

Epicutaneous sensitization results in IgE-dependent intestinal mast cell expansion and food-induced anaphylaxis

Lisa M. Bartnikas, MD,^{a*} Michael F. Gurish, PhD,^{b*} Oliver T. Burton, PhD,^a Sabine Leisten, MS,^a Erin Janssen, MD, PhD,^a Hans C. Oettgen, MD, PhD,^a Jacqueline Beaupré, BA,^a Christopher N. Lewis, BA,^a K. Frank Austen, MD,^b Stephanie Schulte, MD,^c Jason L. Hornick, MD, PhD,^c Raif S. Geha, MD,^{a*} and Michiko K. Oyoshi, PhD^{a*} *Boston, Mass*

Background: Sensitization to food antigen can occur through cutaneous exposure.

Objective: We sought to test the hypothesis that epicutaneous sensitization with food antigen predisposes to IgE-mediated anaphylaxis on oral allergen challenge.

Methods: BALB/c mice were epicutaneously sensitized by repeated application of ovalbumin (OVA) to tape-stripped skin over 7 weeks or orally immunized with OVA and cholera toxin (CT) weekly for 8 weeks and then orally challenged with OVA. Body temperature was monitored, and serum mouse mast cell protease 1 levels were determined after challenge. Tissue mast cell (MC) counts were examined by using chloroacetate esterase staining. Levels of serum OVA-specific IgE and IgG₁ antibodies and cytokines in supernatants of OVA-stimulated splenocytes were measured by means of ELISA. Serum IL-4 levels were measured by using an *in vivo* cytokine capture assay.

Results: Epicutaneously sensitized mice exhibited expansion of connective tissue MCs in the jejunum, increased serum IL-4 levels, and systemic anaphylaxis after oral challenge, as evidenced by decreased body temperature and increased serum mouse mast cell protease 1 levels. Intestinal MC expansion and anaphylaxis were IgE dependent because they did not occur in epicutaneously sensitized IgE^{-/-} mice. Mice orally immunized with OVA plus CT did not have increased serum IL-4 levels, expanded intestinal MCs, or anaphylaxis after oral challenge, despite OVA-specific IgE levels and splenocyte cytokine

production in response to OVA stimulation, which were comparable with those of epicutaneously sensitized mice. **Conclusion:** Epicutaneously sensitized mice, but not mice orally immunized with antigen plus CT, have expansion of intestinal MCs and IgE-mediated anaphylaxis after single oral antigen challenge. IgE is necessary but not sufficient for food anaphylaxis, and MC expansion in the gut can play an important role in the development of anaphylaxis. (*J Allergy Clin Immunol* 2013;131:451-60.)

Key words: Food allergy, epicutaneous sensitization, IgE, mast cells, anaphylaxis

Anaphylaxis to food results from IgE-mediated sensitivity to a food allergen. However, IgE antibodies to foods can exist in subjects who can ingest the foods without experiencing anaphylaxis,¹ suggesting that factors other than IgE might be required. In many cases allergic reactions to foods occur on the first known ingestion, suggesting that routes other than the oral one might be important in sensitization. Epidemiologic data suggest that sensitization to peanut protein can occur in children through the application of peanut oil to inflamed skin,² which is consistent with the skin being an important route of allergen sensitization.

Altered skin barrier function in patients with atopic dermatitis (AD) is thought to promote cutaneous sensitization to environmental antigens, including food proteins, potentially leading to the development of food allergies. Little is known about how to prevent the development of food allergy in atopic patients, and presently, there is no cure for it. Current therapy relies on allergen avoidance and treatment of severe reactions with epinephrine. We have used a mouse model of allergic skin inflammation with many features of AD^{3,4} to demonstrate that epicutaneous sensitization, but not oral immunization, with the food antigen ovalbumin (OVA) results in IgE-dependent expansion of intestinal mast cells (MCs) and IgE-mediated anaphylaxis after oral challenge.

METHODS

Mice

BALB/c mice were purchased from Charles River Laboratories (Wilmington, Mass). IgE^{-/-} mice on a BALB/c background were previously reported.⁵ All mice were housed in a specific pathogen-free environment and fed an OVA-free diet. All procedures were performed in accordance with the Animal Care and Use Committee of Boston Children's Hospital.

Epicutaneous sensitization and oral immunization

Epicutaneous sensitization of mice was performed, as previously described.³ Each mouse had a total of three 1-week exposures to OVA (grade V; Sigma, St Louis, Mo) applied as a patch to tape-stripped skin and separated by 2-week rest intervals.

From ^athe Division of Immunology, Boston Children's Hospital, and the Department of Pediatrics, Harvard Medical School; ^bthe Division of Rheumatology, Immunology, and Allergy, Brigham and Women's Hospital, and the Department of Medicine, Harvard Medical School; and ^cthe Department of Pathology, Brigham and Women's Hospital and Harvard Medical School.

*These authors contributed equally to this work.

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Corresponding author: Michiko K. Oyoshi, PhD or Raif S. Geha, MD, Karp 10, Division of Immunology, Boston Children's Hospital, 300 Longwood Ave, Boston, MA 02115. E-mail: Michiko.Oyoshi@childrens.harvard.edu Or: Raif.Geha@childrens.harvard.edu.

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Abbreviations used

AD:	Atopic dermatitis
CAE:	Chloroacetate esterase
CT:	Cholera toxin
CTMC:	Connective tissue mast cell
IL-4R:	IL-4 receptor
MC:	Mast cell
MLN:	Mesenteric lymph node
mMCP-1:	Mouse mast cell protease 1
OVA:	Ovalbumin
Treg:	Regulatory T
WT:	Wild-type

Oral immunization of mice was performed, as previously described.⁶ Briefly, 4- to 6-week-old mice were enterally (subsequently referred to as orally) immunized by means of gavage once a week for 7 weeks with 5 mg of OVA and 10 μ g of cholera toxin (CT; azide free; List Biological Laboratories, Campbell, Calif) in 150 μ L of normal saline or placebo (10 μ g of CT alone in 150 μ L of normal saline) by using a ball-ended mouse feeding needle.

Induction and measurement of systemic anaphylaxis

At week 7 (epicutaneous sensitization model) or week 8 (oral immunization model), mice received a bolus oral challenge with 100 mg of OVA or intravenous challenge with 100 μ g of OVA. Temperature changes were measured by using the DAS-6006 Smart Probe and transponders (Biomedical Data Systems, Seaford, Del) injected subcutaneously. Mice were killed at 60 minutes after challenge to collect serum and harvest tissues.

Serum antibody measurement

OVA-specific IgG₁ and IgE levels were determined by means of ELISA, as previously described.³

In vitro cytokine production and proliferation assay

Spleen single-cell suspensions were cultured at 2×10^6 /mL in the presence of OVA (200 μ g/mL) for 96 hours, as described previously.⁷ Cytokine secretion in supernatants was measured by means of ELISA per the manufacturer's instructions (IL-4 and IFN- γ , eBioscience, San Diego, Calif; IL-13, R&D Systems, Minneapolis, Minn). Splenocyte proliferation was measured by using tritiated thymidine incorporation after 72 hours of culture.

Serum mouse mast cell protease 1 levels

Mouse mast cell protease 1 (mMCP-1) concentrations were measured in sera collected 1 day before and 60 minutes after oral challenge by means of ELISA per the manufacturer's instructions (eBioscience).

Histologic analysis of MCs

Tissue specimens were fixed in 4% paraformaldehyde and embedded in glycolmethacrylate, and sections were stained with chloroacetate esterase (CAE) for quantification of MCs, as previously described.⁸ Tissue sections were examined by investigators who were blind to the identities of the samples. MCs were counted in 10 high-power fields at a magnification of $\times 400$.

In vivo cytokine capture assay for IL-4

The *in vivo* cytokine capture assay for IL-4 was performed, as previously described.⁹ Briefly, mice were intravenously injected with 10 μ g of biotin

anti-IL-4 mAb (BVD6-24G2, eBioscience) and bled 16 hours later. Serum IL-4 levels were determined by using ELISA.

IL4 mRNA expression in mesenteric lymph nodes

Total RNA was extracted from homogenized mesenteric lymph nodes (MLNs) with the RNAqueous extraction kit (Ambion, Austin, Tex). cDNA was generated with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, Calif). Quantitative real-time PCR was done with the TaqMan Gene Expression Assay, universal PCR master mix, and ABI Prism 7300 sequence detection system (Applied Biosystems, Foster City, Calif). Fold induction of IL-4 gene expression was calculated by using the comparative method for relative quantitation with normalization to the internal control β_2 -microglobulin, as described previously.⁶

Statistical analysis

Statistical significance was determined by using GraphPad Prism, version 4.0a (GraphPad Software, La Jolla, Calif). Statistical differences were calculated by using the Student *t* test (between 2 groups) and 2-way ANOVA (between curves). A *P* value of less than .05 was considered statistically significant.

