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Original Article

**Title: Skin inflammation exacerbates food allergy symptoms in epicutaneously sensitized mice.**

Short title: Skin inflammation exacerbates food allergies

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## ABSTRACT

**Background:** Cutaneous exposure to food antigen through impaired skin barrier has been shown to induce epicutaneous sensitization, thereby causing IgE-mediated food allergies.

**Objective:** We examined whether skin barrier impairment following epicutaneous sensitization exacerbates food allergies.

**Methods:** BALB/c mice were epicutaneously sensitized by repeated application of ovalbumin (OVA) to MC903-pretreated ear skin for 48 hours weekly and then intragastrically challenged with OVA. After the first oral challenge, the skin barrier was disrupted with topical application of MC903 or by tape-stripping. Mice were monitored for changes in body temperature and the occurrence of diarrhea after undergoing the second oral challenge. Serum levels of mouse mast cell protease-1 (mmcp1) and OVA-specific IgE, IgG1, IgG2a antibodies and OVA-specific IgA levels in intestinal lavage fluid were measured by ELISA. Tissue accumulation of eosinophils was determined histologically.

**Results:** Epicutaneously sensitized mice developed anaphylaxis after intragastric challenge, as evidenced by diarrhea, decreased body temperature and increased serum mmcp1 levels. Skin barrier disruption by MC903 treatment or tape-stripping exacerbated allergic reactions induced by oral challenge. MC903 treatment increased serum baseline and post-challenge mmcp1 levels. Topical pretreatment with dexamethasone alleviated allergic reactions that were exacerbated by MC903-treatment.

**Conclusion:** Even after eliminating exposure to the antigen, inflammation from skin barrier disruption can exacerbate the severity of food allergy symptoms. Serum baseline mmcp1 levels might be an effective marker for predicting the severity of antigen-induced allergic symptoms.

(226 words)

**Key Words:** food allergy; epicutaneous sensitization; mast cell protease 1; basophils

## INTRODUCTION

Accumulating evidence indicates pivotal roles for the IgE-mast cell axis in the pathogenesis of food allergies (1). However, when and how sensitization to food allergen occurs remains unknown. A certain portion of infants with food allergies experience an allergic reaction to food after the first consumption, suggesting that routes besides oral might be important to sensitization. Epidemiologic studies suggest that sensitization to peanut protein can occur in children by the application of peanut oil to inflamed skin or exposure to peanut antigen in house dust through an impaired skin barrier (2, 3) and that cutaneous inflammation associated with atopic dermatitis (AD) is a significant risk factor for the development of food allergies (3, 4). In Japan, soap products containing hydrolyzed wheat proteins have been reported to cause wheat-dependent exercise-induced anaphylaxis (5). These observations support the hypothesis that skin barriers disrupted by inflammation or application of soap could serve as ignition for food antigen sensitization.

The concept of skin barrier defects contributing to allergic sensitization is further supported by animal studies demonstrating that epicutaneous sensitization can cause a predisposition to antigen-induced allergic inflammation at other barrier sites. Noti et al. reported that epicutaneous sensitization on atopic dermatitis-like skin lesions, in which the skin barrier was disrupted by the application of the vitamin D3 analogue, MC903, was associated with accumulation of TSLP-elicited basophils. This was necessary and sufficient to promote antigen-induced intestinal food allergies by inducing Th2-type immune reactions (6). Galand et al. reported that IL-33 released locally after mechanical skin injury by tape-stripping enhanced IgE-mediated mast cell degranulation and thereby promotes oral anaphylaxis (7).

Effective management methods for food allergies include the avoidance of causative foods and the immediate use of self-injectable adrenaline in emergency situations. However, these measures may impair patients' quality of life and cannot completely resolve severe allergic reactions such as anaphylaxis. Food avoidance was also shown to be associated with a paradoxically increased risk of developing immediate reactions to that food in patients with food-triggered AD (8).

Using a mouse model for food allergies initiated by epicutaneous antigen sensitization through MC903-treated skin, we demonstrate that repetitive oral antigen exposure after establishment of sensitization modulated IgE-mediated food allergy symptoms and that long-term elimination of antigen exposure improved food allergies. The persistence of AD-like skin lesion and mechanical skin

barrier disruption by tape-stripping thwarted the remission of food allergies even in the absence of additional epicutaneous antigen exposure.

## MATERIALS AND METHODS

### Food allergy animal models

The BALB/c mice were bred under standard pathogen-free conditions. All animal experiments were performed in accordance with institutional guidelines and approved by the Animal Care Review Board of the University of Fukui.

For epicutaneous sensitization, 5-7-week-old female mice were topically pretreated with 1.2 nmol of MC903 (calcipotriol; Sigma-Aldrich Japan, Tokyo, Japan) dissolved in 20  $\mu$ l ethanol (EtOH) on both ears to induce atopic dermatitis-like skin lesions on day -46, -44, -42, -32, -30, -28, -18, -16, and -14 (6). A patch impregnated with 200  $\mu$ g of OVA (Sigma-Aldrich Japan) in 60  $\mu$ l of PBS was applied to the ear skin for 48 hours on days -42, -28, and -14. For intraperitoneal sensitization, mice were sensitized to OVA by intraperitoneal injection of 100  $\mu$ g OVA in alum (Imject Alum; Thermo Scientific, Rockford, IL) on days -28 and -14, as described previously (9).

