the Animal Experiment Committees of Gifu Pharmaceutical University and the University of the Ryukyus and with the *Guide for the Care and Use of Laboratory Animals* by the National Institutes of Health. Given the risk of mortality due to anaphylaxis in this study, we established the endpoint for euthanasia as persistent hypothermia ( $>5^{\circ}\text{C}$  for 5 h).

### 2.2. Creation of murine food allergy and oral tolerance models

The FA and OT models were developed in the same way as described in our previous study (intraperitoneal [IP] model) (Fig. 1A, supplementary Table 1).<sup>2,8</sup> Briefly, we induced OT in BALB/c mice by administering 1 mg of OVA (Merck KGaA, Darmstadt, Germany) once

a day for 5 consecutive days. Moreover, the mice were sensitized via IP injection of 1  $\mu\text{g}$  OVA or phosphate-buffered saline (PBS), both mixed with Imject™ alum (Thermo Fisher Scientific, Rockford, IL, USA). Further, to induce an allergic response, 30 mg OVA was orally administered thrice a week for 3 weeks. In the epicutaneous (EC) model, OVA solution (1 mg OVA in 20  $\mu\text{L}$  PBS) or PBS was placed on the filter paper of the Finn chamber (Smart Practice, Phoenix, AZ, USA) for 3 days; it was then applied to the dorsocervical shaved skin with tape stripping and fixed at the site using the film dressing (Fig. 1B, supplementary Table 1). Furthermore, to create the intradermal (ID) model, 1  $\mu\text{g}$  OVA in 20  $\mu\text{L}$  PBS was injected intradermally into the back skin of each mouse without shaving or tape stripping (Fig. 1C, supplementary Table 1). Notably, ID injection was administered once a day for 3 consecutive days in a week over 4 weeks.

### 2.3. Evaluation of OVA-induced anaphylaxis

FA induction was estimated based on a decrease in body temperature using a rectal thermometer (KN-91, Natsume, Tokyo) for

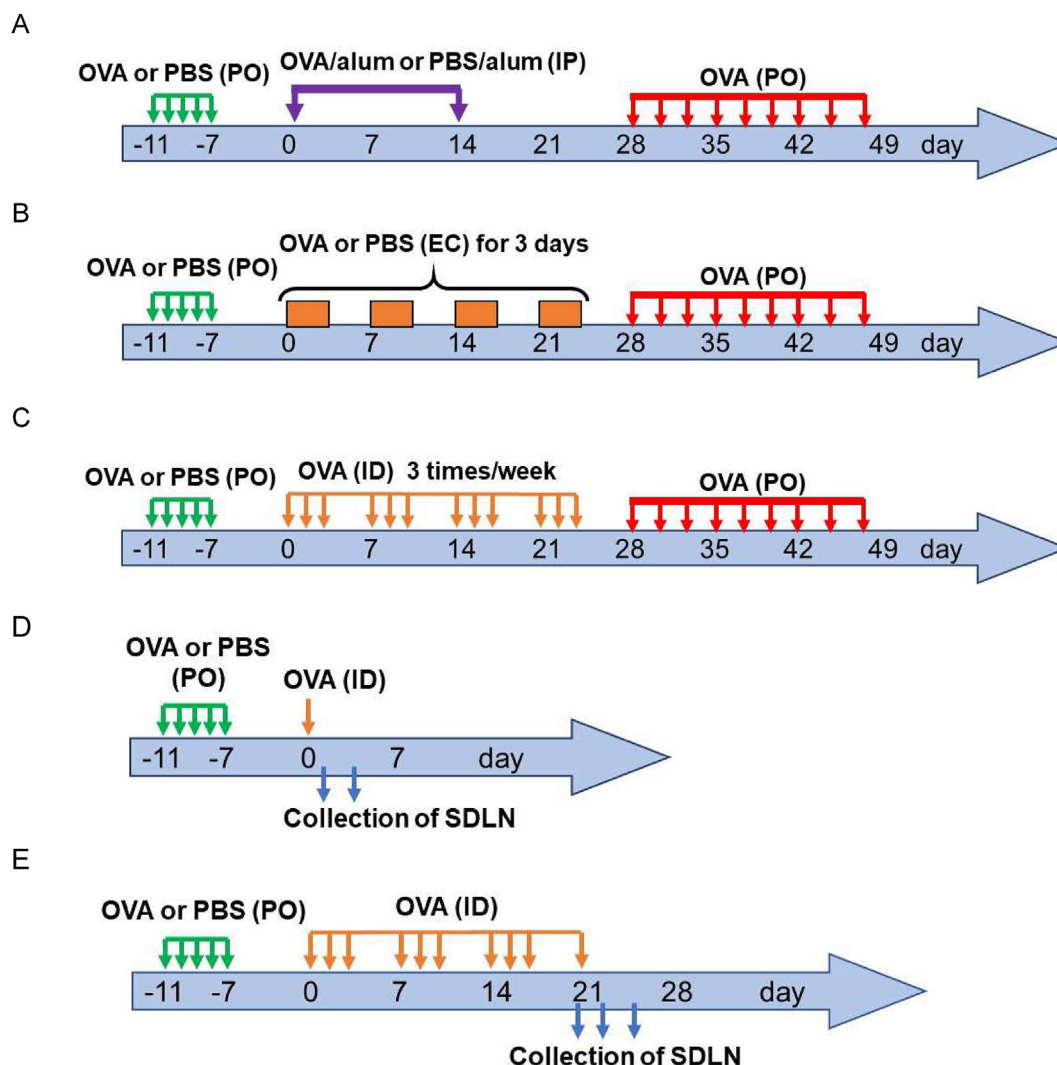


**Fig. 1.** The protocols of murine models of food allergy and oral tolerance. Intraperitoneal (IP) model: On days 0 and 14, BALB/c mice were sensitized via intraperitoneal injection with OVA or PBS mixed with adjuvant (alum). The oral administration of 30 mg OVA three times a week for 3 weeks (days 28, 31, 33, 35, 38, 40, 42, 45, and 47) caused a food allergy. Oral tolerance was induced by the oral administration of 1 mg OVA for 5 consecutive days (days -11, -10, -9, -8, and -7) (A). Epicutaneous sensitization (EC) model: Filter paper with OVA or PBS was attached to the back skin four times for 3 consecutive days a week (days 0–2, days 7–9, days 14–16, and days 21–23) (B). Intradermal (ID) model: ID injections were administered once a day for 3 consecutive days a week for 4 weeks (days 0, 1, 2, 7, 8, 9, 14, 15, 16, 21, 22, and 23) (C). On day 0, the mice were intradermally injected with 1  $\mu\text{g}$  OVA. On days 1 and 3, skin-draining lymph nodes (SDLN) were collected (D). The mice were intradermally injected with 1  $\mu\text{g}$  of OVA on days 0, 1, 2, 7, 8, 9, 14, 15, 16, and 21. SDLN were collected on days 21 (before the tenth injection), 22 (1 day after the tenth injection), and 24 (3 days after the tenth injection) (E).

