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Original Article

The optimal age for epicutaneous sensitization following tape-stripping in BALB/c mice

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DCs	dendritic cells
LCs	Langerhans cells
OVA	ovalbumin
qPCR	quantitative PCR
FITC	fluorescein isothiocyanate

ABSTRACT

Background: Direct contact of food proteins with eczematous lesions is thought to be the main cause of epicutaneous sensitization. To further investigate the development and pathogenesis of food allergy *in vivo*, a good mouse model of epicutaneous sensitization is needed. However, a fundamental problem in that regard is that the optimal age for epicutaneous sensitization of mice is unknown. In this study, we attempted to elucidate that optimal age.

Methods: Dorsal skin of wild-type BALB/c female mice (1, 3, 8 and 24 weeks old) was shaved, depilated and tape-stripped. A Finn chamber containing a 20-μl-aliquot of 20-mg/ml (OVA) was applied to the tape-stripped skin on 3 consecutive days/week, for 3 weeks. The body temperature was measured after intraperitoneal OVA challenge. Serum OVA-specific IgE titers and OVA-induced cytokine production by spleen cells were measured by ELISA. Dendritic cells (DCs) that migrated to the draining lymph nodes were quantified by FITC-labeled OVA and flow cytometry. The mRNA expression levels in the dorsal skin were measured by qPCR.

Results: A significant age-dependent body temperature decline was observed after OVA challenge. The serum OVA-specific IgE titer, OVA-induced cytokine production (i.e., IL-4, IL-5 and IL-13) by spleen cells, and number of FITC-OVA-engulfing DCs increased with age. In addition, mRNA for IL-33, but not TSLP or IL-25, was significantly induced in the skin by tape-stripping and increased with age.

Conclusions: Twenty-four-week-old mice showed the greatest DC migration, Th2 polarization, IgE production and body temperature decline. Skin-derived IL-33 is likely to play key roles in those changes.

Introduction

The prevalence of food allergy has increased in recent decades all over the world, especially in industrialized countries.[1] Because of that increased prevalence and occasional fatal anaphylaxis, food allergy has become a major health burden in children.[2] Almost 99% of food allergies are mediated by antigen-specific IgE antibodies; thus, IgE sensitization is a critical event in the development and pathogenesis of food allergies.

Recent studies have shown that atopic dermatitis is a robust risk for IgE-mediated food allergies.[3] In a mouse model, exposure of intact skin to antigens induced tolerance, whereas exposure of damaged skin to antigens induced sensitization,[4] suggesting that direct contact of eczematous lesions with food proteins is likely to be the main mechanism for epicutaneous sensitization.[5]

A good mouse model of epicutaneous sensitization is needed in order to further investigate the pathogenesis of food allergy *in vivo*.[6] It was reported that several factors, including the animal strain, microbiome, age and others, are involved in IgE sensitization in general.[7] With respect to age, mouse skin in early infancy (13 days old) is reportedly rich in regulatory T cells and tends to tolerate

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skin commensal microbes,[8] whereas older mice (18 months old) showed impaired production of IgE to epicutaneously exposed proteins.[9] However, the optimal age for epicutaneous sensitization in a mouse model is unknown. Clarifying the optimal age and the underlying mechanisms would be useful for establishing a better murine model of epicutaneous sensitization and that information may provide some hints for future intervention strategies.

In this study, we attempted to elucidate the optimal age for the mice for induction of food allergy through epicutaneous sensitization, and the mechanisms behind this sensitization.

Methods

Mice

Wild-type BALB/c female mice at 1, 3, 8 and 24 weeks of age were purchased from SLC Japan (Shizuoka, Japan). They were housed under specific-pathogen-free conditions at the National Research Institute for Child Health and Development. The animal protocols were approved by the Institutional Review Boards of the National Research Institute for Child Health and Development and the Institute of Medical Science, The University of Tokyo.

Epicutaneous sensitization

Epicutaneous sensitization to ovalbumin (OVA) was performed based on a previous study,[10] with some modification. In brief, the dorsal skin of mice was shaved with electric clippers, depilated using a depilatory cream (Veet®; Reckitt Benckiser, Slough, Berkshire, UK), and then tape-stripped using a transparent film dressing (Cellotape; Nichiban, Tokyo, Japan) on the same day. After that, a 20-μl-aliquot of 20-mg/ml OVA (grade V; Sigma–Aldrich, St. Louis, MO, USA) in a Finn Chamber (Finn Chamber disk; 8-mm diameter; Smart Practice, Phoenix, AZ, USA) was applied to the tape-stripped dorsal skin for 3 consecutive days/week for 3 weeks.

Measurement of body temperature

Five days after the final epicutaneous exposure, each mouse was intraperitoneally challenged with OVA (1 mg/ml solution in sterile saline) at 10 μg/g mouse body weight. The rectal temperature of each mouse was measured using a digital thermometer (TD-300®; Shibaura, Saitama, Japan) at 0, 10, 20, 30, 40, 50 and 60 min after OVA challenge.