RESULTS

Epicutaneous sensitization with OVA results in anaphylaxis after oral antigen challenge

Wild-type (WT) BALB/c mice were subjected to 3 cycles of epicutaneous sensitization with OVA or saline as a control and then orally challenged with OVA the day after the last cycle of sensitization (Fig 1, A). Epicutaneous sensitization with OVA resulted in the generation of OVA-specific IgE and IgG₁ antibodies (Fig 1, B), as previously published.^{3,10} After oral challenge, mice epicutaneously sensitized with OVA exhibited a significant decrease in core body temperature (Fig 1, C) and significantly increased β -chymase mMCP-1 levels expressed in mucosal MCs^{8,11,12} compared with values seen in control animals epicutaneously sensitized with saline (Fig 1, D). Oral challenge of epicutaneously sensitized mice with saline caused no detectable changes in core body temperature or mMCP-1 levels (Fig 1, C and D). WT BALB/c mice epicutaneously sensitized with 100 μ g of peanut extract over 7 weeks and then orally challenged with 100 mg of peanut flour also exhibited a significant decrease in temperature and an increase in serum mMCP-1 levels (see Fig E1 in this article's Online Repository at www.jacionline.org). These results indicate that cutaneous introduction of antigen promotes anaphylaxis and MC degranulation after oral antigen challenge.

Anaphylaxis in epicutaneously sensitized mice is IgE dependent

Both IgE and IgG₁ antibodies can mediate anaphylaxis in mice.¹² We used IgE^{-/-} mice to examine the role of IgE in our model. After epicutaneous sensitization with OVA, IgE^{-/-} mice exhibited comparable levels of OVA-specific IgG₁ compared with WT mice (Fig 2, A) but no detectable OVA-specific IgE (data not shown). IgE^{-/-} mice epicutaneously sensitized with OVA did not experience decreased body temperature (Fig 2, B) or increased serum mMCP-1 levels (Fig 2, C) after oral OVA challenge. These results suggest that IgE is necessary for the development of anaphylaxis in our model.

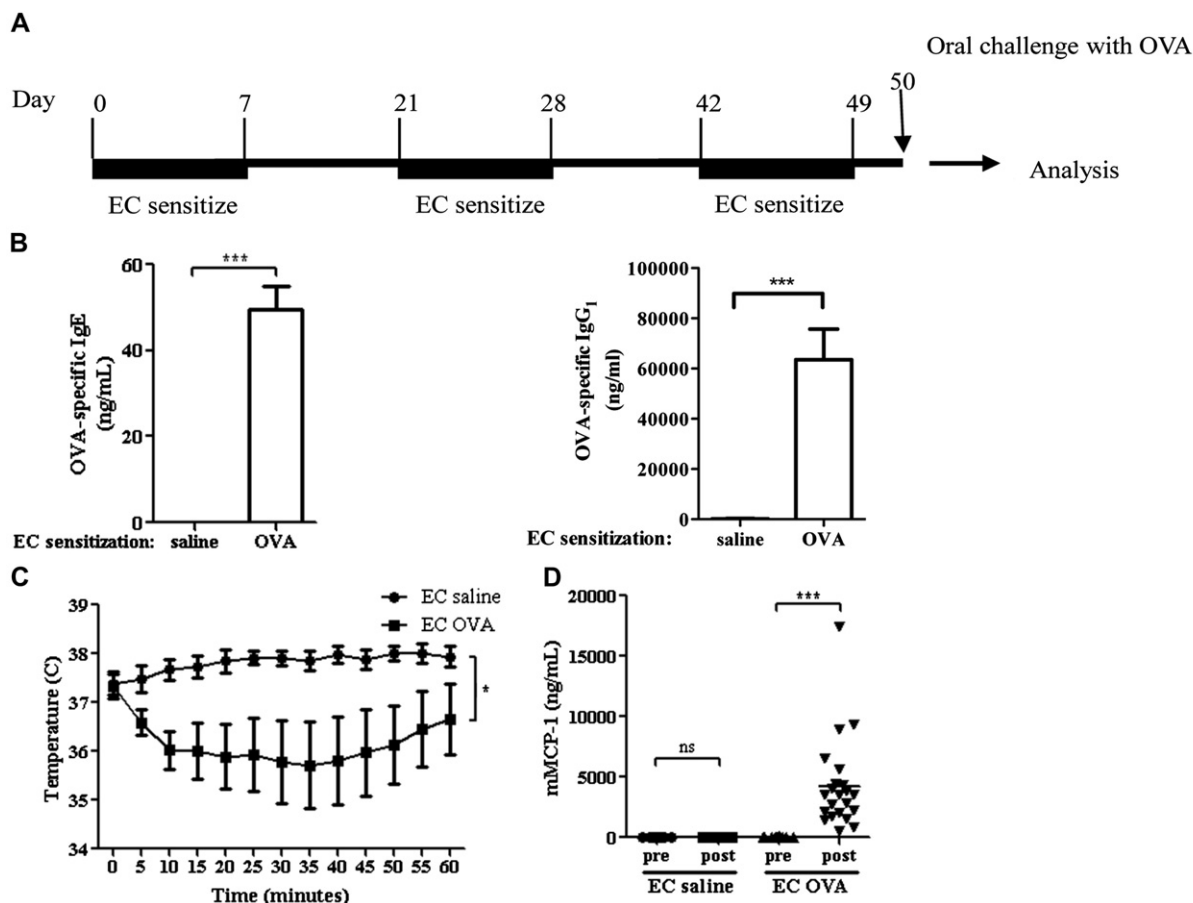


FIG 1. Epicutaneous (EC) sensitization with OVA results in anaphylaxis after oral OVA challenge. **A**, Experimental protocol. **B**, OVA-specific IgE and IgG₁ levels. **C**, Core body temperature after challenge. **D**, Serum mMCP-1 levels before and 60 minutes after challenge. Columns and bars represent means and SEMs (n = 8-20 mice per group). **P* < .05 and ****P* < .001. ns, Not significant.

Oral immunization with OVA plus CT does not result in anaphylaxis to oral challenge, despite eliciting an IgE antibody response comparable with that elicited by epicutaneous sensitization

We have previously shown that oral immunization with OVA plus the mucosal adjuvant CT (OVA plus CT) elicits a robust T_H2-dominated response to OVA with IgG₁ and IgE antibodies.⁶ We used this model to investigate whether a robust IgE antibody is sufficient for oral anaphylaxis. WT BALB/c mice were orally immunized with OVA plus CT or CT alone as a negative control by means of weekly gavage for 8 weeks and then orally challenged with OVA (Fig 3, A). Oral immunization with OVA plus CT elicited detectable serum levels of OVA-specific IgE and IgG₁, which were in the same range as those elicited by means of epicutaneous sensitization with OVA (Fig 3, B). Unlike epicutaneously sensitized mice, orally immunized mice did not experience decreased body temperature after antigen challenge (Fig 3, C). Serum mMCP-1 levels modestly increased after challenge in mice orally immunized with OVA plus CT (Fig 3, D) but were significantly less than in those mice epicutaneously sensitized with OVA (mean, 310.7 ng/mL and 4214.9 ng/mL, respectively; *P* = .001). In contrast to their resistance to oral anaphylaxis, mice orally immunized with OVA plus CT readily underwent anaphylaxis to intravenous administration of OVA

antigen, as evidenced by a marked decrease in body temperature (Fig 3, E). This decrease was comparable with that observed after intravenous challenge of mice epicutaneously sensitized with OVA (Fig 3, F). This result indicates that resistance of orally immunized mice to oral anaphylaxis is not due to a generalized inability to undergo anaphylaxis. These results indicate that IgE antibody is sufficient to cause oral anaphylaxis.

T_H2 cytokines have been shown to play an important role in anaphylaxis, in part by promoting MC expansion and activation. Splenocytes from orally immunized and epicutaneously sensitized mice proliferated to a comparable extent in response to *in vitro* stimulation with OVA (see Fig E2, A, in this article's Online Repository at www.jacionline.org) and secreted comparable amounts of the T_H2 cytokines IL-4 and IL-13, as well as the T_H1 cytokine IFN- γ , which reciprocally regulates the effect of IL-4 on mast cells (see Fig E2, B-D).¹³ Furthermore, mRNA expression in the jejunum for *Il4*, *Il13*, and *Ifng* was comparable in mice epicutaneously sensitized with OVA and mice orally immunized with OVA plus CT (data not shown).