1 h after the oral administration of 30 mg OVA and the symptoms of diarrhea.<sup>2,9</sup> Furthermore, allergic diarrhea was evaluated based on the shape and consistency of the feces, and it was assigned a severity score from 0 to 3, with 0, 1, 2, and 3 representing solid, semi-solid, slurry, and watery states, respectively.

#### 2.4. Detection of immunoglobulins, mast cell protease-1, and OVA in plasma

OVA-specific IgE and IgG1 levels in the plasma collected at the time points indicated in each graph were determined using an ELISA modified from the previously described methods.<sup>10</sup> Notably, the expression of murine mast cell protease-1—which is expressed in intestinal mucosal mast cells and promotes mucosal permeability in intestinal allergic hypersensitivity reactions<sup>11</sup>—against plasma collected 1 h after the last oral administration was measured using the ELISA kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Moreover, ELISA was used to determine the concentration of OVA in plasma in each model using the previously described methods.<sup>9</sup>

#### 2.5. Flow cytometry evaluation of dendritic cell migration into skin-draining lymph nodes and regulatory T-cells

Skin-draining lymph nodes (SDLN) (i.e., axillary and brachial lymph nodes) were collected from ID model mice 1 and 3 days after the first ID injection (days 1 and 3 in Fig. 1D, supplementary Table 2) as well as before and 1 and 3 days after the tenth injection (days 21, 22 and 24 in Fig. 1E, supplementary Table 2). Supplementary Figure 1 shows the classification of dendritic cells (DCs). DCs gated by the positive expression of CD11c (clone N418, BioLegend Inc., San Diego, CA, USA) were classified as CD11b (clone M1/70, BioLegend Inc.), CD207 (clone 4C7, BioLegend Inc.), and CD103 (clone 2E7, BioLegend Inc.). Anti-CD16/32 (clone 93, BioLegend Inc.) was used as an Fc blocker. Fixability Viability Dye eFluor520 (FVD520, Thermo Fisher Scientific) was used for the elimination of dead cells. We defined DC with intermediate expression of CD11b, positive expression of CD207, and low negative expression of CD103 (CD11b<sup>int</sup>CD207<sup>+</sup>CD103<sup>Lo/−</sup>DCs) as Langerhans cells (LCs), and CD11b<sup>int</sup>CD207<sup>+</sup> and high expression of CD103 (CD11b<sup>int</sup>CD207<sup>+</sup>CD103<sup>Hi</sup>DCs) as CD207<sup>+</sup>demal DCs (DDCs),<sup>12,13</sup> CD11b<sup>Hi</sup>CD207<sup>Lo/−</sup>CD103<sup>Lo/−</sup>DCs as CD11b<sup>+</sup>DDCs, and CD11b<sup>−</sup>CD207<sup>−</sup>CD103<sup>+</sup>DCs as CD103<sup>+</sup>DDCs.<sup>14,15</sup> Notably, data on LCs and DDCs were presented as the proportion of the number of cells divided by the number of CD11c<sup>+</sup> cells. Moreover, we defined CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>−</sup>T cells as Tregs to use anti-CD3 (clone 145-2C11, BioLegend Inc.), anti-CD4 (clone GK 1.5, BioLegend Inc.), anti-CD25 (clone PC61, BioLegend Inc.), and anti-CD127 (clone SB/199, BioLegend Inc.). Previously, it has been reported that there is an inverse correlation between the expression of FoxP3 and CD127 in CD4<sup>+</sup>CD25<sup>+</sup>T cells.<sup>16</sup> Furthermore, the proportion of Tregs was analyzed in SDLN collected 3 days after the first and the tenth ID injections.

#### 2.6. Statistical methods

Data are presented as mean ± standard error of the mean. Statistically significant differences between the study groups were determined using the Student's *t*-test, Welch's *t*-test, and Dunnett's, Tukey's, or Turkey–Kramer test. Moreover, the diarrheal scores for each mouse are presented as dots with the average score for each group. Furthermore, the statistical significance of the score was evaluated using the Mann–Whitney *U* test and Steel's or Steel–Dwass test.

### 3. Results

#### 3.1. Oral tolerance in the EC model

Regarding the comparison between the IP and EC models, body temperature decreased after the fifth administration (day 38) of OVA in the IP model FA group and after the seventh administration (day 42) in the EC model FA group (Fig. 2A). Moreover, after the last oral administration of OVA (day 47), the decrease in body temperature of the IP and EC model mice remained the same (Fig. 2B). Notably, similar results were found for allergic diarrhea (Fig. 2C). In the EC model OT group, following the last oral administration of OVA (day 47), few mice developed mild diarrhea (Fig. 2D). Fig. 2E presents changes in plasma levels of OVA-specific IgE. Higher levels of OVA-specific IgE were detected in the IP model FA group than in the EC model FA group on days 13 (before the second IP injection or the third EC sensitization), 27 (before the first oral administration), and 36 (before the fifth oral administration). Notably, the IgE levels were the same in both FA groups of the IP and EC models on day 46 (before the last oral administration) (Fig. 2F). OVA-specific IgE was detected in all mice in the EC model OT group but only one mouse in the IP model OT group (Fig. 2F).

#### 3.2. Oral tolerance in the ID model

We compared the IP, EC, and ID models to determine whether ID injection for allergen sensitization is an effective alternative to EC application. Notably, some mice in the FA groups died due to severe anaphylaxis following oral administration of OVA. Fig. 3A shows the survival curve of the analyzed mice. Interestingly, in the OT group of the ID model, one mouse died after the last administration (day 47). Fig. 3B, C and 3D present the body temperature data by the time course (Fig. 3B) as well as the data on the fifth (Fig. 3C, day 38) and the last oral administration (Fig. 3D, day 47). The figure also demonstrates severe hypothermia at the fifth oral administration was followed by a gradual decrease in body temperature, which further decreased in the IP and ID model FA groups. Notably, this symptom relief was caused by the death of several mice in each group after the fifth oral treatment (day 38). The number of mice is indicated by the numbers presented below in the column in Fig. 3C and D. The decrease in body temperature of the ID model OT group was more severe than that of the OT groups of IP model and EC model. (Fig. 3D). Remarkably, the onset of diarrhea occurred around the same time as the onset of hypothermia in the FA groups of each model (Fig. 3E). During the fifth oral administration (day 38), diarrhea observed in the OT group was lower than that observed in the FA group of the ID model (Fig. 3F). However, in the last oral administration (day 47), severe diarrhea was observed in the ID model OT group (Fig. 3G). Moreover, the IgE levels in the FA groups of the EC and ID models increased from day 27 (before the first oral administration, Fig. 4A) to 46 (before the last administration, Fig. 4B). Furthermore, elevated IgE levels were observed in the OT groups of the EC and ID models (Fig. 4A and B). Compared with the IgE levels in the OT groups of the IP model, the levels in the OT groups of the EC and ID models were significantly increased (Fig. 4B). As systemic anaphylaxis in mice was also mediated by IgG via macrophages and basophils,<sup>17,18</sup> we measured IgG1 levels in our analysis (Fig. 4C and D). Notably, the transition of IgG1 was similar to that of IgE. Moreover, the levels of mast cell protease-1—an indicator of degranulation of the intestinal mast cells—were elevated in all mice in the ID model OT group (Fig. 4E).