Beginning on day 0, the sensitized mice received challenges by oral gavage of 50 mg OVA dissolved in 0.2 ml PBS or PBS alone every other day for up to six doses. For some experiments, after the first challenge, the mice were treated with application of MC903, EtOH, or a combination of MC903 and dexamethasone (DEX; Sigma-Aldrich Japan) on both ears, or with tape-stripping on the back on day 2, 4, 6, and 8, and then received the second OVA challenge on day 10. Before each intragastric challenge, mice were deprived of food for 3 hours. Diarrhea and body temperature was monitored for up to 1 hour following intragastric challenge. Diarrhea was assessed according to a score, as previously described at Dunker *et al.* (score 0 = no changes; score 1 = soft, but well-formed; score 2 = soft, non-formed; score 3 = one episode of liquid diarrhea; score 4 = at least two episodes of liquid diarrhea (10). Serum was collected one day before and one hour after the first and the last intragastric OVA challenges. Ear thickness was measured at indicated time points after treatment by measuring the entire width of the ear in micrometers.

## **OVA-specific antibody and mouse mast cell protease-1 measurements**

Serum levels of OVA-specific IgG1, IgG2a, and IgE and the concentration of OVA-specific secretory IgA in intestinal lavage fluids, obtained by washing 10 cm of intestine with 1 ml of PBS, were determined by ELISAs, as described previously (9). Serum levels of mouse mast cell protease-1 (mmcp1) were measured by using Mouse MCPT-1 ELISA Ready-SET-Go! (eBioscience Inc., San Diego, CA), according to the manufacturer's instructions.

## **Histopathologic analysis**

Alimentary tracts were fixed in formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin, or toluidine blue for histological assessment. The numbers of eosinophils and mast cells were evaluated by counting at high-power field area at  $\times 400$  magnification. At least 10 fields were examined, and number was expressed as the mean number of cells per 10 high power fields.

## **Microarray analysis and real-time RT-PCR**

Total RNAs were isolated from skin and jejunum of epicutaneous sensitized mice after the second oral OVA challenge using RNeasy lipid tissue mini kit (Qiagen K. K., Tokyo Japan) according to the manufacture's instruction. RNA integrity was verified with an Agilent 2100 Bioanalyzer (Agilent Technologies, Tokyo, Japan). Sense-strand DNA was generated from 200 ng total RNA isolated from skin, fragmented and labeled using a GeneChip 3'IVT Plus Regent Kit (Thermo Fisher Scientific Inc, Tokyo, Japan). Hybridization with the GeneChip Mouse Genome 430 2.0array, scanning and generation of probe cell intensity data were carried out using an Affymetrix fluidics station 450 and GeneChip Scanner (GCS 3000-7G Scanner, Thermal Fisher Scientific Inc) imported into the Subio platform version 1.20 (Subic, Kagoshima, Japan) for database management and quality control. All samples were assayed in two different biological replicates. Microarray data were published in the Gene Expression Omnibus (GEO) database with accession number GSE95278.

Total RNA was isolated from jejunum and skin after the second intragastric OVA challenge, and mRNA levels were quantified by real-time quantitative PCR using TaqMan Pre-Developed Assay Reagents according to the manufacturer's instructions as described previously (9). Results were

normalized to the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (Gapdh), and the  $\Delta\Delta$  cycle threshold method was used.

### Statistical analysis

Results are expressed as means  $\pm$  standard errors of mean. Comparison of two groups used the unpaired two-tailed Student's *t*-test, unless an *F*-test revealed that the variances were significantly different. When variances were significantly different, Mann-Whitney's *u*-test was used. A *p*-value  $< 0.05$  was considered statistically significant.

## RESULTS

### Characteristics of food allergy developed in epicutaneously sensitized mice.

As previously reported (9), intraperitoneally sensitized mice did not present with any allergic symptoms upon the initial intragastric OVA challenge, whereas epicutaneously sensitized mice developed allergic diarrhea accompanied by hypothermia even after the initial challenge (Fig. 1B, C). Moreover, the ear became thick immediately after intragastric challenge without topical exposure to OVA (data not shown). Consistent with allergic symptoms, the OVA-challenge induced a significant increase of serum mmcp1 levels in the epicutaneously but not intraperitoneally sensitized mice. The epicutaneously sensitized mice exhibited higher levels of mmcp1 than intraperitoneally sensitized mice even before the intragastric OVA challenge ( $199.75 \pm 37.07$  ng/ml vs  $94.11 \pm 14.07$  ng/ml,  $p < 0.05$ ) (Fig. 1D).

To elucidate the underlying mechanisms of different response to the initial challenge, we measured OVA-specific immunoglobulins. Epicutaneously sensitized mice had higher levels of OVA-specific IgE and lower levels of OVA-specific IgG1, IgG2a and secretory IgA than intraperitoneally sensitized mice. This indicated that epicutaneous sensitization preferentially induced antigen-specific IgE compared with intraperitoneal sensitization.