Non-T cells, including basophils and various populations of innate lymphoid helper cells, secrete type 2 cytokines.^{14,15} To assess IL-4 systemic output *in vivo*, we examined IL-4 serum levels and *Il4* mRNA expression in MLNs. Serum IL-4 levels were significantly higher in mice epicutaneously sensitized with OVA

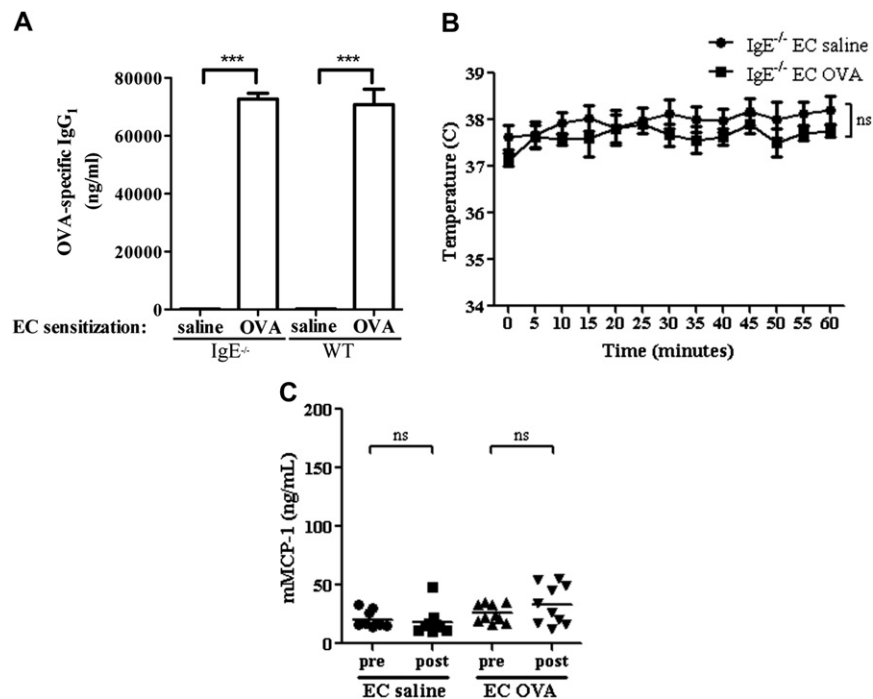


FIG 2. Anaphylaxis after oral OVA challenge in epicutaneously (EC) sensitized mice is IgE dependent. **A**, OVA-specific IgG₁ in epicutaneously sensitized IgE^{-/-} mice and WT control animals. **B**, Core body temperature after oral OVA challenge. **C**, Serum mMCP-1 level before and 60 minutes after challenge. Columns and bars represent means and SEMs (n = 4-10 mice per group). ***P < .001. ns, Not significant.

than in mice epicutaneously sensitized with saline (Fig 4, A). In addition, serum IL-4 levels were significantly higher in mice epicutaneously sensitized with saline than in unmanipulated control animals. In contrast, there was no significant increase in circulating IL-4 levels in mice orally sensitized with OVA plus CT or saline plus CT compared with that seen in unmanipulated control animals. *Il4* mRNA levels were significantly higher in MLNs of mice epicutaneously sensitized with OVA compared with those seen in mice epicutaneously sensitized with saline (Fig 4, B). In addition, *Il4* mRNA levels were significantly higher in MLNs from mice epicutaneously sensitized with saline than in MLNs from unmanipulated mice. These results suggest epicutaneous sensitization is associated with increased systemic output of IL-4 *in vivo*.

Epicutaneous sensitization induces MC expansion in the jejunum

MCs play an important role in IgE-mediated anaphylaxis.¹² We counted the number of MCs in jejunum of BALB/c mice at the end of the 7-week epicutaneous sensitization period and in unmanipulated BALB/c control animals by staining for CAE reactivity which is present in both mucosal and submucosal MCs.¹⁶ We detected a robust and significant increase in the number of MCs in the jejunum of mice epicutaneously sensitized with OVA compared with those seen in unmanipulated control subjects (Fig 5, A and B). Unexpectedly, mice epicutaneously sensitized with saline also exhibited a robust increase in the number of jejunal MCs. There was no detectable increase in the number of jejunal MCs in mice orally immunized with OVA plus CT or CT alone (Fig 5, A and B). The expansion in MC numbers in the jejunum of

epicutaneously sensitized mice occurred predominantly in the submucosa. Analysis of MC numbers in CAE-stained sections revealed a modest but not significant 1.5- to 1.8-fold increase in the numbers of mucosal MCs in epicutaneously sensitized mice (Fig 5, C). In contrast, there was a significant 2.7- to 3.2-fold increase in the numbers of submucosal connective tissue mast cells (CTMCs) in these mice (Fig 5, D). CTMCs in epicutaneously sensitized mice did not express mMCP-1 (data not shown), suggesting that systemic activation of mucosal MCs in various tissues was responsible for the increased serum mMCP-1 levels after oral challenge.

The increase in intestinal MC numbers in epicutaneously sensitized mice was selective. Consistent with previous reports, epicutaneous sensitization with OVA, but not saline, resulted in a 2.4-fold increase in MC numbers at the site of OVA sensitization (see Fig E3, A, in this article's Online Repository at www.jacionline.org).³ However, there was no significant increase in the number of MCs in the ears, spleens, tracheas, or lungs (see Fig E3, B-E). These results indicate that repeated tape stripping of the skin with or without application of antigen causes selective expansion of intestinal submucosal MCs.

The comparable intestinal MC expansion in mice epicutaneously sensitized with saline versus OVA prompted us to examine whether CTMC expansion in the gut was sufficient for IgE-dependent oral anaphylaxis. Intravenous administration on day -1 before challenge of 4 μ g of anti-OVA IgE per mouse to mice epicutaneously sensitized with saline did not result in anaphylaxis in response to oral challenge with OVA (see Fig E4 in this article's Online Repository at www.jacionline.org). In contrast, it resulted in anaphylaxis after intravenous challenge with OVA (see Fig E4), indicating that the anti-OVA IgE we used