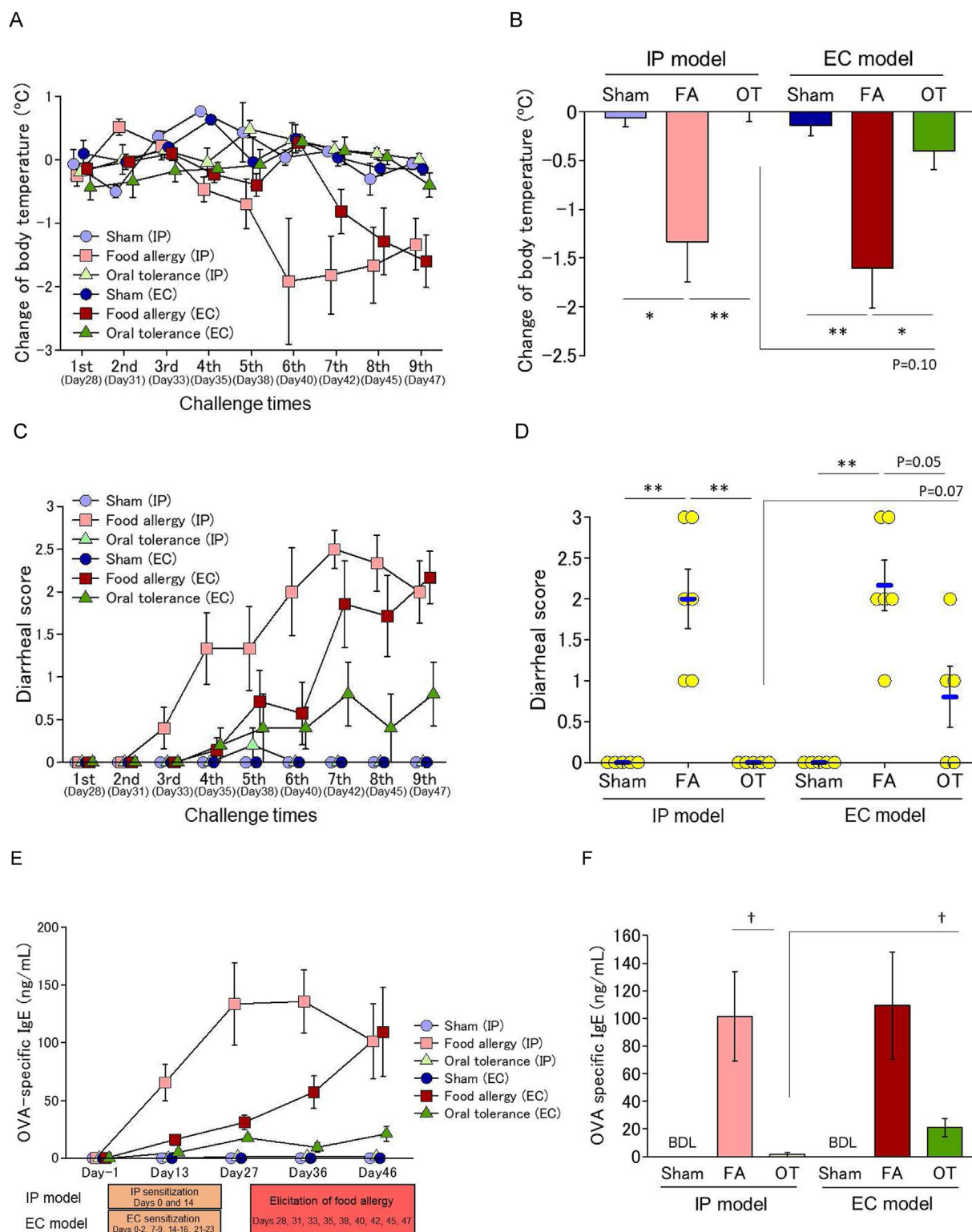


**Fig. 2.** Comparison of the intraperitoneal and epicutaneous sensitization models. A food allergy (FA) was induced in mice sensitized via intraperitoneal (IP) injection of OVA or OVA application to damaged skin (EC) by oral administration of 30 mg OVA (days 28, 31, 33, 35, 38, 40, 42, 45, and 47). Oral tolerance (OT) was induced before sensitization by oral administration of 1 mg OVA (days -11, -10, -9, -8, and -7). Anaphylaxis was evaluated by monitoring a decrease in rectal temperature (A, B) and by scoring the severity of allergic diarrhea (C, D). Panels A and C show the time course. Panels B and D show the data from the last oral administration (day 47). OVA-specific IgE levels in the plasma were measured using ELISA (E, time course; F, day 46). The values are shown as the mean  $\pm$  SEM ( $n = 5$  or  $6$ ). In panel D, each dot represents an individual datum, and the bars represent the mean values. \* $p < 0.05$ , \*\* $p < 0.01$ , or the directly indicated  $p$ -value are calculated for the FA group of each model using the Dunnett's or Steel's test.  $ip < 0.05$  and  $p$ -values are presented for data analyzed using the Student's or Welch's  $t$ -test or Mann-Whitney  $U$  test. BDL, below the detection limit.