### **Repetitive oral Ag challenge modified food allergy symptoms.**

Since intraperitoneally sensitized mice gradually developed allergic diarrhea after repetitive Ag challenges (9), we sought to determine whether repetitive intragastric challenges exacerbated allergic symptoms in epicutaneously sensitized mice. Hypothermia and diarrhea were transiently alleviated upon the second to fourth challenges. When compared with the first challenge, hypothermia observed in the sixth challenge was milder and more prolonged (Fig. 2). The diarrhea observed in the sixth challenge was more severe than observations in the first challenge. The serum levels of mmcp1 before and after the sixth OVA challenge were significantly higher than levels before and after the first OVA challenge, respectively. The mRNA expression levels of mouse mast cell protease 1 (*Mcpt1*) and *Mcpt2* in the jejunum before the sixth OVA challenge were significantly higher than expression levels before the first OVA challenge. There was also a higher accumulation of eosinophils and mast cells in the intestinal mucosa after the sixth challenge. After six challenges, serum levels of OVA-specific IgG1 and IgG2a and secretory IgA levels in intestinal lavage fluid were elevated, whereas serum levels of OVA-specific IgE were not significantly upregulated.

### **Skin barrier impairment exacerbated food allergy symptoms.**

We next asked whether skin barrier impairment alone modified food allergy symptoms of epicutaneously sensitized mice. As shown in Fig. 3, the whole ear thickness was increased and decreased after topical application of MC903 and control EtOH, respectively. The hypertrophy of epidermis and the accumulation of inflammatory cells in the dermis were observed in MC903-applied ear skin. When compared the first and second OVA challenges, induced hypothermia, diarrhea and changes in the ear thickness were worse in the MC903-applied mice, whereas these symptoms were alleviated in the control EtOH-applied mice.

Consistent with the severity of allergic symptoms, serum mmcp1 levels were dramatically increased after the second challenge in the MC903-treated mice. The serum baseline mmcp1 levels before the second challenge were higher in MC903-applied mice than control EtOH-applied mice (Fig. 3F, pre-challenge). Meanwhile, there were no significant differences in the serum levels of OVA-specific IgG1, IgE, or secretory IgA levels in intestinal lavage fluid between MC903-applied mice and control EtOH-applied ones.

To exclude the possibility that remaining OVA, which was applied to the ear during the sensitization phase, boosted the sensitization in concert with MC903, the back of the epicutaneously sensitized mice were treated with depilatory cream and then tape-stripped without exposure of OVA to the skin. As shown in Fig. 4, the tape-stripped mice developed significantly more severe hypothermia and tended to express higher levels of *Mcpt1* and *Mcpt2* mRNA after the second challenge when compared to the control mice. There were no significant differences in the diarrhea score and the serum mmp1 levels between the tape-stripped mice and the controls.

### **Treatment with topical glucocorticoid prevented the exacerbation of food allergy symptoms.**

We next asked whether topical glucocorticoid treatment for skin inflammation alleviated intragastric challenge-induced allergic symptoms. As shown in Fig. 5, treatment with topical dexamethasone alleviated the MC903-application-induced exacerbation of hypothermia and diarrhea as well as the elevation of serum mmcp1 levels after the second challenge. The treatment with dexamethasone also suppressed the OVA challenge-induced accumulation of eosinophils in the intestinal mucosa and the dermis of ear skin.

To clarify the underlying mechanisms involved in the suppressive effect of topical glucocorticoid, we analyzed mRNA expression in the skin and jejunum. The microarray analysis revealed that the application of MC903 significantly enhanced *Mcpt8*, TSLP, IL-4, and IL-3 mRNA expression in the ear skin of MC903-treated mice compared with the ear skin of EtOH-treated mice. High levels of IL-33 mRNA were constitutively expressed in the skin but were not affected by MC903-treatment (Fig. 6A, B). Topical application of dexamethasone suppressed the MC903-induced upregulation of IL-4, IL-3, *Mcpt8* and *Mcpt1* mRNA but not that of TSLP mRNA in the skin (Fig 6B).

The expression levels of *Mcpt1* mRNA in the jejunum were much higher than that in the skin. Among the examined mRNA in the jejunum, only *Mcpt1* and *Mcpt2* mRNA was enhanced and suppressed by MC903 and dexamethasone treatments, respectively (Fig. 6C). Consistent with mRNA expression, the accumulation of mast cells in the intestinal mucosa was suppressed by dexamethasone treatment in MC903-treated mice (Fig. 6D).

## DISCUSSION

In this study, we demonstrated that the phenotype of food allergies observed in epicutaneously sensitized mice was different from that in intraperitoneally sensitized mice. The food allergies in epicutaneously sensitized mice were exacerbated by chemical or mechanical skin barrier impairment without additional epicutaneous antigen exposure and were alleviated by topical treatment with glucocorticoid. Moreover, among the examined mRNA that were upregulated by MC903 pretreatment, topical glucocorticoid suppressed mRNA expression of Mcpt8, IL-3 and IL-4 in the skin and that of Mcpt1 in the jejunum, suggesting that IL-4 expression and basophil activation in the skin and mucosal mast cell activation in the jejunum were correlated with the severity of food allergy.