Measurement of OVA-specific IgE titers in sera

One day before intraperitoneal OVA challenge, a serum sample was collected from each mouse. The OVA-specific IgE titer in each was measured by ELISA, as described elsewhere.[11] HRP (horseradish peroxidase)-conjugated anti-mouse IgE antibody was obtained from Bethyl Laboratories (Montgomery, TX, USA).

OVA-induced cytokine production by spleen cells

OVA-induced cytokine production by spleen cells was measured as described elsewhere, with slight modification.[12] In brief, the spleen was harvested from each mouse 2 days after OVA challenge, and a spleen cell suspension was filtered through a 70-μm cell strainer (Falcon®; Corning, NY, USA), followed by erythrocyte lysis with a lysing buffer (Red blood cell lysing buffer®; Sigma–Aldrich) for 5 min at 37 °C. The spleen cells were re-suspended in RPMI1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% FCS (fetal calf serum: Biological Industries; Beit Haemek, Israel) and cultured with and without OVA (200 μg/ml) for 72 h. The supernatants were harvested, and the cytokine concentrations (IL-4, IL-5, IL-13 and IFNγ) in them were measured by ELISA (eBioscience, Thermo Fisher Scientific, Kanagawa, Japan).

Quantification of dendritic cell (DC) and Langerhans cell (LC) migration to draining lymph nodes

Dorsal skin of each mouse was shaved, depilated and tape-stripped. After these procedures, each mouse was exposed to 400 μg fluorescein isothiocyanate (FITC)-conjugated OVA[13] in a Finn Chamber on the tape-stripped dorsal skin for 24 h. After FITC treatment, axillary and inguinal lymph nodes were collected and single cell suspensions were incubated with anti-mouse CD16/CD32 mAb (clone 93; eBioscience). Cells were incubated with Brilliant violet 510-conjugated anti-mouse CD45 mAbs (clone 30-F11; Biolegend, San Diego, CA, USA), APC (allophecocyanin)-conjugated anti-mouse CD11c mAbs (clone N418; Biolegend), PE (phycoerythrin)-cy7-conjugated anti-mouse MHC class II proteins IA/IE (clone M5/114.15.2; Biolegend), PE (phycoerythrin)-conjugated anti-CD207 mAb (clone 4C7; Biolegend) and Dye eFluor 780 (Affymetrix, Thermo Fisher Scientific, Kanagawa, Japan). FITC-positive dendritic cells (DCs) in the draining lymph nodes were quantified by flow cytometry, and the number of FITC-positive DCs/1,000,000 cells was shown. DCs and LCs were determined as $CD45^{+}CD11c^{+}$MHC class II $(IA/IE)^{high}$ $CD207^{-}$ and $CD45^{+}CD11c^{+}$MHC class II $(IA/IE)^{high}$ $CD207^{+}$ cells, respectively.

Quantitative measurement of mRNA expression and histological examination of the dorsal skin

Dorsal skin of mice was harvested before shaving, after shaving and depilation, and 4 h after tape-stripping. Total RNA was extracted from the dorsal skin samples using an RNeasy kit (Qiagen, Valencia, CA, USA).[14] Quantitative PCR (qPCR) for cytokine mRNA expression was performed as described elsewhere.[15] The mRNA expression levels were normalized to the GAPDH level in each sample. Before and after 3-times tape-stripping, the epidermis was stained with hematoxylin–eosin, and its thickness was measured using a light microscope (IX70; Olympus, Tokyo, Japan) equipped with a digital camera (DP11; Olympus).

Statistics

The data are shown as the median and the ranges unless otherwise noted. Differences between groups were examined for statistical significance by the Kruskal–Wallis test followed by Dunn's multiple comparisons test. Correlations between groups were examined by Spearman's rank correlation test. Statistical evaluations were performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA), and $P < 0.05$ was regarded as significant.

Results

Successful epicutaneous sensitization to OVA in one-week-old mice

Dorsal skin of one-week-old mice was exposed to OVA after shaving, depilating and tape-stripping (Fig. 1A). Mice whose skin had been tape-stripped three times before exposure to OVA showed a significant body temperature decline after intraperitoneal OVA challenge (Fig. 1B). However, mice tape-stripped only one time before exposure to OVA showed only a modest body temperature decline. Those 2 tape-stripping groups showed almost comparable serum OVA-specific IgE titers, both of which were significantly higher than in the control mice (Fig. 1C).