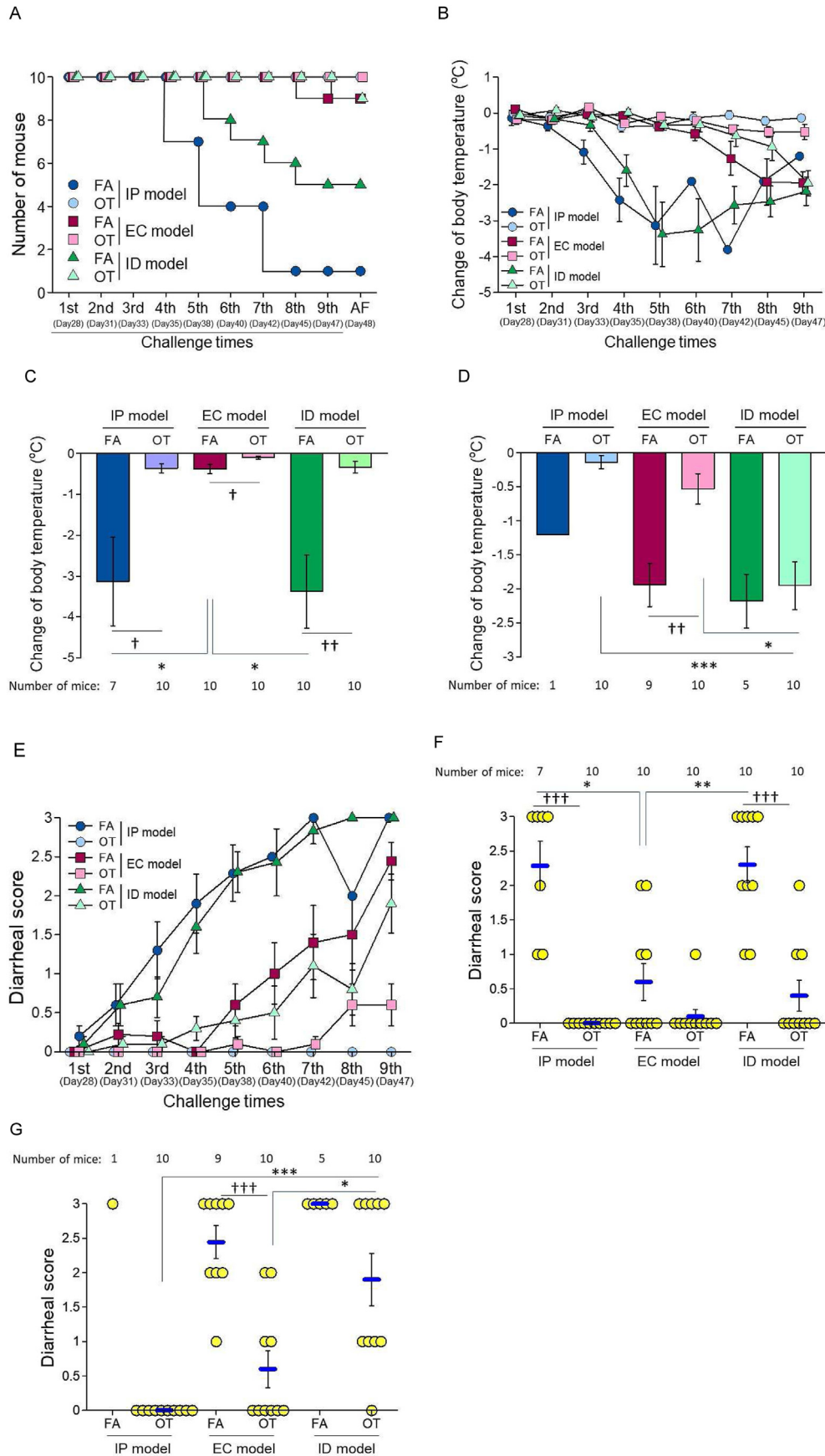


**Fig. 3.** Food allergy and oral tolerance in the intraperitoneal, epicutaneous, and intradermal models. The development of food allergy (FA) and the overriding of oral tolerance (OT) were estimated in the intraperitoneal (IP), epicutaneous (EC), and intradermal (ID) models. Data were compiled from two independent studies. Initially, there were 10 mice in each

### 3.3. The migration of dendritic cells into skin-draining lymph nodes and Treg proportion in the ID model

We attempted to elucidate the mechanism of OT overrides using the ID model. We analyzed the migration of DC into SDLN in the ID model at two time points: after the first injection in the first week (Fig. 1D) and before the first injection in the fourth week (Fig. 1E). Further, we estimated the expression of CD103 in the three cell populations (I, CD11b<sup>+</sup>CD207<sup>+</sup> cells; II, CD11b<sup>int</sup>CD207<sup>+</sup> cells; and III, CD11b<sup>hi</sup>CD207<sup>lo/-</sup> cells) on the contour line plot (X-axis, CD11b; Y-axis, CD207) gated in CD11c (Supplementary Fig. 1). Following the first injection in the first week, the proportion of LC in the OT group was significantly lower than that in the group sensitized via OVA injection (Sens group, Fig. 5A, day 3). Notably, there was no difference in CD207<sup>+</sup>DDC counts between the Sens and OT groups after the first injection (Fig. 5B). CD11b<sup>+</sup>DDC counts were decreased in all OT groups after 1 day of the first injection (Fig. 5C, day 1). In contrast, CD103<sup>+</sup>DDC counts were increased in all OT groups after 3 days of the first injection (Fig. 5D, day 3). However, in the fourth week, we were unable to confirm the differences in LC counts between the Sens and OT groups (Fig. 5E). We also found CD207<sup>+</sup>DDC counts increased in response to repeated OVA injections in the OT groups, with increases being more pronounced after 3 days of each injection (day 24) than after 1 day of each injection (Fig. 5F, day 22). A similar trend was observed in CD11b<sup>+</sup>DDC counts (Fig. 5G), and CD103<sup>+</sup>DDC counts with greater increases were observed 1 and 3 days after injections in the OT groups than in the Sens groups (Fig. 5H, days 22 and 24). Subsequently, we evaluated the proportion of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup>T cells defined as Tregs in SDLN of the ID model at 3 days after the first (Fig. 5I, day 3) and the tenth ID injections (Fig. 5J, day 24). Notably, we were able to confirm the differences between the Sens and OT groups in the first injection but not in the tenth injection.

## 4. Discussion

OT is characterized by the suppression of an excessive immune response to food. In particular, the development of FA is seen as overriding of OT. According to the dual allergen exposure hypothesis, tolerance occurs because of oral exposure to food, and allergic sensitization is caused by cutaneous exposure.<sup>19</sup> In particular, several epidemiological studies have reported that intentional feeding of allergenic foods to young children can prevent the development of FA.<sup>20–23</sup> A birth cohort study reported the use of neonatal moisturizers reduces the risk of developing atopic dermatitis and protein sensitization.<sup>24</sup> According to the results of these studies, the prevention of FAs requires early acquisition of OT for each allergen in childhood before skin contact sensitization. Furthermore, preliminary oral treatment with OVA prevented the humoral immune response of OVA-specific IgE and IgG in a mouse FA model.<sup>5</sup>

According to the abovementioned studies, acquired OT is a robust mechanism and its early induction is the most effective way to prevent FAs. However, an exception to this has been observed in cases of adult wheat allergies acquired through skin contact with soap containing hydrolyzed wheat protein. In such a case, percutaneous application of the allergen overrode previously acquired OT.<sup>7</sup> This suggests some degree of plasticity in the mechanisms underlying FA and tolerance development.