Epidemiological studies indicated that AD in infancy is a risk factor for the subsequent development of food allergies (11). Infants with AD sometimes experience the initial symptoms of food allergies upon their first ingestion of the causative food. Several food allergy models using different antigen sensitization routes have been reported (6, 12 - 14). The mice sensitized by oral administration of antigen and cholera toxin or by intraperitoneal immunization have been shown to develop food allergies after repetitive oral antigen exposures. On the other hand, since the epicutaneously sensitized mice exhibited allergic responses after the first ingestion of antigen, the food allergy model in this study is reflective of the onset of food allergy observed in AD infants.

In inflamed skin lesions of atopic dermatitis, IL-4-competent cells, including eosinophils, basophils and group 2 innate lymphoid cells (ILC2s) accumulate and interact to regulate the development of type 2 inflammation (15). Among these cells, basophil-derived IL-4 plays an important role in the accumulation of ILC2s (15). In contrast to ILC2 responses in the intestine, which are critically regulated by IL-33 and IL-25, ILC2 responses in the skin and skin-draining lymph nodes are independent of these cytokines but are critically dependent on TSLP (16). Although a recent study by Hussain *et al.* (17) elegantly demonstrated that that basophil-derived IL-4 is critical for both skin sensitization to food antigens and the simultaneous development of IgE-mediated food allergies, eosinophils and ILC2s may also be the predominant IL-4-competent cells accumulating in the skin in response to MC903 treatment after the establishment of antigen sensitization. IL-4 was shown to induce a marked intestinal mastocytosis in mice, resulting in enhanced anaphylactic reactions (18).

Other studies have proposed that TSLP-elicited basophils play a central role in the induction of Th2 responses, whereas IL-3-elicited basophils act as late-phase effector cells by exhibiting IgE-mediated degranulation and histamine release (19, 20). Topical corticosteroid suppressed the upregulation of

mRNA expression of IL-4 and IL-3 but not that of TSLP in the MC903-treated skin of epicutaneously sensitized mice. Since IL-3 is predominantly produced by activated T cells, topical corticosteroid might suppress IL-3 production by T cells and thereby indirectly suppress the function of basophils, resulting in the alleviation of food allergies.

Galand et al. (7) have recently reported that IL-33 released from tape stripped skin acted on mast cells to promote IgE mediated degranulation just after establishment of epicutaneous sensitization. The different skin area and low frequency of tape stripping could explain mild allergic reactions observed in the tape stripped mice. The control mice but not the tape stripped mice were treated with application of ethanol on ear skin. The EtOH treatment itself might affect allergic reactions in controls, resulting in the small differences in the diarrhea score between the tape stripped mice and the controls.

Intraperitoneally sensitized mice had elevated serum levels of OVA-specific IgE but not mmcp1 before the initial oral challenge (Fig. 1D). After repetitive oral challenges, intraperitoneally sensitized mice developed anaphylaxis and had an expansion of intestinal mast cells, similar to previous observations in mice orally sensitized with antigen plus cholera toxin (12). Since serum mmcp1 is derived from mucosal mast cells (21), serum levels of mmcp1 before the oral challenge could reflect an accumulation or priming status of mast cells in the intestine, supporting the notion that the expansion and pre-activation of intestinal mast cells is essential for the development of food allergies.

Antigen-specific IgG1 and secretory IgA have been shown to function as blocking antibodies to prevent IgE-mediated signaling and to inhibit absorption of luminal antigen, respectively (22, 23). The higher OVA-specific IgE to IgG1 ratio and the lower levels of OVA-specific secretory IgA could favor the elicitation of food allergy symptoms in response to the initial antigen ingestion in epicutaneously sensitized mice.

Maintz et al. (24) reported that changes in the counterregulatory mechanisms between histamine synthesis and degradation might attenuate effects of histamine during the buildup phase of venom immunotherapy. Therefore, repetitive oral challenges might temporally alleviate hypothermia by enhancing histamine metabolism regardless gradually accumulating mast cells. In contrast to venom immunotherapy, mast cell proteases released from the activated mast cells might enhanced permeability of mucosal epithelium, resulting in exacerbation of diarrhea (25).

The fundamental strategy of oral immunotherapy for IgE-mediated food allergies is to deliver gradually increasing doses of specific food allergens. Eosinophilic gastrointestinal diseases have been

shown to develop in some patients undergoing oral immunotherapy (26, 27). Recurrent allergic reactions in alimentary tracts that occur during oral immunotherapy might induce accumulation of eosinophils as observed in this mouse model.

Egg and milk allergies are outgrown in the majority of children by school age. The severity of baseline atopic dermatitis is a predictive factor for the resolution of food allergies as well as food allergen-specific IgE levels (28, 29). A significant number of patients with food-triggered atopic dermatitis has been shown to develop immediate food reactions after beginning an elimination diet (8). Taken together with our findings that epicutaneously sensitized mice exacerbated food allergy by deteriorating skin lesions without additional epicutaneous antigen exposures, control of atopic dermatitis is important for the prevention of deterioration and facilitation of outgrowing of comorbid food allergies.