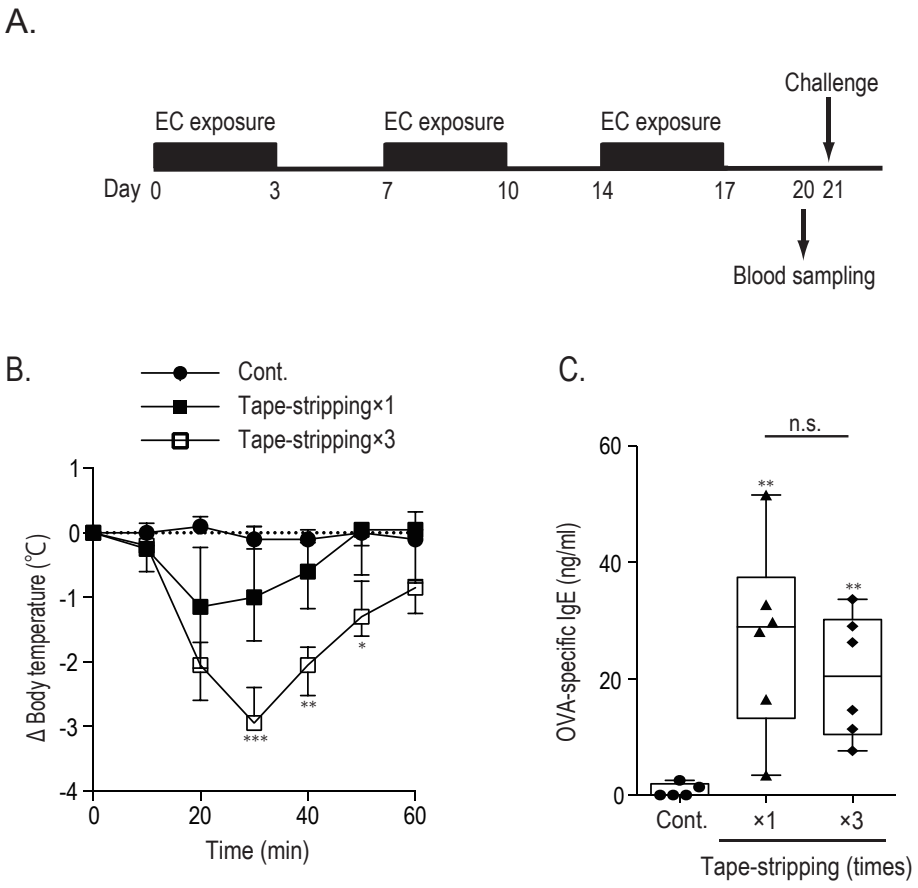


Fig. 1. Remarkable epicutaneous sensitization was induced in a three-times tape-stripped group of one-week-old mice. **A.** The protocol for epicutaneous sensitization by shaving, depilation and tape-stripping. Dorsal skin of wild-type one-week-old BALB/c female mice was shaved and depilated, followed by tape-stripping. A Finn chamber containing a 20-μl-aliquot of 20-mg/ml ovalbumin (OVA) solution was placed on the skin site for 3 consecutive days/week, for 3 weeks. Blood samples were collected from the animals' cheeks on day 20. The rectal temperature was measured after intraperitoneal OVA challenge (200 μg OVA/mouse) on day 21. EC: epicutaneous. **B.** After epicutaneous sensitization to OVA, the body temperature was measured every 10 min after intraperitoneal OVA challenge. **C.** Blood samples were collected from the cheeks on the day before OVA challenge. OVA specific IgE and titers were measured by ELISA. The data are shown as the median ± 75 and 95 percentiles as box plots. Saline (control), n = 4; one-time tape-stripping before OVA exposure, n = 6; three-times tape-stripping before OVA exposure, n = 6. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. indicated group.

Induction of epicutaneous sensitization following tape-stripping was profound with age

Four groups of wild-type BALB/c female mice (1, 3, 8, and 24 weeks old) were sensitized with OVA epicutaneously by the aforementioned procedures (Fig. 2A). After intraperitoneal OVA challenge, mice in all groups showed a significant body temperature decline (Fig. 2B). The magnitude of that decline was age-dependent ($P < 0.0001$ for the peak body temperature decline and the age group, by Spearman's rank correlation test, Fig. 2C) and was largest in the 24-week-old mice. The serum OVA-specific IgE titer also increased significantly with age ($P < 0.0001$, by Spearman's rank correlation test), and it was highest in the 24-week-old mice (Fig. 2D).

OVA-dependent Th2 cytokine (IL-4, IL-5 and IL-13) production by the spleen cells increased significantly with age ($P = 0.0008$, $P < 0.0001$ and $P < 0.0001$ for IL-4, IL-5 and IL-13, respectively, by Spearman's rank correlation test); the supernatants of spleen cells from 24-week-old mice showed the highest concentrations (Fig. 2E). However, IFNγ production was comparable in each age group. In addition, Th2 cytokine (IL-4, IL-5 and IL-13) concentrations in the culture supernatants of spleen cells cultured without OVA were barely detectable (Fig. 2F).

There was a statistically significant correlation between the body temperature decline in each individual mouse and its OVA-specific IgE titer ($P < 0.0001$, Fig. 3A). Both the body temperature decline (Fig. 3B) and the OVA-specific IgE titer in the serum correlated well with the level of each Th2 cytokine in the culture supernatant (IL-4, IL-5 and IL-13) (Fig. 3C).