Based on the asthma model, we established an FA model via EC sensitization.<sup>25</sup> We then compared our standard FA model sensitized via IP injection (IP model) with the EC model. In the FA group of the IP model, the first IP injection of OVA with adjuvant led to an early rise in OVA-specific IgE, and the IgE level was peaked before the first oral administration (Day 13 and Day 27 in Fig. 2E). In the FA group of the EC model, IgE was upregulated later and had not risen compared to the IP model before the first oral administrations (Day 27 in Fig. 2E), but it eventually reached the same level as that in the FA group in the IP model (Fig. 2F). Notably, it is thought that the earlier increasing in IgE in the FA group of the IP model compared to EC model was involved in the manifestation of FA symptoms (Fig. 2A and C) and the survival (Fig. 3A). IP injection of the allergen in combination with an adjuvant as well as its administration quickly resulted in a strong immune response. Interestingly, although the strong systemic response in the IP model could not override OT, topical exposure could induce a mild increase in IgE level in the EC model (Fig. 2F). Moreover, although it has already been established murine FA models can be developed via EC sensitization,<sup>26</sup> we believe the clear detection of OVA-specific IgE in the OT group of the EC model is a novel finding. However, we were unsure whether EC sensitization overrode OT because IgE levels were low and FA symptoms were minimal (Fig. 2B and D). We have confirmed several times that the invalidation of OT in the EC model is weak and unstable (Figs. 2, 3 and 4, and unpublished data). We hypothesized the lack of stable FA symptoms was due to a variation in the rate of plasma IgE levels in the EC model OT group. Furthermore, the variation was dependent on successful EC sensitization. During 3 days of EC sensitization, the OVA filters attached to some mice were removed by self-scratching and biting by other mice. Biting the filter may affect the acquisition or interruption of OT. Therefore, we established the ID model.

Notably, IL-33 and thymic stromal lymphopoietin (TSLP), produced in response to skin damage, are known to accelerate Th2 responses.<sup>27,28</sup> In a murine asthma model, ID injection of OVA with TSLP or IL-33 increased IgE production and worsened asthma-like airway inflammation.<sup>29,30</sup> As the cytokines were required for the development of allergic symptoms in these reports, we thought adjuvant treatment will be necessary to initially induce ID sensitization. Accordingly, we administered ID injections of OVA with IL-33 as a strong adjuvant (data not shown). However, a single ID injection of OVA without IL-33 was sufficient to cause percutaneous sensitization and increase OVA-specific IgE levels (Fig. 4A and B). Notably, ID-sensitized mice in the FA group experienced severe hypothermia and some died (Fig. 3A and D). One of our most interesting findings was that ID injection of OVA without adjuvant had a strong sensitizing effect.

To clarify the difference in sensitization between the IP, EC, and ID models, we compared plasma OVA concentrations (Supplementary Fig. 2). Although the OVA concentration became high for a short time upon EC sensitization, it did not persist (Supplementary Fig. 2A). In our ID model, 3 consecutive days of peak OVA concentration and a stable concentration on each day were observed (Supplementary Fig. 2B and C). We believe FA symptoms were induced in our ID model due to the lower amount of OVA and higher frequency of injections.

The experimentally induced OT was robust and lethal anaphylactic shock was rare, but one mouse died due to hypothermia in the ID model OT group (Fig. 3A). Furthermore, IgE levels increased

group. Some mice died due to anaphylaxis; a survival graph is shown (A). The upper and lower numbers of the columns denote the numbers of the mice. Anaphylaxis was indicated by a decrease in rectal temperature (B–D) and by scoring allergic diarrhea (E–G). Panels B and E show the time course. Panels C and F provide data from the fifth oral administration (day 38), and panels D and G provide data from the last oral administration (day 47). Statistical analyses were conducted using the Tukey and Tukey–Kramer tests, Steel–Dwass test (\**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001 among the OT groups), Student's and Welch's *t* tests, or Mann–Whitney *U* test. (†*p* < 0.05, ††*p* < 0.01, and †††*p* < 0.001, FA vs. OT). AF, after the last administration.