A retrospective study demonstrated that proactive treatment with intermittent applications of topical corticosteroids and daily applications of emollients decreased serum IgE levels in patients with severe atopic dermatitis (30). Topical corticosteroid treatment failed to decrease serum OVA-specific IgE levels in epicutaneously sensitized mice. Thus, long-term corticosteroid treatment might be required to inhibit specific IgE production. Suppression of serum food allergen-specific IgE levels is not essential to alleviate food allergies.

In conclusion, epicutaneously sensitized patients could present with initial symptoms of food allergies upon the first ingestion of causative food. Inflammation associated with skin barrier disruption may deteriorate the severity of food allergies in patients with an atopic dermatitis comorbidity. Although current therapeutic strategies for food allergies rely on avoidance of causative foods, our data suggest that controlling skin lesions is important for patients with atopic dermatitis to resolve comorbid food allergy.

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**Conflict of interest:**

The authors declare that they have no conflict of interest.

**Author contribution:** AK wrote the manuscript. YO organized the study. Each named author in this report contributed to its content either through being integral to the experimental planning and/or its implementation at the bench. All authors read and approved the final manuscript.

## FIGURE LEGENDS

**Figure 1.** Epicutaneously but not intraperitoneally sensitized mice developed allergic reactions upon the initial oral Ag challenge. (A) Experimental protocol. (B) Body temperature changes after OVA challenge in epicutaneously sensitized mice (closed circles) and intraperitoneally sensitized mice (open circles). Diarrhea scores (C), pre- and post-challenge serum mmcp1 levels (D), serum OVA-specific IgG1 (E), IgG2a (F), and IgE (G) levels, and OVA-specific IgA levels in intestinal lavage fluid (H) of epicutaneously sensitized mice (closed bars) and intraperitoneally sensitized mice (open bars). Results are means  $\pm$  SE (n = 6) and are representative of 3 - 5 independent experiments. Unpaired Student's *t*-tests, Welch's tests, and Mann-Whitney tests were used for statistical analysis. \**p* < 0.05, \*\**p* < 0.01

**Figure 2.** Repetitive oral Ag challenge tentatively alleviated and then altered allergic symptoms. (A) Experimental protocol. The lowest body temperature (B) and diarrhea scores (C) observed in epicutaneously sensitized mice after the indicated number of OVA (closed squares) or PBS (open squares) challenges. (D) Body temperature changes after the first (closed circles) and last (open circles) challenge. Numbers of infiltrated eosinophils (E), and mast cells (F), and the relative mRNA expression levels of *Mcpt1* (G) and *Mcpt2* (H) in the jejunum of epicutaneously sensitized mice before the first and the last OVA or PBS challenge. Pre- and post-challenge serum mmcp1 levels (I), serum OVA-specific IgG1 (J), IgG2a (K), and IgE (L) levels, and OVA-specific IgA levels in intestinal lavage fluid (M) of OVA (closed bars and squares) or PBS (open bars and squares) challenged mice. Results are means  $\pm$  SE (n = 6) and are representative of 3 independent experiments. Unpaired Student's *t*-tests, Welch's tests and Mann-Whitney tests were used for statistical analysis. \**p* < 0.05, \*\**p* < 0.01

**Figure 3** Skin inflammation deteriorated oral allergen induced allergic reactions. (A) Experimental protocol. Histological findings (B) and ear thickness (C) of MC903-treated mice (closed bars) or vehicle ethanol-treated mice (open bars) after OVA challenges. (D) Body temperature changes after OVA challenge in MC903-treated mice (closed circles) and ethanol-treated mice (open circles). Diarrhea scores (E), pre- and post-challenge serum mmcp1 levels (F), serum OVA-specific IgG1 (G) and IgE (H) levels, and OVA-specific IgA levels in intestinal lavage fluid (I) of MC903-applied mice (closed circles and bars) and ethanol-applied mice (open squares and bars). Results are means  $\pm$  SE (n = 6) and are representative of 3 - 4 independent experiments. Unpaired Student's *t*-tests, Welch's tests and Mann-Whitney tests were used for statistical analysis. \**p* < 0.05, \*\**p* < 0.01

**Figure 4.** Tape stripping deteriorated oral allergen induced allergic reactions. (A) Experimental protocol. Body temperature changes after OVA challenge (B), diarrhea scores (C), post-challenge serum mmcp1 levels (D), and the relative mRNA expression levels of *Mcpt1* and *Mcpt2* in the jejunum (E) in MC903-treated mice (closed circles and bars), tape stripping mice (closed squares and hatched bars) and ethanol-treated mice (open circles and bars). Results are means  $\pm$  SE (n = 6) are representative of 3 independent experiments. Unpaired Student's *t*-tests, Welch's tests and Mann-Whitney tests were used for statistical analysis. \**p* < 0.05