Migration of DCs and LCs to the draining lymph nodes by age

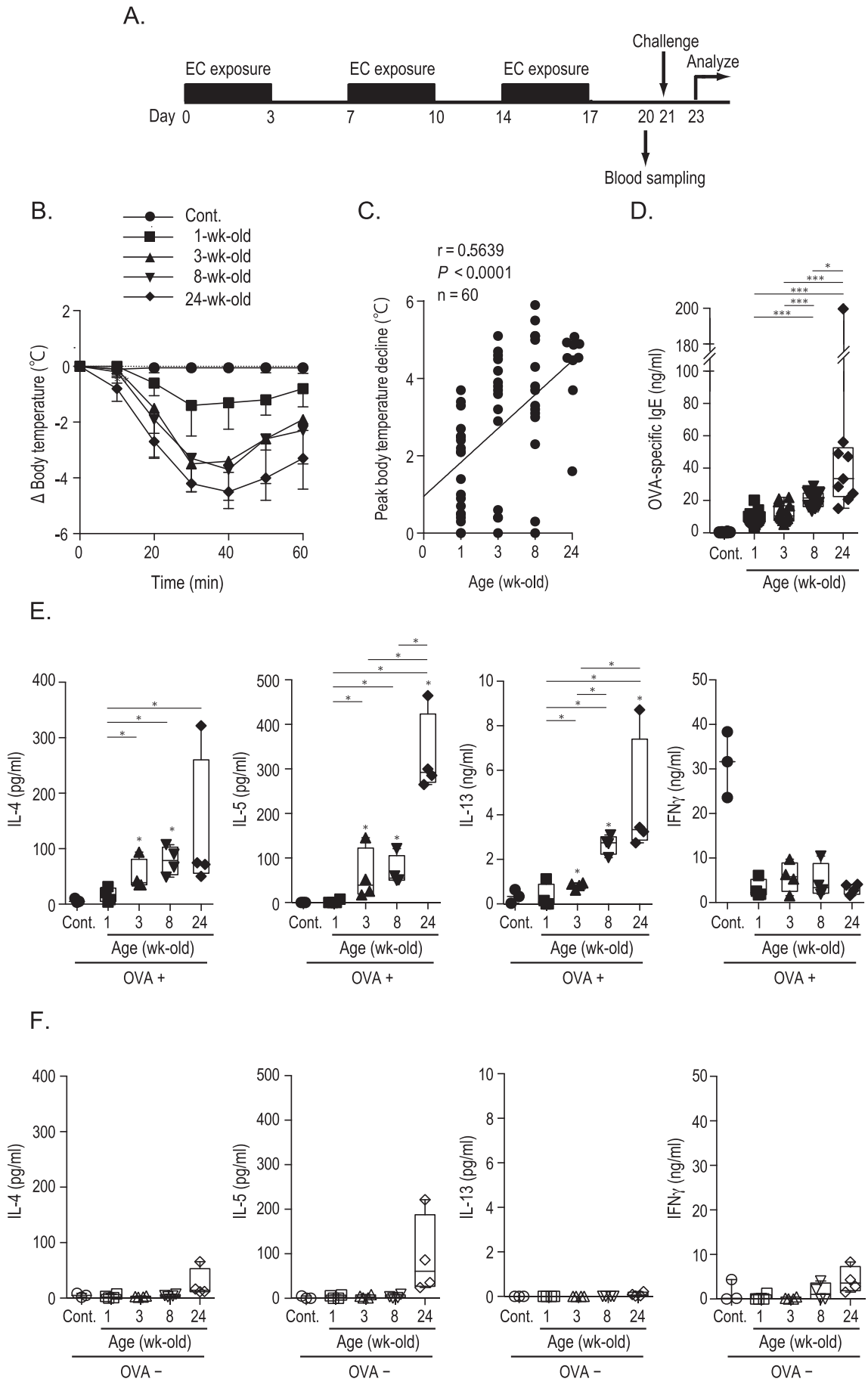
The number of FITC-OVA-engulfing (FITC-positive) DCs in the draining lymph nodes increased significantly with age ($P < 0.0018$, by Spearman's rank correlation test) and was largest in the 24-week-old mice (Fig. 4A). The number of FITC-OVA-engulfing LCs in the draining lymph nodes showed exactly the same pattern (Fig. 4B), but their numbers were fairly small compared with DCs (approximately 1/17 of DCs).

Expression of mRNA for IL-33, TSLP and IL-25 in dorsal skin

The mRNA expression levels for DC-activating cytokines, i.e., IL-33, TSLP and IL-25, in the dorsal skin before shaving, after shaving and depilation, and after tape-stripping were measured by qPCR. IL-33 mRNA expression increased significantly after shaving and tape-stripping, in an age-dependent manner (Fig. 5). However, expression of TSLP and IL-25 mRNA was barely detectable.