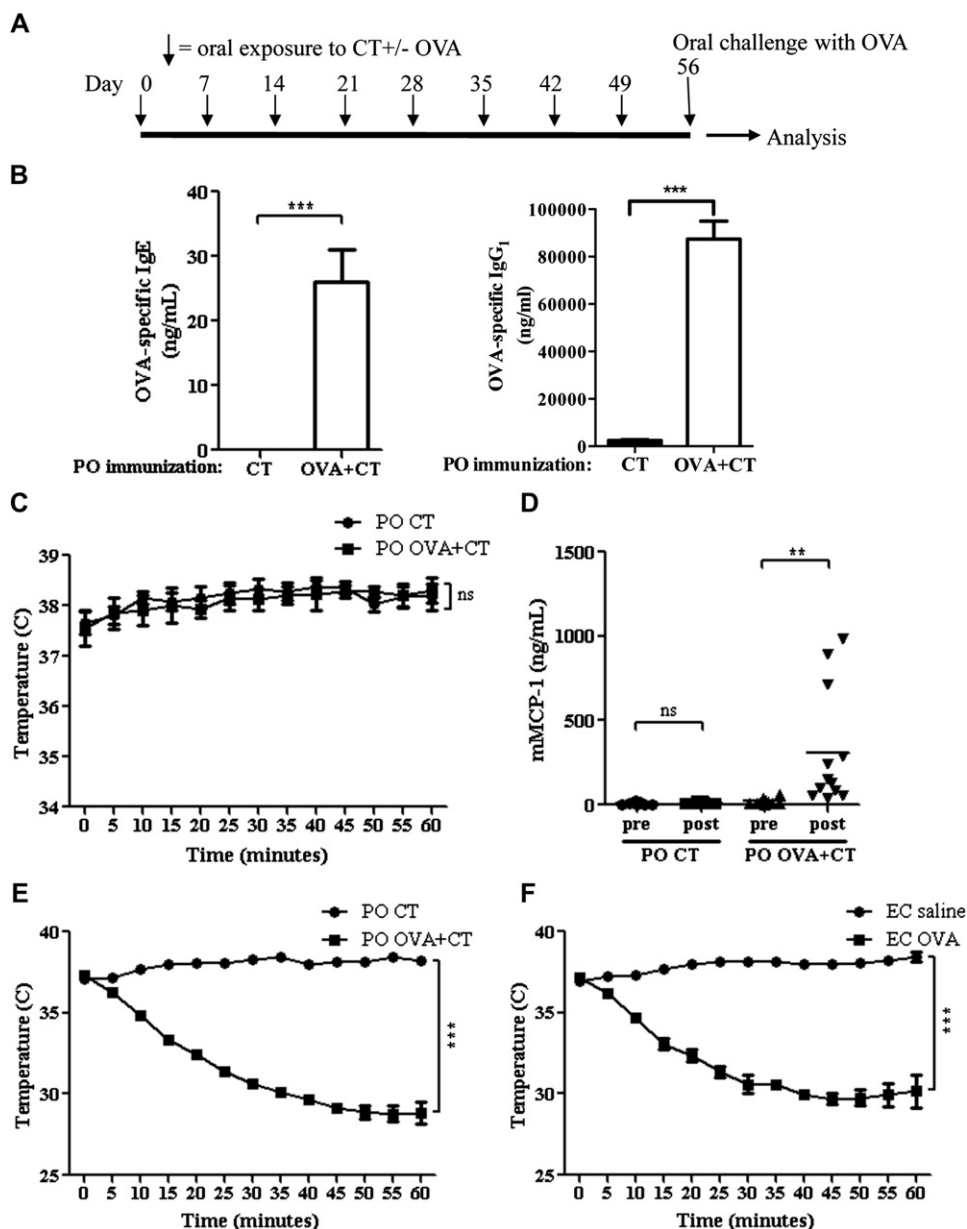


FIG 3. Response of orally immunized mice to antigen challenge. **A**, Experimental protocol. **B**, OVA-specific IgE and IgG₁. **C**, Core body temperature after oral challenge. **D**, Serum mMCP-1. **E** and **F**, Core body temperature after intravenous challenge. Columns and bars represent means and SEMs (n = 10-12 per group in Fig 3, A-D, and 4 in Fig 3, E and F). **P < .01 and ***P < .001. EC, Epicutaneous; ns, not significant; PO, per oral.

was functional. These results suggest that factors other than the number of intestinal MCs are also important for the development of oral anaphylaxis.

Expansion of intestinal MCs is IgE dependent

IgE plays an important role in MC homeostasis and has been shown to drive MC expansion and sustain the survival of mature MCs.¹⁷ Histologic examination of the jejunum from IgE^{-/-} mice epicutaneously sensitized with OVA or saline revealed no significant increase in the numbers of MCs compared with those seen in unmanipulated IgE^{-/-} controls (Fig 6). These results indicate that IgE plays an essential role

in driving the expansion of intestinal MCs in mice subjected to repeated tape stripping of the skin with or without application of antigen.

DISCUSSION

We have demonstrated that epicutaneous sensitization with the food antigen OVA results in IgE-mediated anaphylaxis after oral antigen challenge and IgE-dependent expansion of intestinal MCs. These findings support the hypothesis that cutaneous sensitization to food allergens plays an important role in the development of food allergy in human subjects and that IgE and intestinal MCs are critical to this pathology.

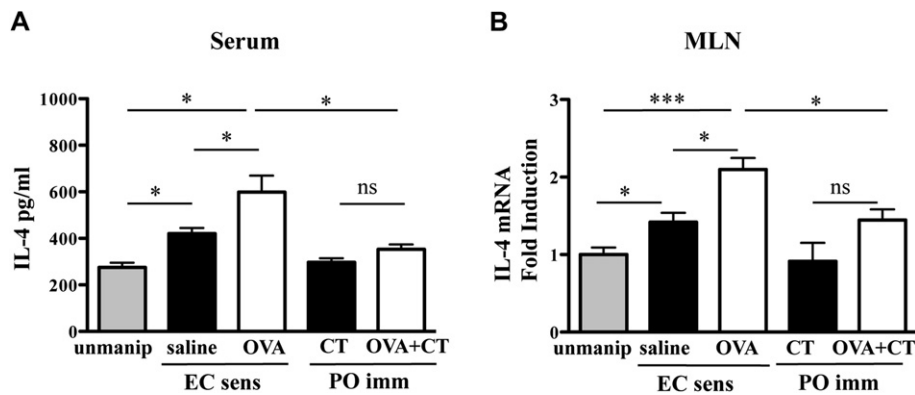


FIG 4. Increased IL-4 serum levels in epicutaneously (EC) sensitized mice. **A**, Serum levels of IL-4 determined by using the *in vivo* cytokine capture assay. **B**, *Il4* mRNA levels in MLNs determined by using quantitative PCR. Columns and bars represent means and SEMs. * $P < .05$ and *** $P < .001$. ns, Not significant; PO, per oral.

Mice epicutaneously sensitized by means of repeated application of OVA to tape-stripped skin underwent anaphylaxis on oral food challenge, as evidenced by decreased body temperature and increased serum mMCP-1 levels, which is indicative of mucosal MC degranulation. The development of oral anaphylaxis in epicutaneously sensitized mice is in agreement with the results of Hsieh et al,¹⁰ who reported that oral challenge of mice epicutaneously sensitized with OVA according to our protocol resulted in anaphylaxis, with an increase in symptom score and serum histamine levels.

Tape stripping, which can be considered a surrogate for scratching, disrupts the normal skin barrier to antigens and elicits a local cutaneous inflammatory response.¹⁸ Thus our data are likely relevant to the potential cutaneous sensitization of children with AD, a disease characterized by scratching of dry skin with a disrupted barrier, high incidence of food-specific IgE antibodies, and high prevalence of food allergies.^{19,20} The observation that loss-of-function variants in the filaggrin gene are a significant risk for peanut allergy,²¹ together with the known role of filaggrin in maintaining an intact skin barrier,²² further supports the relevance of our model to food allergy. In contrast to epicutaneous sensitization of tape-stripped skin, antigen uptake through intact skin downregulates the allergen-specific response in previously sensitized mice.²³

The fact that epicutaneously sensitized mice had anaphylaxis to a single oral challenge in an IgE-dependent manner makes our model highly relevant to human IgE-mediated food anaphylaxis. In previous reports of IgE-mediated anaphylaxis in WT mice,^{24,25} the mice were intraperitoneally immunized, and systemic anaphylaxis, as evidenced by a temperature decrease, was observed in only one of these reports and required multiple oral challenges. We previously reported IgE-dependent anaphylaxis in orally immunized IL-4 receptor (IL-4R) α F709Y chain mutant mice in response to a single oral challenge. The corresponding mutation in the human IL-4R α F709Y chain has not been described.

In contrast to epicutaneous sensitization, oral immunization with OVA plus CT did not elicit anaphylaxis in response to food challenge. This is in agreement with several published studies on oral immunization with a variety of antigens, including OVA and peanut antigens, by using CT as an adjuvant.²⁶⁻²⁸ However, other groups have reported anaphylaxis after oral challenge of mice orally immunized with OVA plus CT.^{29,30} Differences in the doses

and schedules of immunization and in the intestinal flora might be important in determining whether oral immunization leads to anaphylaxis.³¹⁻³³ It has been reported that antimicrobial regimens promote anaphylaxis after oral immunization, possibly by modulating Toll-like receptor signaling by microbially derived Toll-like receptor ligands or by altering cytokine production by T cells and the activity of regulatory T (Treg) cells,^{34,35} which can suppress MC mediator release.³⁶ Nevertheless, under certain conditions of immunization, CT could act both as an adjuvant and a suppressor of anaphylaxis, possibly by promoting Treg cells.³⁷⁻³⁹

The failure of oral immunization to result in anaphylaxis to oral challenge despite induction of OVA-specific IgE antibody levels, which were comparable with those induced by means of epicutaneous sensitization, clearly indicates that although IgE is necessary, it is not sufficient for the development of food anaphylaxis. It is well established that many subjects, including some patients with AD, have circulating IgE antibodies to foods but demonstrate no clinical evidence of food allergy.²⁰ We have ruled out differences between the orally immunized mice and epicutaneously sensitized mice in antigen-driven T-cell production of IL-4, which promotes IgE-mediated anaphylaxis.⁴⁰ However, circulating IL-4 levels and *Il4* mRNA expression in MLNs were significantly increased in epicutaneously sensitized mice but not in orally immunized mice, suggesting that increased systemic IL-4 output, which was associated with intestinal CTMC expansion, might have played an important role in the oral anaphylaxis of epicutaneously sensitized mice. Immunohistochemistry studies did not reveal differences in the numbers of forkhead box protein 3-positive Treg cells in the gut, and forkhead box protein 3 mRNA analysis revealed comparable levels in the jejunum of epicutaneously sensitized and orally immunized mice (data not shown). However, further studies are needed to assess the generation of OVA-specific Treg cells in the 2 immunization protocols.