**Figure 5** Topical steroid treatment inhibited the skin inflammation-associated deterioration of oral allergen induced allergic reactions. (A) Experimental protocol. Changes in ear thickness (B) and body temperature (C) of MC903-treated mice (closed circles), dexamethasone-pretreated and MC903-treated mice (gray squares) or vehicle ethanol-treated mice (open circles) after OVA challenges. Diarrhea scores (D), pre- and post-challenge serum mmcp1 levels (E), infiltration of eosinophils into the jejunum and ear skin (F) of MC903-treated mice (closed circles and bars), dexamethasone-pretreated and MC903-treated mice (gray squares and hatched bars) or ethanol-treated mice (open circles and bars). Results are means  $\pm$  SE (n = 6) and are representative of 3 independent experiments. Unpaired Student's *t*-tests, Welch's tests and Mann-Whitney tests were used for statistical analysis. \**p* < 0.05, \*\**p* < 0.01

**Figure 6.** MC903-induced skin inflammation and dexamethasone treatment modulated mRNA expression in skin and jejunum. (A) Comparison of mRNA expression in the skin between MC903-treated mice and vehicle ethanol-treated mice by microarray analysis. Cytokines, *Mcpt8*, and *Mcpt1* mRNA expression in the skin (B) and the jejunum (C) of MC903-treated mice (closed bars), MC903/dexamethasone-treated mice (hatched bars), and ethanol-treated mice (open bars) after the second challenge were quantified by real-time quantitative PCR. Toluidine blue staining from the jejunum (D). Arrows indicate mast cells. Results are means  $\pm$  SE (n = 6) and are representative of 3 independent experiments. Unpaired Student's *t*-tests, Welch's tests and Mann-Whitney tests were used for statistical analysis. \*\**p* < 0.01