Discussion

In order to elucidate the optimal age for mice in an epicutaneous sensitization model, we first needed to select a single epicutaneous


A.
EC exposure
EC exposure
EC exposure
Challenge
Analyze
Day 0 3 7 10 14 17 20 21 23
Blood sampling
B.
Cont.
1-wk-old
3-wk-old
8-wk-old
24-wk-old
Δ Body temperature (°C)
Time (min)
C.
r = 0.5639
P < 0.0001
n = 60
Peak body temperature decline (°C)
Age (wk-old)
D.
OVA-specific IgE (ng/ml)
Cont. 1 3 8 24
Age (wk-old)
E.
IL-4 (pg/ml)
IL-5 (pg/ml)
IL-13 (ng/ml)
IFNγ (ng/ml)
Cont. 1 3 8 24
Age (wk-old)
OVA +
F.
IL-4 (pg/ml)
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IL-13 (pg/ml)
IFNγ (ng/ml)
Cont. 1 3 8 24
Age (wk-old)
OVA −

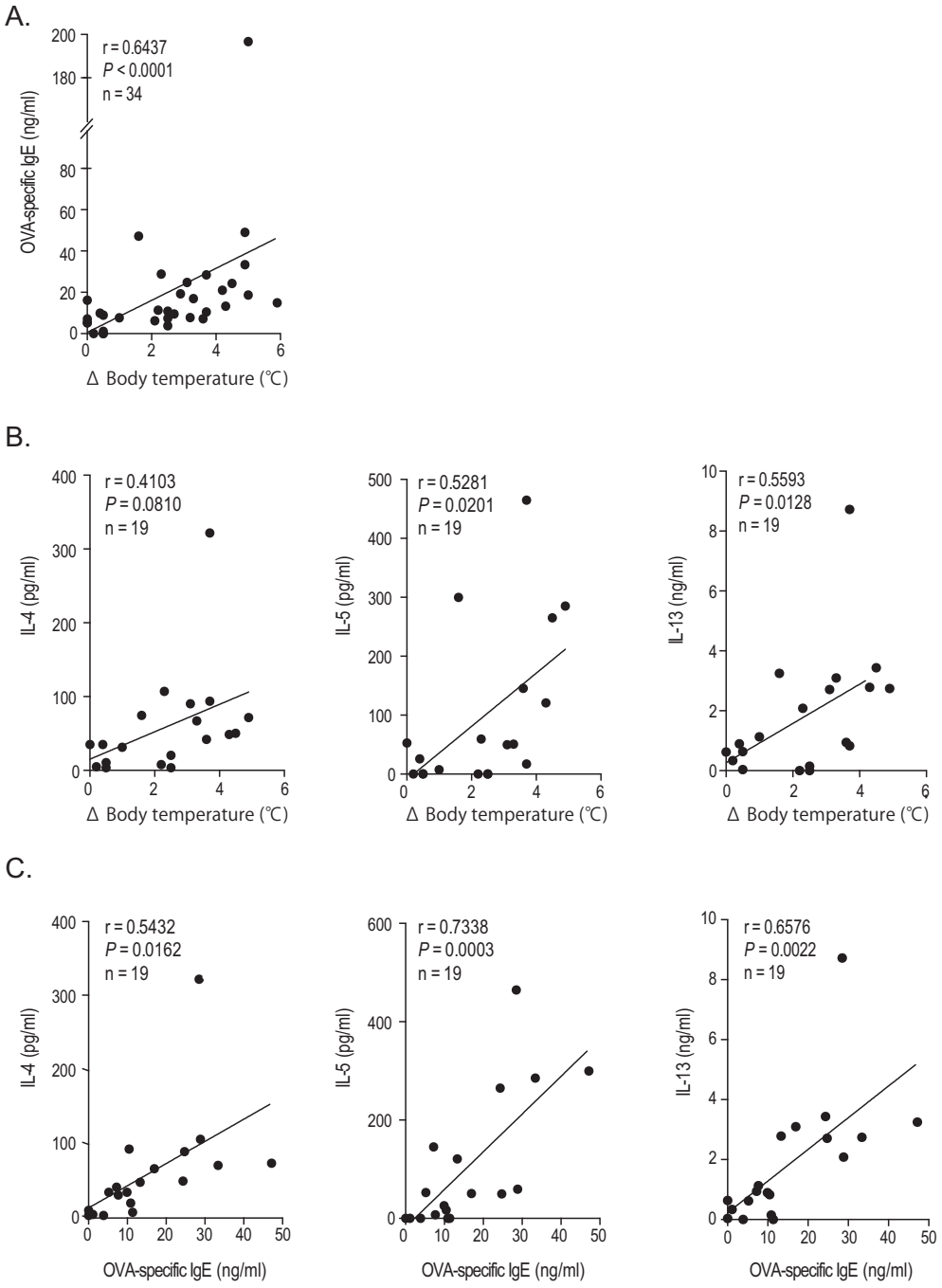

Fig. 3. Correlations among body temperature decline, serum OVA-specific IgE titer and OVA-induced Th2 cytokine production by spleen cells. **A.** Correlation between body temperature decline and OVA-specific IgE titer. **B.** Correlation between body temperature decline and OVA-dependent Th2 cytokine (IL-4, IL-5 and IL-13) production by spleen cells. **C.** Correlation between serum OVA-specific IgE titer and OVA-dependent Th2 cytokine (IL-4, IL-5 and IL-13) production by spleen cells (n = 19–34/group). All data were analyzed using Spearman's correlation. Lines represent linear regression fits.