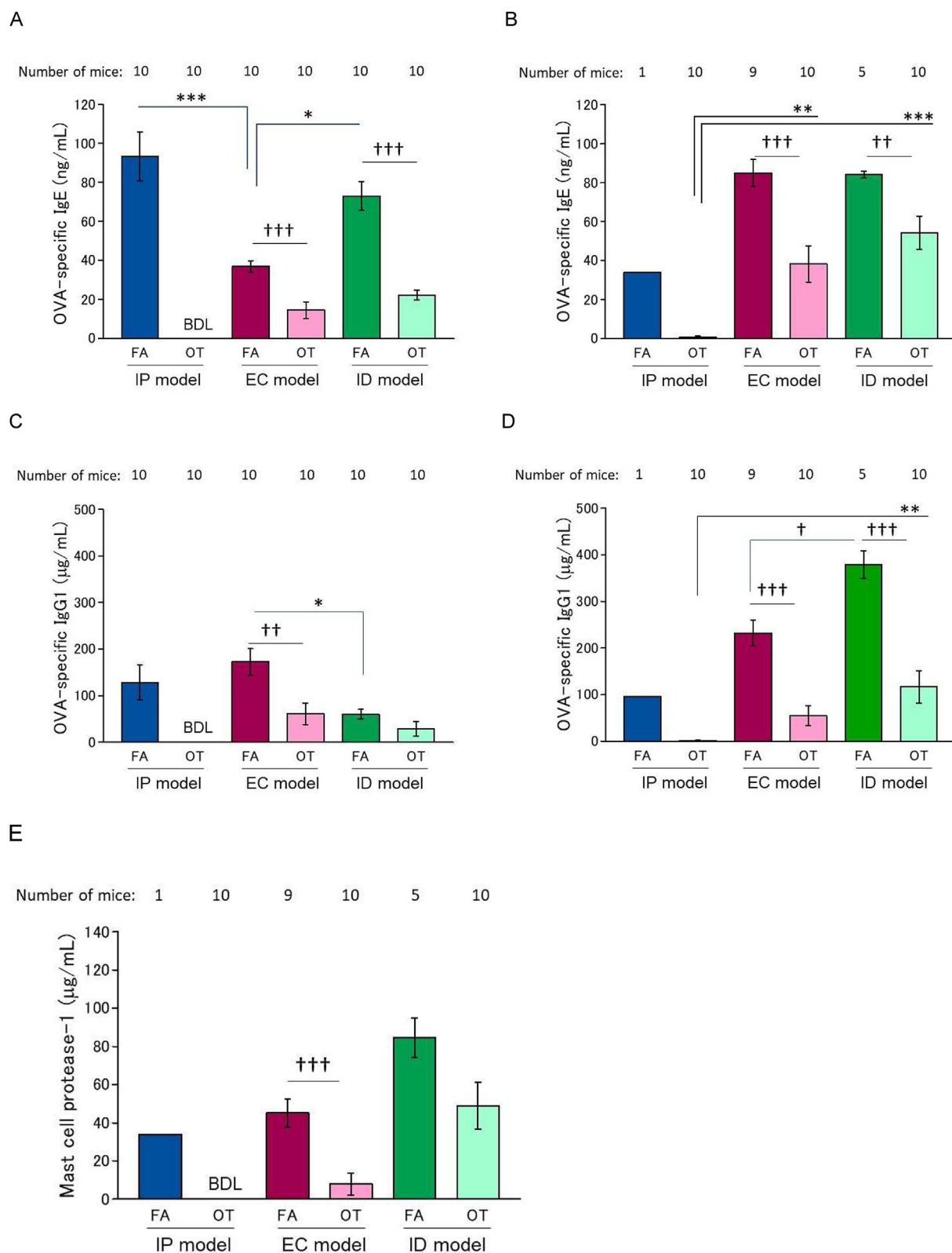


**Fig. 4.** Immunoglobulin and mast cell protease-1 levels in the FA and OT groups of each model. OVA-specific IgE (A, B) and IgG1 (C, D) levels in the plasma were measured using ELISA (A and C on day 27, B and D on day 46). Mast cell protease-1 levels were measured using ELISA in the plasma collected 1 h after the last oral administration on day 47 as an indicator of intestinal mast cell degranulation (E). The upper numbers in the columns represent the number of mice. Statistical analyses were conducted using the Tukey and Tukey–Kramer test (\* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  among the OT groups) or Student's and Welch's  $t$ -tests ( $\dagger p < 0.05$  and  $\dagger\dagger\dagger p < 0.001$ , FA vs. OT). BDL, below the detection limit.

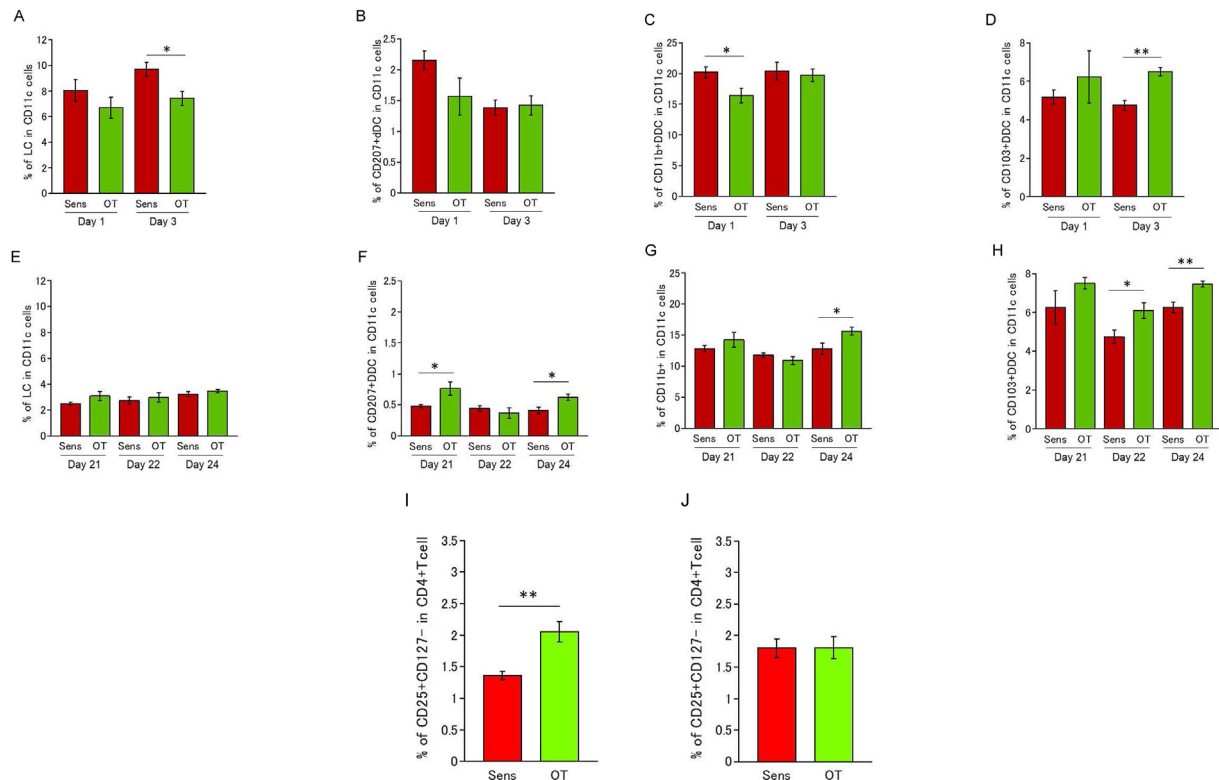


**Fig. 5.** Migration of dendritic and regulatory T cells into skin-draining lymph nodes. We measured the number of dendritic cells (DCs) that migrated into skin-draining lymph nodes (SDLN) 1 and 3 days after the first intradermal injection of OVA in the first week of ID model protocol (Day 1 and Day 3, A–D), and before or 1 and 3 days after the fourth-week injection of ID model protocol (Day 21, Day 22, and Day 24, E–H). DC gated by the positive expression of CD11c were classified as CD11b, CD207, and CD103. Notably, we defined  $CD11b^{int}CD207^{+}CD103^{Lo/-}$  DC as Langerhans cells (LC) (A, E) and  $CD11b^{int}CD207^{+}CD103^{Hi}$  DC as CD207<sup>+</sup>dermal DC (DDC) (B, F). We defined  $CD11b^{Hi}CD207^{Lo/-}CD103^{Lo/-}$  DC as  $CD11b^{+}DDC$  (C, G) and  $CD11b^{-}CD207^{-}CD103^{+}DC$  as  $CD103^{+}DDC$  (D, H). Data were adjusted for the number of  $CD11c^{+}$  cells. The proportion of regulatory T cells (Treg) defined as  $CD3^{+}CD4^{+}CD25^{+}CD127^{-}$  in SDLN was analyzed on day 3 of the first and fourth week (Day 3 and Day 24, I, J). (\* $p < 0.05$  and \*\* $p < 0.01$ , Sens vs. OT).

in the OT group of the ID model compared to the IP model (Fig. 4B). Notably, the data of the ID model OT group indicated obvious anaphylaxis (Figs. 3D, G, and 4E). Some symptoms in the OT group of the ID model were mild compared to the FA group, but the observation of clear symptoms was an important finding for elucidating the mechanism of OT invalidation. Thus, we were able to create a stable FA model through percutaneous sensitization that could override the previously acquired OT via ID injection. Effective overriding of OT in the ID sensitization might depend on long-term optimal blood OVA concentration (Supplementary Fig. 2).