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Figure 1

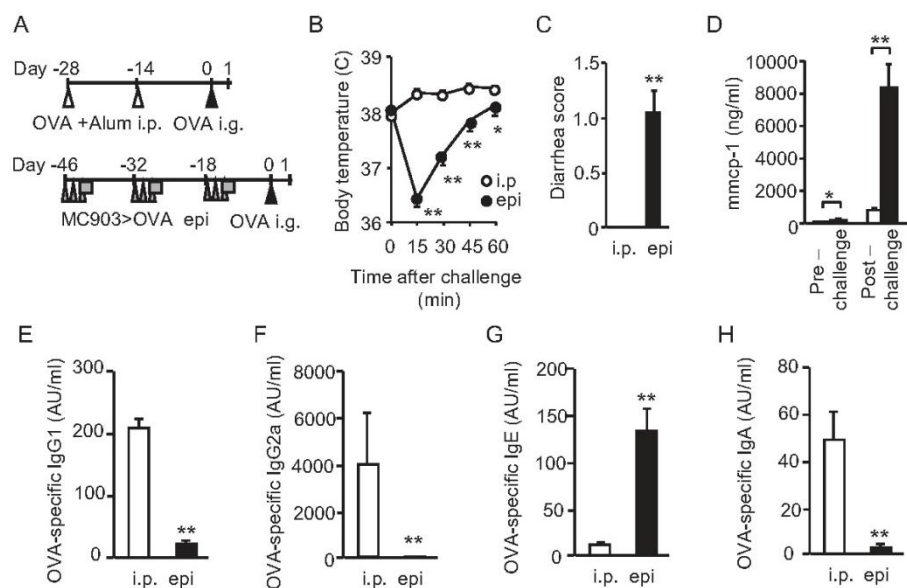


Figure 2

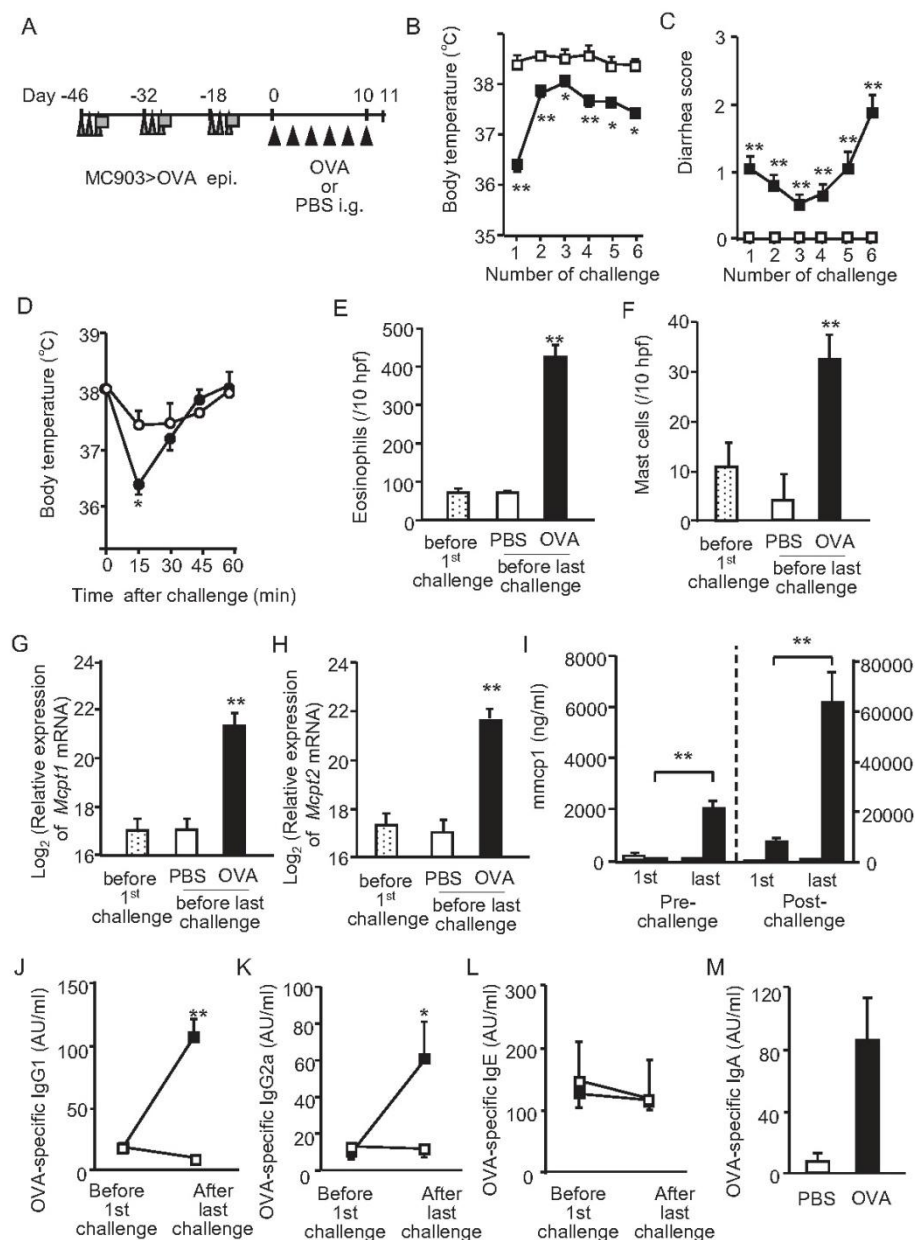