sensitization method that could be performed over a wide age range. However, there have been few reports of methods for epicutaneous sensitization of mice as young as 1 week. Therefore, we initially attempted to establish such a model using one-week-old mice. As a result, we successfully sensitized mice by epicutaneous exposure after shaving, depilation and tape-stripping (Fig. 1C). The body temperature after intraperitoneal OVA challenge decreased significantly in animals that had been tape-stripped 3 times

Fig. 2. Induction of epicutaneous sensitization following tape-stripping was profound with age. **A.** Protocol for epicutaneous sensitization. Dorsal skin of wild-type BALB/c female mice (1, 3, 8 and 24 weeks old) was shaved, depilated and 3-times tape-stripped. A Finn chamber containing a 20-μl aliquot of 20-mg/ml OVA solution was placed on the skin site for 3 consecutive days/week, for 3 weeks. Blood samples were collected from the animals' cheeks on day 20. The body temperature was measured after intraperitoneal OVA challenge (10 μg OVA/g mouse body weight) on day 21. All mice were sacrificed on day 23. **B.** The body temperature of each animal was measured every 10 min after intraperitoneal OVA challenge. The data are shown as the median ± 75 and 95 percentiles as box plots (saline, n = 10; 1 week old, n = 21; 3 weeks old, n = 15; 8 weeks old, n = 15; 24 weeks old, n = 9). **C.** Correlation between peak body temperature decline and the age of mice (n = 60). **D.** Blood samples were collected from the animals' cheeks on the day before OVA challenge. OVA-specific IgE titers were measured by ELISA. **E.** OVA-induced cytokine production (IL-4, IL-5, IL-13 and IFNγ) by spleen cells was measured by ELISA. The data are shown as the median and the ranges, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. indicated group. **F.** Baseline Th2 cytokine (IL-4, IL-5 and IL-13) concentrations in the culture supernatants of spleen cells cultured without OVA.

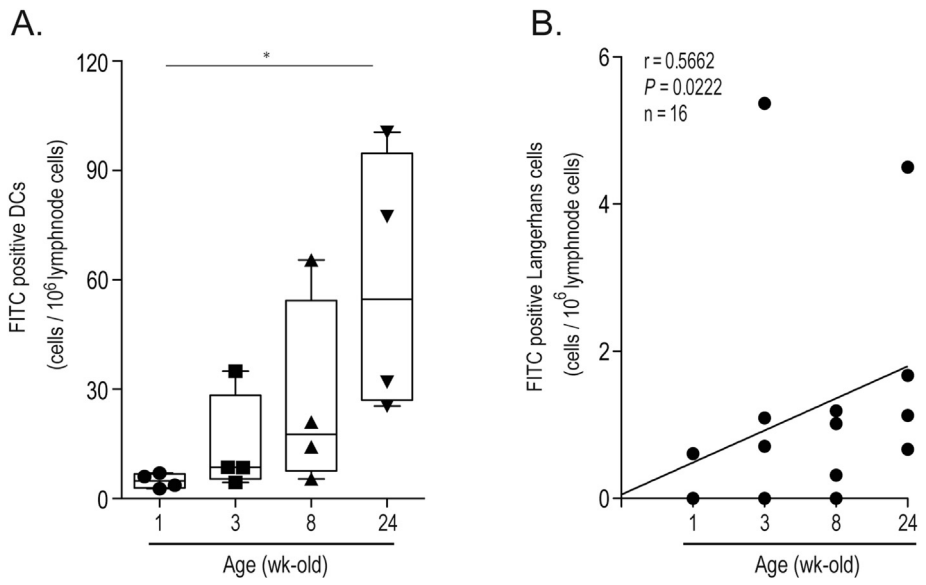

Fig. 4. Migration of OVA-engulfing DCs to the draining lymph nodes. The dorsal skin of wild-type BALB/c mice (1, 3, 8 and 24 weeks old) was shaved, depilated and tape-stripped. Each mouse was exposed to 400 μg of FITC-conjugated OVA in a Finn chamber placed on the tape-stripped dorsal skin for 24 h. FITC-positive cells in the draining lymph nodes were quantified by flow cytometry. FITC-OVA-engulfing DCs (**A**) and LCs (**B**) were identified as $CD45^{+}CD11c^{+}$MHC class II $(IA/IE)^{high}CD207^{-}$ cells and $CD45^{+}CD11c^{+}$MHC class II $(IA/IE)^{high}CD207^{+}$ cells, respectively. The data are shown as the median and ranges (n = 4 per group). $^{*}P < 0.05$ vs. indicated group.