Two underlying pathways of anaphylaxis have been defined in mice: the classical IgE-mediated pathway and the alternative IgG-mediated pathway.<sup>31</sup> Thus, we measured OVA-specific IgG1 levels in addition to IgE levels (Fig. 4C and D). Notably, after sensitization, the IgE level of the ID model FA group was higher than that of the EC model; however, the result for the IgG1 level was the opposite (Fig. 4A and C). On the date before the last administration, a similar trend was observed in the IgG1 and IgE levels in the FA and OT groups in each model (Fig. 4B and D). These data prohibited us to assess IgE- or IgG-dependent anaphylaxis in the EC and ID models.

It would be better to analyze the EC model to elucidate the mechanism of overriding OT for using hydrolyzed wheat-containing soap. However, the OT invalidation in the EC model was weak and unstable, therefore we estimated the overriding OT in the ID model to analyze DC migration into SDLN. DCs are heterogeneous leukocytes found in the epidermis and dermis and are involved in both the allergic response and immune tolerance.<sup>14</sup> LC is a subset of DCs found in the epidermis. Murine LC has been characterized by the expression of surface markers, such as langerin (CD207), CD11c, CD11b, and epithelial cell adhesion molecules.<sup>32</sup> Another CD207<sup>+</sup>DC subgroup is

CD207<sup>+</sup>dermal DC (CD207<sup>+</sup>DDC), which expresses CD103 in the dermis.<sup>13,32</sup> In our models, LC was found to be related to the initiation of allergic responses after percutaneous sensitization in the first week; however, it may not be related after removing OT from data in the fourth week (Fig. 5A and E). CD207<sup>+</sup>DDC increased in the OT groups in the fourth week, suggesting that it plays a role in overriding OT (Fig. 5F). CD103<sup>+</sup>DDC and CD11b<sup>+</sup>DDC are subsets found in the dermis.<sup>14,33</sup> The CD103<sup>+</sup>DC subset can promote OT in an FA model according to activation of regulatory T-cells in the intestines.<sup>9,34</sup> CD11b<sup>+</sup>DC subset can activate CD4<sup>+</sup>T-cells and accelerate Th2-differentiation.<sup>35</sup> As tape stripping induce long-lasting CD103<sup>+</sup>DDC (equivalent to CD11b<sup>+</sup>DDC) migration into SDLN,<sup>36</sup> CD11b<sup>+</sup>DC might have initiated sensitization to food antigens in the percutaneous sensitization. Moreover, CD11b<sup>+</sup>DC migration into mesenteric lymph nodes was related OT invalidation.<sup>9</sup> Therefore, in FA induced by percutaneous sensitization, CD103<sup>+</sup>DDC may be associated with OT for food antigens, whereas CD11b<sup>+</sup>DDC may be associated with food antigen sensitization. We concluded that the immune tolerance induced by CD103<sup>+</sup>DDC subset was broken by the induction of inflammatory CD11b<sup>+</sup>DDC subset by the repeated immune stimulations through the skin.

In addition to the timing of DC analyses, we examined the proportion of Tregs in SDLN collected 3 days after the first (day 3) and the tenth oral administration (day 24). We found the proportional difference between the Sens and OT groups on the first week was reduced on the fourth week (Fig. 5I and J). Notably, the effect of repeated ID injection on DC migration overrode OT, depending on Treg.

We were unable to identify the mechanisms through which the percutaneous administration of OVA overrode more effectively

than systemic IP injection of OVA. In the IP model, macrophages<sup>37</sup> and inflammatory monocytes<sup>38</sup> could capture the antigen in the systemic immune system. We hypothesized differences in antigen-capturing cells and pathways are related to the differential effects of EC and IP allergen administration on established OT.

The primary aim of this study was to reproduce the case of wheat soap in animal models. Therefore, we developed the EC model for OT. However, we were not able to obtain a stable refractive OT in the EC model. We then developed the ID model, which obtained stable results using a simple technique, but it was an artificial model. However, we believe both models share similar sensitization mechanisms. OVA adhered to the injured skin is absorbed through the dermis and be captured by the DDCs in the EC sensitization.

We developed an FA model in which sensitization to food allergens was induced via ID injection. We found ID injection of an allergen could efficiently override a previously acquired OT. We believe further analysis of our ID models of FA and OT will help elucidate the causes of FAs.

### Ethics statement

This study was conducted in accordance with the guidelines of the Animal Committee of Gifu Pharmaceutical University, Japan, and the study protocol was approved by the Institutional Review Board of Gifu Pharmaceutical University (approval numbers: 2015-128, 2016-040, 2017-121, and 2017-270). The study also followed the guidelines of the Animal Committee of the University of the Ryukyus, Japan, and was approved by the Institutional Review Board of the University of the Ryukyus (approval number: A2020021). Given the risk of mortality due to anaphylaxis in this study, we established the endpoint for euthanasia as persistent hypothermia ( $>5^{\circ}\text{C}$  for 5 h).

### Author contributions

HM collected and analyzed the data. HT, NI, and MT participated in the design of the study and drafting of the manuscript. HY conceived the study and participated in the design and drafting of the manuscript.

### Declaration of competing interest

The authors declare no conflicts of interest.

### Acknowledgments

This study was supported by funding from Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research (JP19K0761) and the Nipponham Foundation for the Future of Food, both awarded to HY. We would like to thank Enago for the English language review.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jphs.2023.03.003>.