MCs are important in food-induced anaphylaxis. MC depletion caused by treatment with a c-kit mAb²⁴ and treatment with the MC stabilizer cromolyn⁴¹ ameliorate food-induced anaphylaxis in mice. A major difference between epicutaneously sensitized and orally immunized mice was the marked expansion in CTMCs in the jejunum of epicutaneously sensitized mice, which was not detected in their orally immunized counterparts. The lack of MC expansion in the guts of orally immunized mice might explain their resistance to anaphylaxis.

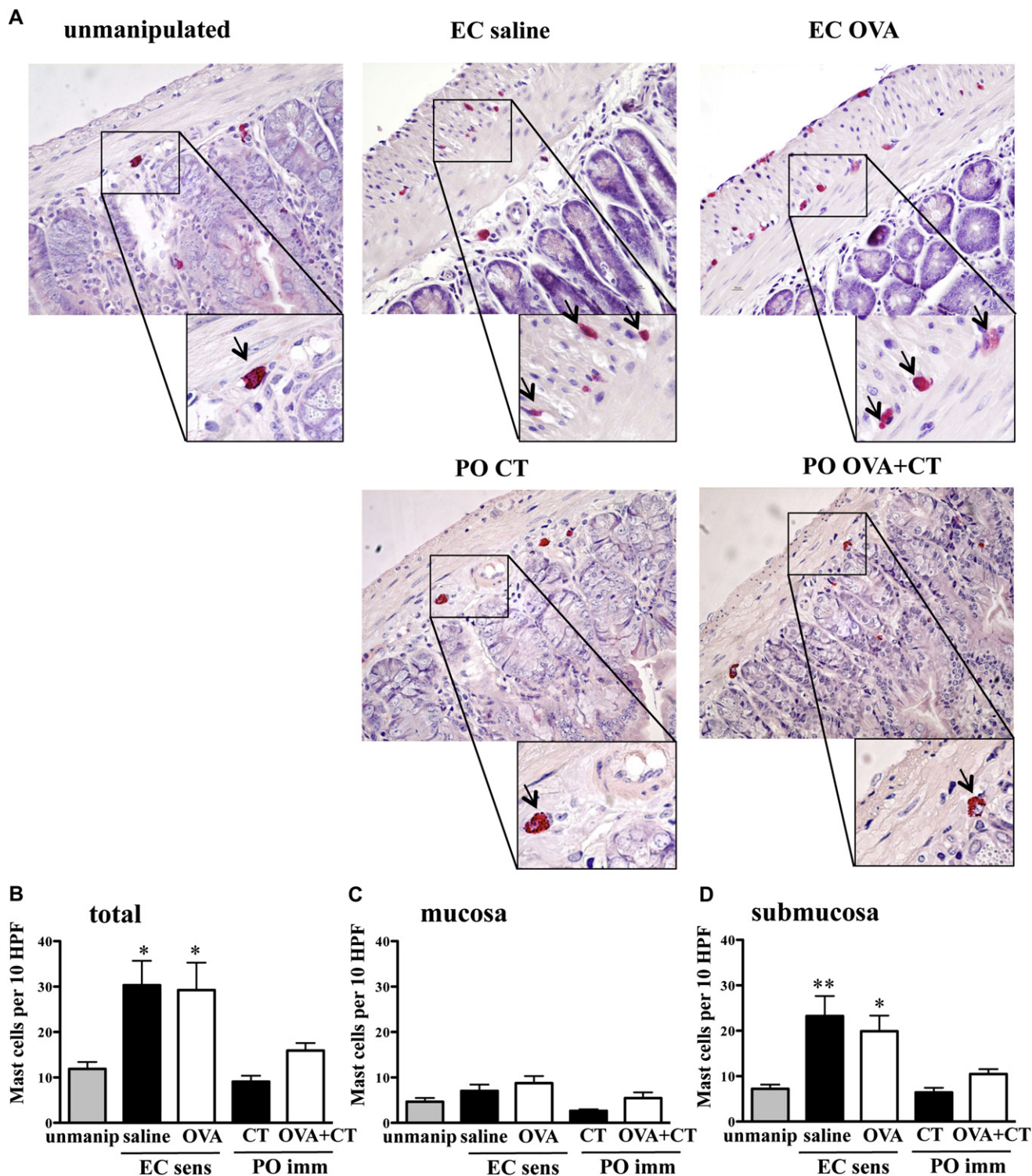


FIG 5. Expansion of submucosal MCs in the jejunum of epicutaneously (*EC*) sensitized mice. **A**, CAE staining of jejunum from epicutaneously sensitized and orally immunized mice (magnification $\times 200$, inset magnification $\times 400$). **B-D**, Total (Fig 5, *B*), mucosal (Fig 5, *C*), and submucosal (Fig 5, *D*) MCs in jejunum of epicutaneously sensitized, orally immunized, and unmanipulated mice. *Columns* and *bars* represent means and SEMs ($n = 5-10$ mice per group). Statistical significance was calculated relative to the unmanipulated group. * $P < .05$ and ** $P < .01$. *HPF*, High-power field; *PO*, per oral.

MC expansion in epicutaneously sensitized mice involved predominantly the submucosal MCs. Furthermore, except for a modest 2.4-fold expansion of MCs in OVA-sensitized skin sites,

there was no evidence of MC expansion in several other organs of epicutaneously sensitized mice. In a recent study from our group,²⁸ mice carrying a gain-of-function mutation in the

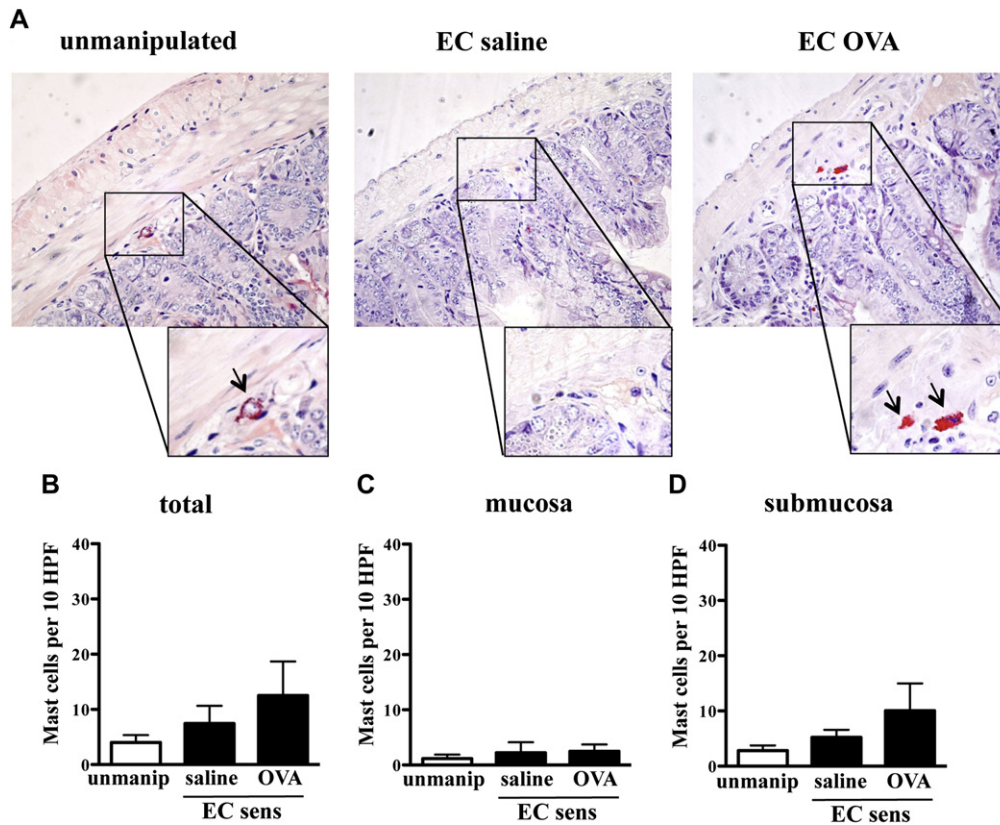


FIG 6. A-D, Expansion of intestinal MCs in epicutaneously (EC) sensitized mice is IgE dependent. Quantitation of MCs in CAE-stained jejunal tissue sections from epicutaneously sensitized IgE^{-/-} mice and unmanipulated IgE^{-/-} control animals. Columns and bars represent means and SEMs (n = 5 mice per group). HPF, High-power field.

IL-4R α chain (Y709F) were found to be susceptible to food allergy after oral immunization and had a modest expansion of MCs, which appeared to involve predominantly the mucosal MCs and to be IgE independent.²⁸ The differential requirement for IgE in intestinal MC expansion in the 2 models points to important differences. These might include differences in responsiveness to IL-4 and routes of antigen sensitization. The observation that Fc ϵ RI promotes survival of CTMCs but not mucosal MCs *in vitro*⁴² supports our finding that IgE is necessary for CTMC expansion.