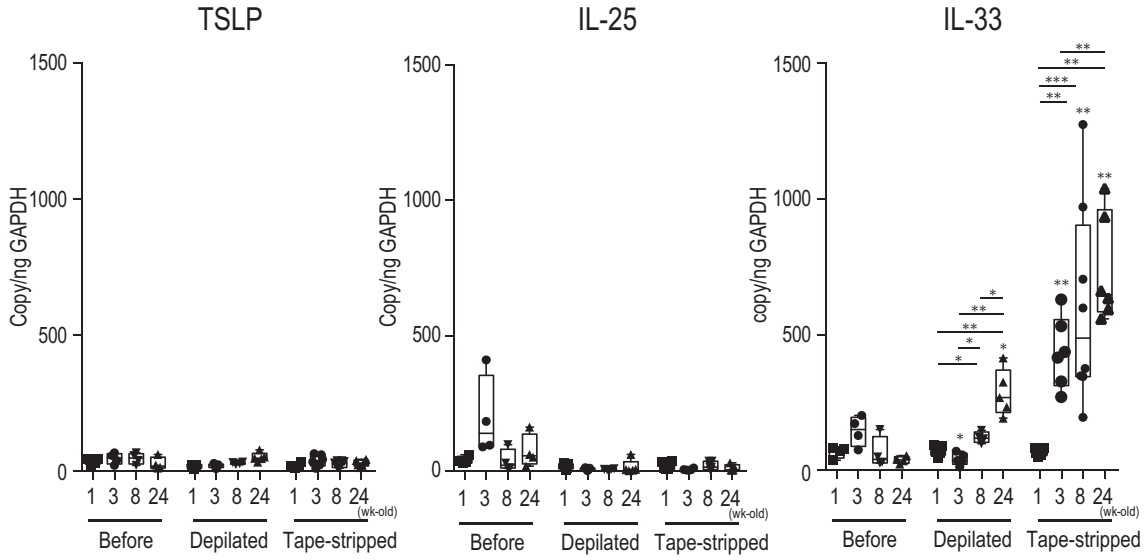

Fig. 5. mRNA expression levels for TSLP, IL-25 and IL-33, before shaving, after shaving and depilation, and after 3-times tape-stripping. Dorsal skin was collected before shaving, after shaving and depilation, and 4 h after tape-stripping. The mRNA expression levels for TSLP, IL-25 and IL-33 were measured by qPCR. The data are shown as the median and range (n = 4–8 per group) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. indicated group; and $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. corresponding values for the 'before shaving' samples.

compared with only 1 time (Fig. 1B). Thus, we performed tape-stripping 3 times for subsequent experiments (Fig. 1A, 2A).

Intraperitoneal OVA challenge after epicutaneous sensitization resulted in an age-dependent body temperature decline that was greatest in the 24-week-old mice (Fig. 2B). The decline in each mouse correlated significantly with that animal's serum OVA-specific IgE titer (Fig. 3A) and OVA-induced spleen cell production of Th2 cytokines (IL-4, IL5 and IL-13; Fig. 3B). The serum OVA-specific IgE titer also correlated with those Th2 cytokine levels (Fig. 3C). Those findings indicate that the profound body temperature decline in older mice was unlikely to be due to differences in effector-phase factors, such as responses to chemical mediators or release of chemical mediators from mast cells. It was also unlikely to be due to some part of the induction phase, such as antigen-specific IgE production or induction of Th2 differentiation. Therefore, a factor upstream of Th2 differentiation, that is to say, DCs, may play a key role in the differences in body temperature decline observed in this study.

When we measured DC migration to the draining lymph nodes after FITC-OVA exposure, the numbers of FITC-OVA–engulfing DCs and LCs detected in the draining lymph nodes increased with age, and were highest in the 24-week-old mice (Fig. 4A, B). The majority of migrating antigen presenting cells were DCs, presumably because the samples were obtained only 24 h after epicutaneous

FITC exposure; a previous study demonstrated that it takes 24 and 72 h for DCs and LCs, respectively, to migrate into the draining lymph nodes.[16] This finding suggests that factors involved in activation of DCs, *per se*, may play key roles in this model. IL-33, TSLP and IL-25 are known to be the main factors involved in priming and activation of DCs to induce Th2 polarization,[17–19] and in induction of DC migration to the draining lymph nodes.[20] In our model, we detected a high level of mRNA expression only for IL-33, but not TSLP or IL-25, in the skin samples after tape-stripping (Fig. 5). In addition, the IL-33 expression level correlated significantly with age. These findings suggest that IL-33 expressed in the skin played key roles in the DC migration, Th2 polarization, OVA-specific IgE production and profound body temperature decline seen in the older mice.