### References

1. Lack G. Update on risk factors for food allergy. *J Allergy Clin Immunol*. 2012;129:1187–1197.
2. Yamashita H, Takahashi K, Tanaka H, Nagai H, Inagaki N. Overcoming food allergy through acquired tolerance conferred by transfer of Tregs in a murine model. *Allergy*. 2012;67:201–209.
3. Chen Y, Inobe J, Kuchroo VK, Baron JL, Janeway Jr CA, Weiner HL. Oral tolerance in myelin basic protein T-cell receptor transgenic mice: suppression of autoimmune encephalomyelitis and dose-dependent induction of regulatory cells. *Proc Natl Acad Sci U S A*. 1996;93:388–391.
4. Galliaerdes V, Desvignes C, Peyron E, Kaiserlian D. Oral tolerance to haptens: intestinal epithelial cells from 2,4-dinitrochlorobenzene-fed mice inhibit hapten-specific T cell activation in vitro. *Eur J Immunol*. 1995;25:1385–1390.
5. Russo M, Nahori MA, Lefort J, et al. Suppression of asthma-like responses in different mouse strains by oral tolerance. *Am J Respir Cell Mol Biol*. 2001;24:518–526.
6. Mucida D, Kutchukhidze N, Erazo A, Russo M, Lafaille JJ, Curotto de Lafaille MA. Oral tolerance in the absence of naturally occurring Tregs. *J Clin Invest*. 2005;115:1923–1933.
7. Fukutomi Y, Taniguchi M, Nakamura H, Akiyama K. Epidemiological link between wheat allergy and exposure to hydrolyzed wheat protein in facial soap. *Allergy*. 2014;69:1405–1411.
8. Yamashita H, Hayashi T, Saneyasu K, et al. Immune suppression of food allergy by maternal IgG in murine models. *Allergol Int*. 2018;67:506–514.
9. Yamashita H, Matsuhara H, Miotani S, et al. Artificial sweeteners and mixture of food additives cause to break oral tolerance and induce food allergy in murine oral tolerance model for food allergy. *Clin Exp Allergy*. 2017;47:1204–1213.
10. Nagai H, Maeda Y, Tanaka H. The effect of anti-IL-4 monoclonal antibody, rapamycin and interferon-gamma on airway hyperreactivity to acetylcholine in mice. *Clin Exp Allergy*. 1997;27:218–224.
11. Wastling JM, Knight P, Ure J, et al. Histochemical and ultrastructural modification of mucosal mast cell granules in parasitized mice lacking the beta-chymase, mouse mast cell protease-1. *Am J Pathol*. 1998;153:491–504.
12. Henri S, Poulin LF, Tamoutounour S, et al. CD207+ CD103+ dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. *J Exp Med*. 2010;207:189–206.
13. Chu CC, Di Meglio P, Nestle FO. Harnessing dendritic cells in inflammatory skin diseases. *Semin Immunol*. 2011;23:28–41.
14. Heath WR, Carbone FR. The skin-resident and migratory immune system in steady state and memory: innate lymphocytes, dendritic cells and T cells. *Nat Immunol*. 2013;14:978–985.
15. Zanna MY, Yasmin AR, Omar AR, et al. Review of dendritic cells, their role in clinical immunology, and distribution in various animal species. *Int J Mol Sci*. 2021;22:8044.
16. Seddiki N, Santner-Nanan B, Martinson J, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med*. 2006;203:1693–1700.
17. Strait RT, Morris SC, Yang M, Qu XW, Finkelman FD. Pathways of anaphylaxis in the mouse. *J Allergy Clin Immunol*. 2002;109:658–668.
18. Tsujimura Y, Obata K, Mukai K, et al. Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis. *Immunity*. 2008;28:581–589.
19. Lack G. Epidemiologic risks for food allergy. *J Allergy Clin Immunol*. 2008;121:1331–1336.
20. Du Toit G, Roberts G, Sayre PH, et al. Randomized trial of peanut consumption in infants at risk for peanut allergy. *N Engl J Med*. 2015;372:803–813.
21. Du Toit G, Sayre PH, Roberts G, et al. Effect of avoidance on peanut allergy after early peanut consumption. *N Engl J Med*. 2016;374:1435–1443.
22. Perkin MR, Logan K, Tseng A, et al. Randomized trial of introduction of allergenic foods in breast-fed infants. *N Engl J Med*. 2016;374:1733–1743.
23. Natsume O, Kabashima S, Nakazato J, et al. Two-step egg introduction for prevention of egg allergy in high-risk infants with eczema (PETIT): a randomised, double-blind, placebo-controlled trial. *Lancet*. 2017;389:276–286.
24. Horimukai K, Morita K, Narita M, et al. Application of moisturizer to neonates prevents development of atopic dermatitis. *J Allergy Clin Immunol*. 2014;134:824–830.e6.
25. Kodama M, Asano K, Oguma T, et al. Strain-specific phenotypes of airway inflammation and bronchial hyperresponsiveness induced by epicutaneous allergen sensitization in BALB/c and C57BL/6 mice. *Int Arch Allergy Immunol*. 2010;152(Suppl 1):67–74.
26. Leyva-Castillo JM, Galand C, Kam C, et al. Mechanical skin injury promotes food anaphylaxis by driving intestinal mast cell expansion. *Immunity*. 2019;50:1262–1275.e4.
27. Muto T, Fukuoka A, Kabashima K, et al. The role of basophils and proallergic cytokines, TSLP and IL-33, in cutaneously sensitized food allergy. *Int Immunol*. 2014;26:539–549.
28. Oyoshi MK, Larson RP, Ziegler SF, Geha RS. Mechanical injury polarizes skin dendritic cells to elicit a T(H)2 response by inducing cutaneous thymic stromal lymphopoietin expression. *J Allergy Clin Immunol*. 2010;126:976–984.e5.
29. Han H, Xu W, Headley MB, et al. Thymic stromal lymphopoietin (TSLP)-mediated dermal inflammation aggravates experimental asthma. *Mucosal Immunol*. 2012;5:342–351.
30. Han H, Ziegler SF. Intradermal administration of IL-33 induces allergic airway inflammation. *Sci Rep*. 2017;7:1706.
31. Escribese MM, Rosace D, Chivato T, Fernández TD, Corbí AL, Barber D. Alternative anaphylactic routes: the potential role of macrophages. *Front Immunol*. 2017;8:515.

32. Merad M, Ginhoux F, Collin M. Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. *Nat Rev Immunol*. 2008;8: 935–947.
33. Ginhoux F, Liu K, Helft J, et al. The origin and development of nonlymphoid tissue CD103+ DCs. *J Exp Med*. 2009;206:3115–3130.
34. Coombes JL, Siddiqui KR, Arancibia-Cárcamo CV, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med*. 2007;204: 1757–1764.
35. Williams JW, Tjota MY, Clay BS, et al. Transcription factor IRF4 drives dendritic cells to promote Th2 differentiation. *Nat Commun*. 2013;4:2990.
36. Tomura M, Hata A, Matsuoka S, et al. Tracking and quantification of dendritic cell migration and antigen trafficking between the skin and lymph nodes. *Sci Rep*. 2014;4:6030.
37. Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature*. 2008;453:1122–1126.
38. Kool M, Soullié T, van Nimwegen M, et al. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J Exp Med*. 2008;205:869–882.