Understanding the factors that drive the expansion of intestinal MCs in epicutaneously sensitized mice is of critical importance in understanding food allergy. IgE is clearly one of these factors because intestinal MCs did not expand in epicutaneously sensitized IgE^{-/-} mice. However, IgE was not sufficient for the expansion of intestinal MCs in mice orally immunized with OVA plus CT. IL-4 has a well-established known role in MC homeostasis.^{43,44} Its increase in the sera and MLNs of epicutaneously sensitized mice suggests that it might play a role in the intestinal expansion of CTMCs in these mice.

Given the lack of MC expansion in other organs except the skin, MC expansion in the guts of epicutaneously sensitized mice might be mediated by cells that traffic from skin to gut, which could include dendritic cells and T cells.^{6,45} We previously reported that mechanical injury caused by tape stripping induces TSLP production in the skin and that dendritic cells derived from skin injured by tape stripping are programmed by thymic stromal lymphopoietin

(TSLP) to drive a T_H2 response and migrate to MLNs.⁴⁶ TSLP levels were increased in epicutaneously sensitized skin (see Fig E5 in this article's Online Repository at www.jacionline.org) and might have contributed to the MC expansion in the intestines of epicutaneously sensitized mice. The precise nature of the cells and the factors that mediate the skin-to-gut cross-talk that results in gut MC expansion remains to be determined.

The finding that MCs underwent expansion in the jejunum of mice epicutaneously sensitized with saline was unexpected. It could have resulted from the skin inflammation caused by tape stripping, which might have released cytokines and cells that promoted MC expansion in the gut, and/or from sensitization to environmental antigens (ie, pathogens and proteins in mouse diet) absorbed through tape-stripped skin. Experiments with germ-free mice and mice maintained on elemental diets should help distinguish between these possibilities.

During oral antigen challenge, antigen traffics across the intestinal epithelium in 2 phases.⁴⁷ In phase 1 antigen transport occurs through the transcellular route in an MC-independent manner and is likely mediated by CD23-dependent IgE-mediated uptake.^{48,49} In phase 2, which occurs several minutes later, massive antigen transport occurs through the transcellular route, and this is dependent on Fc ϵ RI-mediated activation of MCs. Degranulating MCs secrete a variety of mediators that can enhance intestinal permeability, which would promote systemic anaphylaxis. These include mMCP-1, which originates from mucosal MCs, and mMCP-4, which is derived from

submucosal CTMCs.³³ Increased intestinal MC expansion has been reported in adults with food allergy⁵⁰ and could underlie the increased intestinal permeability in these patients.^{33,47}

Our findings demonstrate that introduction of food antigen through the skin causes expansion of intestinal MCs and predisposes to IgE-mediated anaphylaxis. Prevention of cutaneous sensitization by allergen avoidance and aggressive skin barrier protection and therapies targeted at limiting the expansion and activation of intestinal MCs can protect against food allergies.

Clinical implications: The skin might be an important route of sensitization to food antigens. Avoidance of cutaneous sensitization could prevent the development of food anaphylaxis.

REFERENCES

- Hill DJ, Heine RG, Hosking CS, Brown J, Thiele L, Allen KJ, et al. IgE food sensitization in infants with eczema attending a dermatology department. *J Pediatr* 2007;151:359-63.
- Lack G, Fox D, Northstone K, Golding J. Factors associated with the development of peanut allergy in childhood. *N Engl J Med* 2003;348:977-85.
- Spergel JM, Mizoguchi E, Brewer JP, Martin TR, Bhan AK, Geha RS. Epicutaneous sensitization with protein antigen induces localized allergic dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice. *J Clin Invest* 1998;101:1614-22.
- Jin H, He R, Oyoshi M, Geha RS. Animal models of atopic dermatitis. *J Invest Dermatol* 2009;129:31-40.
- Oettgen HC, Martin TR, Wynshaw-Boris A, Deng C, Drazen JM, Leder P. Active anaphylaxis in IgE-deficient mice. *Nature* 1994;370:367-70.
- Oyoshi MK, Elkhali A, Scott JE, Wurbel MA, Hornick JL, Campbell JJ, et al. Epicutaneous challenge of orally immunized mice redirects antigen-specific gut-homing T cells to the skin. *J Clin Invest* 2011;121:2210-20.
- Jin H, Kumar L, Mathias C, Zurakowski D, Oettgen H, Gorelik L, et al. Toll-like receptor 2 is important for the T(H)1 response to cutaneous sensitization. *J Allergy Clin Immunol* 2009;123:875-82.e1.
- Xing W, Austen KF, Gurish MF, Jones TG. Protease phenotype of constitutive connective tissue and of induced mucosal mast cells in mice is regulated by the tissue. *Proc Natl Acad Sci U S A* 2011;108:14210-5.
- Finkelman F, Morris S, Orekhova T, Sehy D. The in vivo cytokine capture assay for measurement of cytokine production in the mouse. In: Coligan JE, Bierer BE, Margulies DH, Shevach EM, Strober W, Brown P. *Current protocols in immunology*. Malden, MA; 2003.
- Hsieh KY, Tsai CC, Wu CH, Lin RH. Epicutaneous exposure to protein antigen and food allergy. *Clin Exp Allergy* 2003;33:1067-75.
- Andersson MK, Pemberton AD, Miller HR, Hellman L. Extended cleavage specificity of mMCP-1, the major mucosal mast cell protease in mouse-high specificity indicates high substrate selectivity. *Mol Immunol* 2008;45:2548-58.
- Finkelman FD. Anaphylaxis: lessons from mouse models. *J Allergy Clin Immunol* 2007;120:506-17.
- Deschoolmeester ML, Eastmond NC, Dearman RJ, Kimber I, Basketter DA, Coleman JW. Reciprocal effects of interleukin-4 and interferon-gamma on immunoglobulin E-mediated mast cell degranulation: a role for nitric oxide but not peroxynitrite or cyclic guanosine monophosphate. *Immunology* 1999;96:138-44.
- Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TK, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 2010;464:1367-70.
- Oliphant CJ, Barlow JL, McKenzie AN. Insights into the initiation of type 2 immune responses. *Immunology* 2011;134:378-85.
- Friend DS, Ghildyal N, Austen KF, Gurish MF, Matsumoto R, Stevens RL. Mast cells that reside at different locations in the jejunum of mice infected with *Trichinella spiralis* exhibit sequential changes in their granule ultrastructure and chymase phenotype. *J Cell Biol* 1996;135:279-90.
- Mathias CB, Freyschmidt EJ, Caplan B, Jones T, Poddighe D, Xing W, et al. IgE influences the number and function of mature mast cells, but not progenitor recruitment in allergic pulmonary inflammation. *J Immunol* 2009;182:2416-24.
- Wood LC, Stalder AK, Liou A, Campbell IL, Grunfeld C, Elias PM, et al. Barrier disruption increases gene expression of cytokines and the 55 kD TNF receptor in murine skin. *Exp Dermatol* 1997;6:98-104.
- Eigenmann PA, Sicherer SH, Borkowski TA, Cohen BA, Sampson HA. Prevalence of IgE-mediated food allergy among children with atopic dermatitis. *Pediatrics* 1998;101:e8.
- Boyce JA, Assa'ad A, Burks AW, Jones SM, Sampson HA, Wood RA, et al. Guidelines for the diagnosis and management of food allergy in the United States: report of the NIAID-sponsored expert panel. *J Allergy Clin Immunol* 2010;126(suppl): S1-58.
- Brown SJ, Asai Y, Cordell HJ, Campbell LE, Zhao Y, Liao H, et al. Loss-of-function variants in the filaggrin gene are a significant risk factor for peanut allergy. *J Allergy Clin Immunol* 2011;127:661-7.
- O'Regan GM, Irvine AD. The role of filaggrin in the atopic diathesis. *Clin Exp Allergy* 2010;40:965-72.
- Dioszeghy V, Mondoulet L, Dhelft V, Ligouis M, Puteaux E, Benhamou PH, et al. Epicutaneous immunotherapy results in rapid allergen uptake by dendritic cells through intact skin and downregulates the allergen-specific response in sensitized mice. *J Immunol* 2011;186:5629-37.
- Brandt EB, Strait RT, Hershko D, Wang Q, Muntel EE, Scribner TA, et al. Mast cells are required for experimental oral allergen-induced diarrhea. *J Clin Invest* 2003;112:1666-77.
- Osterfeld H, Ahrens R, Strait R, Finkelman FD, Renaud JC, Hogan SP. Differential roles for the IL-9/IL-9 receptor alpha-chain pathway in systemic and oral antigen-induced anaphylaxis. *J Allergy Clin Immunol* 2010;125:469-76.e2.
- Bashir ME, Louie S, Shi HN, Nagler-Anderson C. Toll-like receptor 4 signaling by intestinal microbes influences susceptibility to food allergy. *J Immunol* 2004;172: 6978-87.
- Berin MC, Zheng Y, Domaradzki M, Li XM, Sampson HA. Role of TLR4 in allergic sensitization to food proteins in mice. *Allergy* 2006;61:64-71.
- Mathias CB, Hobson SA, Garcia-Lloret M, Lawson G, Poddighe D, Freyschmidt EJ, et al. IgE-mediated systemic anaphylaxis and impaired tolerance to food antigens in mice with enhanced IL-4 receptor signaling. *J Allergy Clin Immunol* 2011; 127:795-805, e1-6.
- Perrier C, Thierry AC, Mercenier A, Corthesy B. Allergen-specific antibody and cytokine responses, mast cell reactivity and intestinal permeability upon oral challenge of sensitized and tolerized mice. *Clin Exp Allergy* 2010;40:153-62.
- Ganeshan K, Neilsen CV, Hadsaitong A, Schleimer RP, Luo X, Bryce PJ. Impairing oral tolerance promotes allergy and anaphylaxis: a new murine food allergy model. *J Allergy Clin Immunol* 2009;123:231-8.e4.
- Du Toit G, Katz Y, Sasieni P, Mesher D, Maleki SJ, Fisher HR, et al. Early consumption of peanuts in infancy is associated with a low prevalence of peanut allergy. *J Allergy Clin Immunol* 2008;122:984-91.
- Diesner SC, Knittelfelder R, Krishnamurthy D, Pali-Scholl I, Gajdzik L, Jensen-Jarolim E, et al. Dose-dependent food allergy induction against ovalbumin under acid-suppression: a murine food allergy model. *Immunol Lett* 2008;121:45-51.
- Groschwitz KR, Hogan SP. Intestinal barrier function: molecular regulation and disease pathogenesis. *J Allergy Clin Immunol* 2009;124:3-22.
- Prioult G, Nagler-Anderson C. Mucosal immunity and allergic responses: lack of regulation and/or lack of microbial stimulation? *Immunol Rev* 2005;206:204-18.
- Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, et al. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* 2011; 331:337-41.
- Gri G, Piconese S, Frossi B, Manfredi V, Merluzzi S, Tripodo C, et al. CD4+CD25+ regulatory T cells suppress mast cell degranulation and allergic responses through OX40-OX40L interaction. *Immunology* 2008;29:771-81.
- Sun JB, Cuburu N, Blomquist M, Li BL, Czerkinsky C, Holmgren J. Sublingual tolerance induction with antigen conjugated to cholera toxin B subunit induces Foxp3+CD25+CD4+ regulatory T cells and suppresses delayed-type hypersensitivity reactions. *Scand J Immunol* 2006;64:251-9.
- Sun JB, Czerkinsky C, Holmgren J. Sublingual "oral tolerance" induction with antigen conjugated to cholera toxin B subunit generates regulatory T cells that induce apoptosis and depletion of effector T cells. *Scand J Immunol* 2007;66:278-86.
- Sun JB, Raghavan S, Sjoling A, Lundin S, Holmgren J. Oral tolerance induction with antigen conjugated to cholera toxin B subunit generates both Foxp3+CD25+ and Foxp3-CD25- CD4+ regulatory T cells. *J Immunol* 2006;177:7634-44.
- Strait RT, Morris SC, Smiley K, Urban JF Jr, Finkelman FD. IL-4 exacerbates anaphylaxis. *J Immunol* 2003;170:3835-42.
- Forbes EE, Groschwitz K, Abonia JP, Brandt EB, Cohen E, Blanchard C, et al. IL-9- and mast cell-mediated intestinal permeability predisposes to oral antigen hypersensitivity. *J Exp Med* 2008;205:897-913.
- Ekoff M, Strasser A, Nilsson G. FcepsilonRI aggregation promotes survival of connective tissue-like mast cells but not mucosal-like mast cells. *J Immunol* 2007;178: 4177-83.
- Lorentz A, Wilke M, Sellge G, Worthmann H, Klempnauer J, Manns MP, et al. IL-4-induced priming of human intestinal mast cells for enhanced survival and Th2 cytokine generation is reversible and associated with increased activity of ERK1/2 and c-Fos. *J Immunol* 2005;174:6751-6.
- Shelburne CP, Ryan JJ. The role of Th2 cytokines in mast cell homeostasis. *Immunol Rev* 2001;179:82-93.