A previous study reported that TSLP was markedly induced in the skin after tape-stripping,[18] whereas in our model, IL-33, but not TSLP or IL-25, was induced in the skin. In order to clarify why TSLP expression was not induced in this model, we investigated in detail the changes in the mRNA levels in the skin (Supplementary Fig. 1). The expression level of TSLP increased slightly after depilation but decreased after tape-stripping in the 24-week-old mice. The lack of induction by tape-stripping was at odds with that previous report. In that report,[18] the dorsal skin of mice were shaved and tape-stripped, while in our model the dorsal skin of mice was shaved, depilated with depilatory-cream and tape-stripped. Thus, our depilation step might be involved in the different expression of TSLP. In fact, our pilot experiments indicated that the presence of short hair after shaving influenced the efficiency of tape-stripping (data not shown).

TSLP protein is known to be expressed predominantly in the upper layer of the epidermis.[21] Therefore, in our model, intense tape-stripping may have removed almost all of the upper layer that expresses TSLP. In fact, the thickness of the epidermis was markedly reduced after 3-times tape-stripping (Supplementary Fig. 2A), and the upper layers were removed (Supplementary Fig. 2B). Unlike in the 24-week-old mice, the expression level of TSLP mRNA decreased only after depilation in the 1, 3 and 8-week-old mice. That suggests that TSLP mRNA was low but constitutively expressed in the hair follicles of mice younger than 8 weeks old. When we measured TSLP protein in the skin lysates from mice at 1, 3, 8 and 22 weeks of age, 5 h after shaving, depilating and tape-stripping, the TSLP concentrations in all skin lysates were below the detection limit (15.6 pg/ml). However, a weak but similar tendency to IL-33 can be seen even below the limit of detection (Supplementary Fig. 3A, B). Note that these concentrations are almost 1/100 of those reported for tape-stripped skin in a previous study.[18]

IL-25 mRNA was expressed at a low level, but constitutively, in the skin (Supplementary Fig. 1), and it decreased after depilation, suggesting that IL-25 protein may also be expressed mainly in the hair follicles in all age groups.

In sharp contrast to TSLP and IL-25, IL-33 mRNA was expressed constitutively but at a low level in the resting skin of all age groups. Also, it was weakly induced after depilation, and strongly induced after tape-stripping, in an age-dependent manner. In our previous study, strong IL-33 induction required six-times tape-stripping one day after shaving when depilation was not performed.[22] The reason for strong induction of IL-33 after only three-times tape-stripping in our present model may be due to the fact that depilation was performed prior to tape-stripping on the same day.

Recent clinical studies found that both IL-33 and TSLP are highly expressed at sites of eczematous lesions and also detected in sera from patients with atopic dermatitis.[23,24] In particular, the degree of excoriation correlated with the serum IL-33 levels,[25] which is consistent with our data that the frequency of tape-stripping correlated significantly with the body temperature decline after intraperitoneal OVA challenge (Fig. 1B). Collectively, our model mimics epicutaneous sensitization in atopic dermatitis patients with severe epidermal excoriation.

Epidemiologically, severe symptoms due to food allergy are more frequent in infants than adults. However, our present finding that older mice experienced a greater body temperature decline than younger mice is not consistent with that clinical picture. The precise reason for this discrepancy is not clear, but one possible explanation might be that the frequency and dose of food proteins coming into contact with eczematous lesions, especially on the face, are much greater in infants than adults. Another explanation may be that treatment of eczema is more effective in adults, whereas complete eradication of facial eczema in infants is not always accomplished.

Thus, this model may be better suited for investigating the pathogenesis of food allergy in adults with severe atopic dermatitis *in vivo*. Crustacean and wheat proteins are common allergens in adult food allergy, and this model might be useful for elucidating the underlying pathogenesis.

This study has several limitations. First, all the results generated in this study indicated that this model is IL-33-dependent, but we could not definitely prove that because IL-33 knockout mice on the BALB/c background are not available. Second, we could not investigate for correlations between IL-33 expression in the skin and the antigen-specific IgE titer or body temperature decline in the same individual mice, because each mouse was sacrificed when the skin samples were harvested. However, there was a statistically significant correlation when we investigated those parameters within each group. Third, we did not clarify how age affects IL-33 induction in the skin. The mechanisms of how aging leads to such changes warrant further study. Finally, we did not investigate the peak age of animals that could be used in the model. Because others reported that 18-month-old mice showed impaired IgE production in response to epicutaneous exposure,[9] the peak age must be between 24 weeks and 18 months. However, practically speaking, mice older than 24 weeks are not commercially available, which means that any model based on use of such older mice would be impractical.

In conclusion, the procedures and murine model we have described found that 24-week-old mice showed the greatest DC migration, Th2 polarization, IgE production and body temperature decline. We surmise that skin-derived IL-33 probably plays key roles in those changes.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.alit.2018.01.003.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

MT, SN, HS, HM, AM and KMa designed the study and wrote the manuscript. KO, KMo, AM and KA contributed to data collection. HS performed interpretation of the results. All authors read and approved the final manuscript.

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