Figure 3

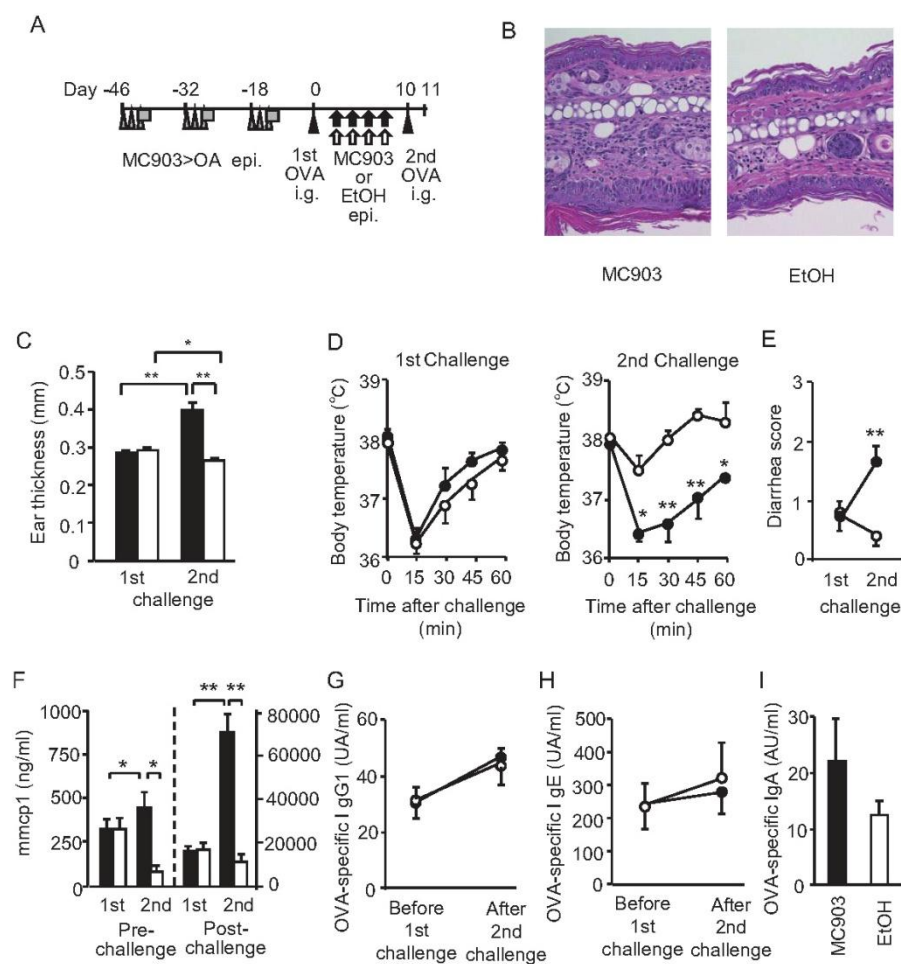


Figure 4

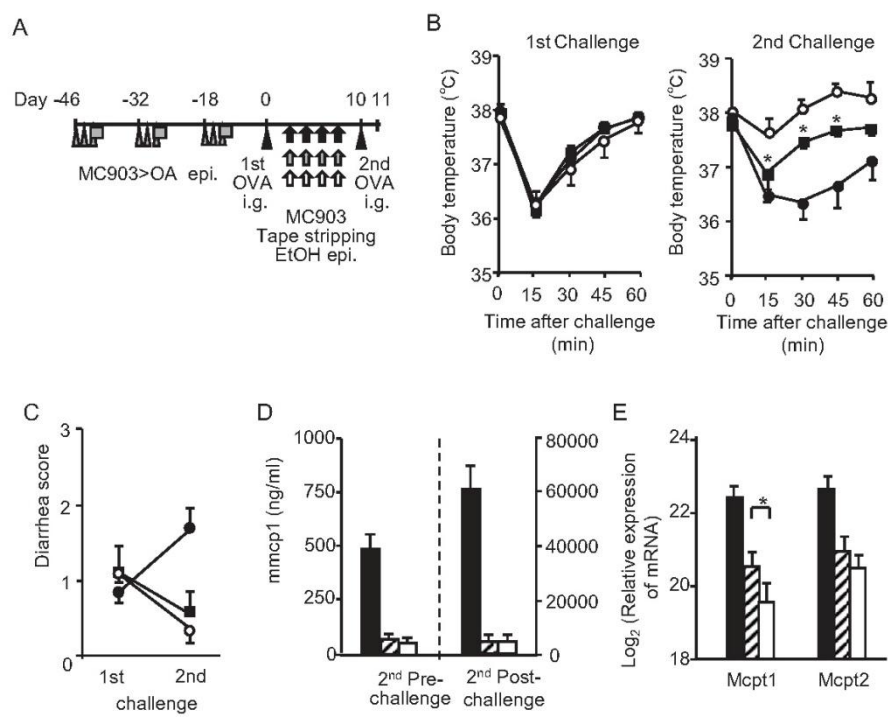


Figure 5

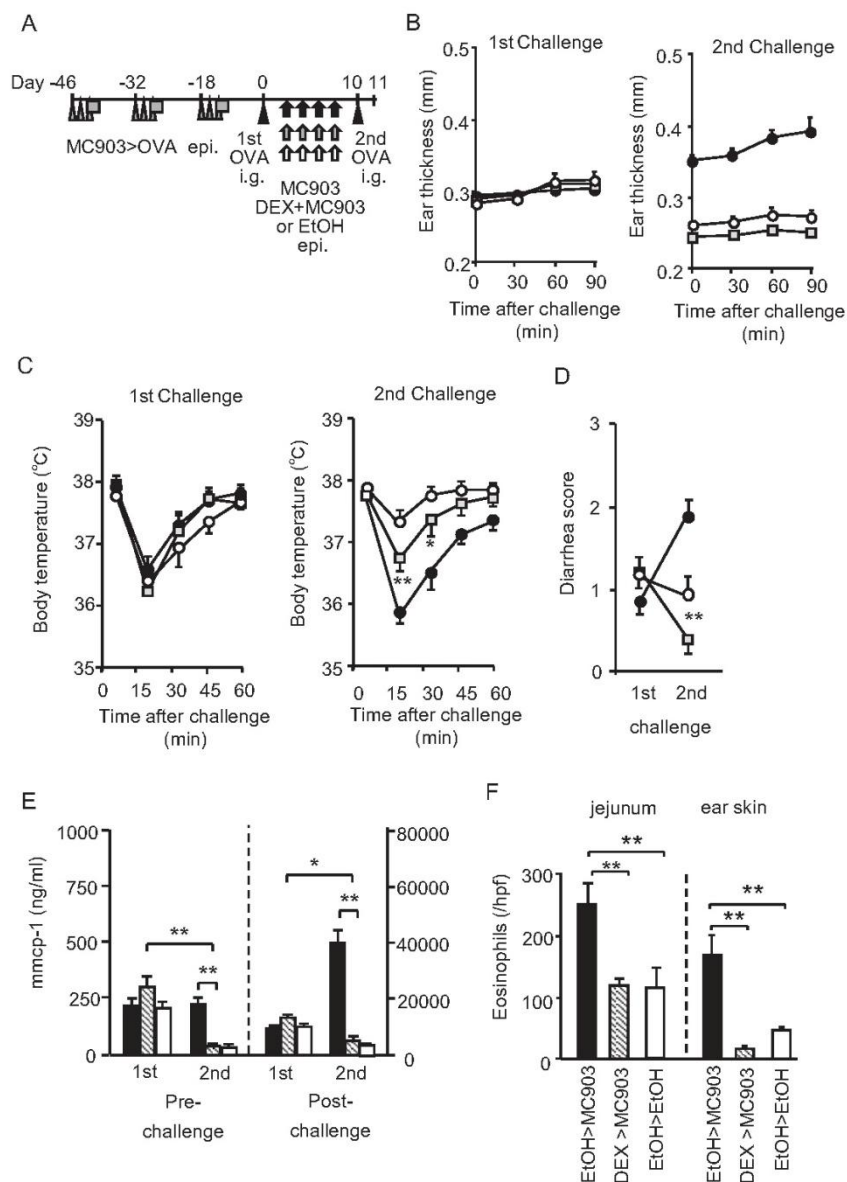


Figure 6