45. Hammerschmidt SI, Friedrichsen M, Boelter J, Lyszkiewicz M, Kremmer E, Pabst O, et al. Retinoic acid induces homing of protective T and B cells to the gut after subcutaneous immunization in mice. *J Clin Invest* 2011;121:3051-61.
46. 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;126:976-84, e1-5.
47. Perrier C, Corthesy B. Gut permeability and food allergies. *Clin Exp Allergy* 2011;41:20-8.
48. Li H, Nowak-Wegrzyn A, Charlop-Powers Z, Shreffler W, Chehade M, Thomas S, et al. Transcytosis of IgE-antigen complexes by CD23a in human intestinal epithelial cells and its role in food allergy. *Gastroenterology* 2006;131:47-58.
49. Bevilacqua C, Montagnac G, Benmerah A, Candalh C, Brousse N, Cerf-Bensussan N, et al. Food allergens are protected from degradation during CD23-mediated transepithelial transport. *Int Arch Allergy Immunol* 2004;135:108-16.
50. Chen X, Song CH, Liu ZQ, Feng BS, Zheng PY, Li P, et al. Intestinal epithelial cells express galectin-9 in patients with food allergy that plays a critical role in sustaining allergic status in mouse intestine. *Allergy* 2011;66:1038-46.

METHODS

Epicutaneous sensitization with peanut extract and measurement of systemic anaphylaxis

EC sensitization of mice was performed, as previously described.^{E1} Each mouse had a total of three 1-week exposures to 100 μ g of peanut extract (Greer Laboratories, Lenoir, NC) applied as a patch to tape-stripped skin and separated by 2-week rest intervals. At week 7, mice received a bolus oral challenge with 100 mg of peanut flour (*nuts.com*). Temperature changes were measured with the DAS-6006 Smart Probe and transponders (Biomedic Data Systems) injected subcutaneously. Mice were killed at 60 minutes after challenge to collect serum and harvest tissues.

Skin TSLP levels

Skin TSLP levels were measured, as previously reported.^{E2} Briefly, skin was homogenized by using the Polytoron RT-3000 (Kinematica AG,

Lucerne, Switzerland) in T-PER tissue protein extraction reagent (Thermo Scientific, Waltham, Mass) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was quantified with Nanodrop (Thermo Scientific). TSLP concentrations were measured by using ELISA (R&D Systems), and the amount of TSLP per 100 μ g of protein was plotted.

REFERENCES

- E1. Spengel JM, Mizoguchi E, Brewer JP, Martin TR, Bhan AK, Geha RS. Epicutaneous sensitization with protein antigen induces localized allergic dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice. *J Clin Invest* 1998;101:1614-22.
- E2. 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;126:976-84, e1-5.

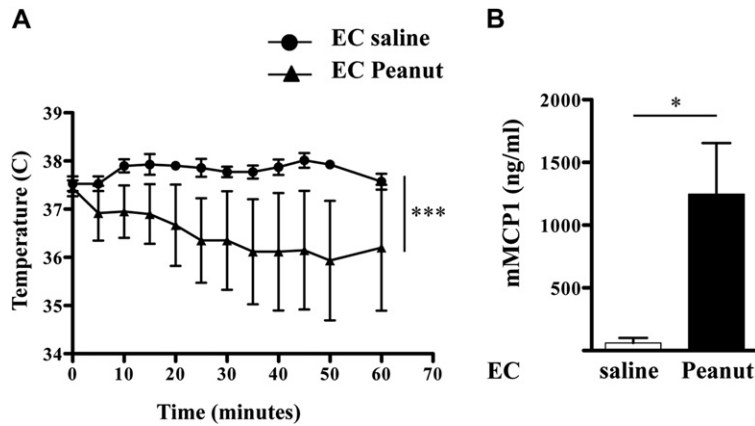


FIG E1. Epicutaneous (EC) sensitization with peanut extract results in anaphylaxis after oral antigen challenge. **A**, Core body temperature of WT BALB/c mice epicutaneously sensitized with peanut extract or saline and orally challenged with peanut flour. **B**, Serum mMCP-1 levels before and 60 minutes after challenge. Columns and bars represent means and SEMs ($n = 7$ mice per group). * $P < .05$ and *** $P < .001$.

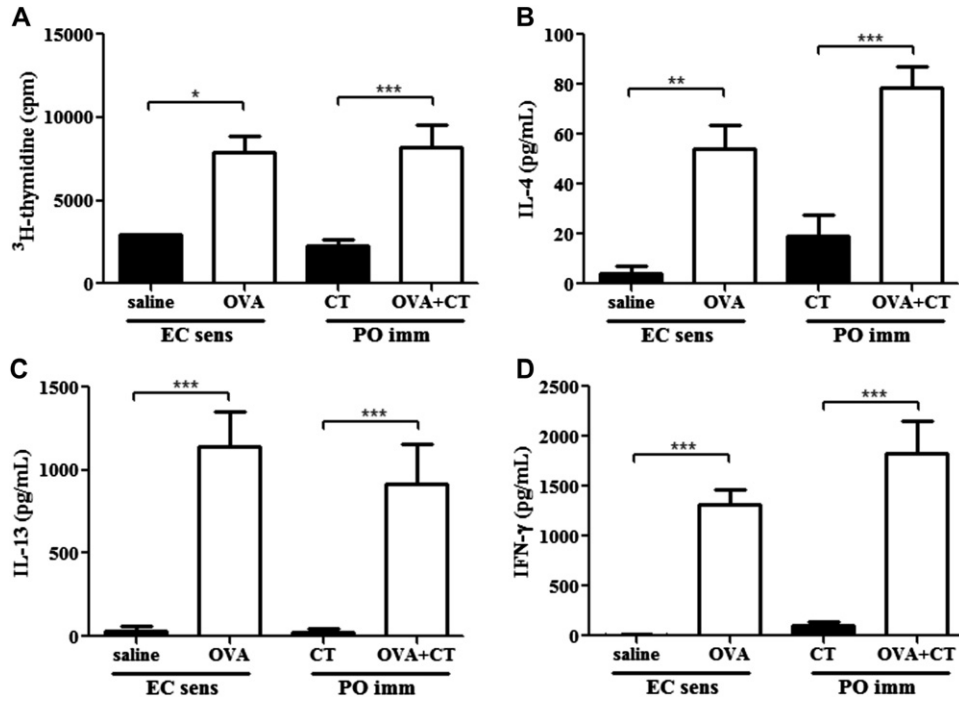


FIG E2. Production of T_H1 and T_H2 cytokines and proliferation by OVA-stimulated splenocytes from epicutaneously (*EC*) sensitized and orally immunized mice. **A**, Proliferation measured by incorporation of tritiated thymidine into DNA ($n = 3-9$ mice per group). **B-D**, Secretion of IL-4 (Fig E2, *B*), IL-13 (Fig E2, *C*), and IFN- γ (Fig E2, *D*). * $P < .05$, ** $P < .01$, and *** $P < .001$. *PO*, Per oral.

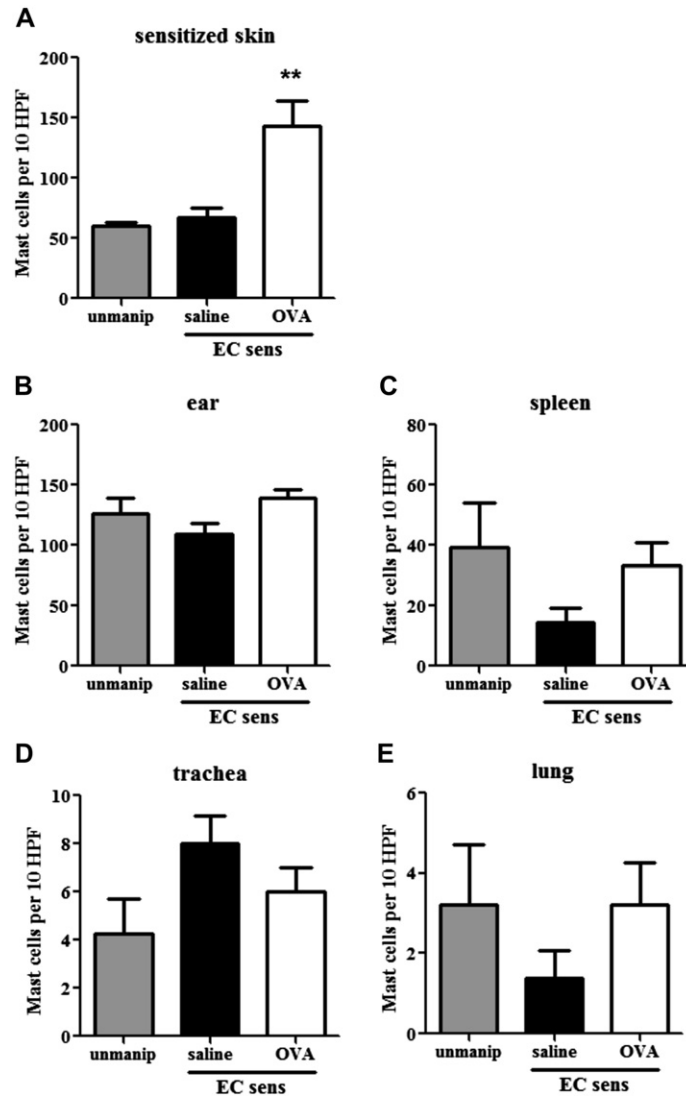


FIG E3. Effect of epicutaneous (EC) sensitization on MC numbers in different tissues. **B**, Quantitation of MCs in tissue sections. Ear sections ($n = 5-12$ mice per group) were stained with Giemsa. **A** and **C-E**, All others ($n = 2-5$ mice per group) were stained for CAE. Columns and bars represent means and SEMs. Statistical significance was calculated relative to the unmanipulated (*unmanip*) group. $**P < .01$.

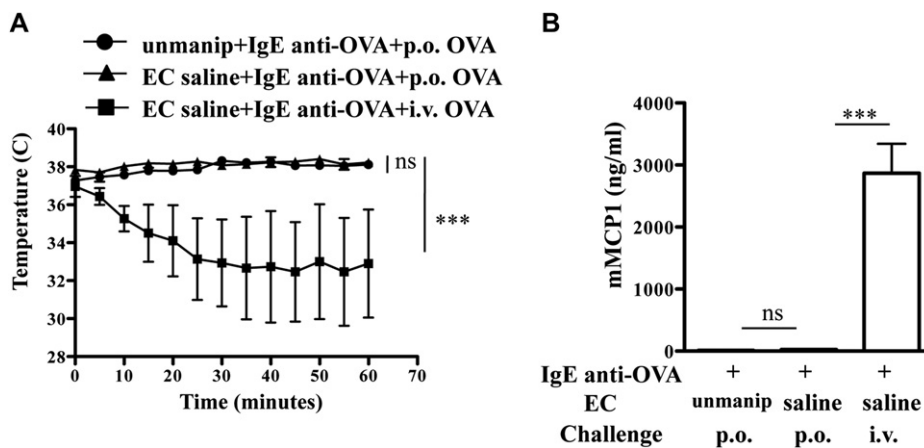


FIG E4. Administration of OVA-specific IgE did not induce oral anaphylaxis in mice epicutaneously (*EC*) sensitized with saline. **A**, Effect of oral OVA challenge on core body temperature of unmanipulated BALB/c mice and BALB/c mice epicutaneously sensitized with saline that had received 4 μ g of intravenous (*i.v.*) anti-OVA IgE 24 hours before challenge. Mice epicutaneously sensitized with saline received anti-OVA IgE and intravenously challenged with 100 μ g of OVA were used as positive controls. **B**, Serum mMCP-1 levels before and 60 minutes after challenge. Columns and bars represent means and SEMs ($n = 5$ mice per group). *** $P < .001$. ns, Not significant; *p.o.*, per oral; *unmanip*, unmanipulated.

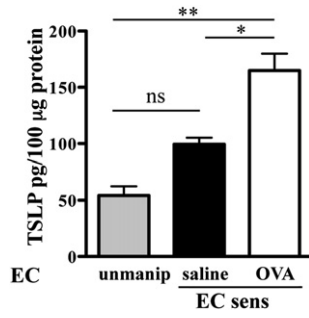


FIG E5. Upregulation of TSLP levels in mouse skin epicutaneously (*EC*) sensitized with OVA. Skin TSLP levels in BALB/c mice that were unmanipulated (*unmanip*) or epicutaneously (*EC*) sensitized with saline or OVA were measured by using ELISA. *Columns and bars* represent means and SEM ($n = 7$ mice per group). $*P < .05$ and $**P < .01$. *ns*, Not significant.