

Prevention of oral food allergy sensitization via skin application of food allergen in a mouse model

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Abstract

Background Treatment options for food allergy remain limited. Development of novel approaches for the prevention and/or treatment of severe peanut allergy and other food allergies is urgently needed. The objective of this study was to test whether skin application of food allergen can be used as a prophylactic and/or therapeutic intervention for food allergy.

Methods Balb/C mice were given 5 weekly cutaneous application of complete peanut extract (CPE) or ovalbumin (OVA) ranging from 10 to 1000 µg on the shaved back skin, followed by 5 weekly treatments with oral CPE or OVA plus cholera toxin to induce allergic reactivity to the food. At various time points, the immunologic responses and allergic clinical manifestations to allergens were examined.

Results Skin application of a 10–1000 µg dose of CPE or OVA to structurally intact skin did not lead to allergic sensitization to peanut or OVA. Rather, cutaneous allergen application blocked, in a dose-dependent fashion, the subsequent induction of the oral sensitization including inhibiting oral sensitization-induced CPE-specific IgE, IgG1, and IgG2a production, suppressing the peanut anaphylaxis, and modulating the oral sensitization-promoted cytokine production. The cutaneous OVA application also resulted in similar results as seen with CPE application.

Conclusion Cutaneous application of intact skin with peanut or OVA can block the development of orally induced corresponding food allergies, suggesting that allergic tolerance to peanuts and OVA might be achieved via allergen cutaneous application.

IgE-mediated food allergy continues to increase and now affects up to ~3.7% of the overall US population and 6–8% of young children (1, 2). In contrast to the expanding array of treatments available for inhalant and cutaneous allergies, the treatment options for food allergy remain very limited. Thus, it is universally agreed that novel approaches for the prevention and/or treatment of severe food allergy are urgently needed.

Failure to induce the normal oral tolerance to a food or a breakdown in an oral tolerance could result in the development

of IgE-mediated food allergy. It is likely that the initial sensitization to food allergens occurs primarily via the gastrointestinal tract (3, 4). However, alternative routes such as inhalation and/or skin contact also have been proposed for peanut sensitization as many peanut-allergic children have no identifiable oral peanut consumption prior to peanut sensitization (1, 2, 5). Animal experiments have confirmed that repeated topical contact with allergens through skin whose barrier function is disrupted could promote allergic sensitization and airway hypersensitivity and prevent oral tolerance induction (6–13).

Cutaneous application of antigens has been shown to be able to induce a state of unresponsiveness to subsequent antigen challenge (14, 15). Skin-induced immune tolerance has been demonstrated in several experiments (14–19).

Abbreviations

CPE complete peanut extract; CT cholera toxin; DIG digoxigenin; ILN inguinal lymph node; MLN mesenteric lymph node; OVA ovalbumin.

Recently, Mondoulet et al. (20–22) reported that established allergic diseases including food allergy could be treated in an allergen-specific manner with a device directly applying allergens onto the intact skin. Thus, manipulation of the skin-based immune network holds the promise of a means to regulate immune and/or allergic responses in either a positive or negative fashion (23).

While establishing a cutaneous sensitization model for peanut allergy (24, 25), we observed that repeated cutaneous administration of complete peanut extract (CPE) or ovalbumin (OVA) to intact skin did not sensitize the animals, but instead rendered the animals resistant to subsequent oral sensitization. Investigation of this effect revealed that cutaneous application of a potential allergen can induce primary allergic tolerance and suggest that appropriate application of allergens to intact skin may serve as novel approach for the prevention/treatment of food allergies.

Materials and methods

Allergen skin application and skin biopsy

Balb/c mice (6–8 weeks old) were fed with a diet free of OVA or peanut. The endotoxin-free CPE was prepared as described (26). CPE or OVA (0, 10, 100, and 1000 µg) was painted onto the dorsal skin at weekly intervals for 5 weeks, followed by the experimental procedures diagrammed in Fig. 1A. The mice were individually housed in separate cages to prevent potential oral cross-contact. All experiments described in this study were approved by the UCLA Animal Research Committee.

Oral allergic sensitization and induction of anaphylaxis

For oral sensitization, mice were administered with 1 mg of CPE or OVA plus 10 µg of cholera toxin (CT) by

intragastric feeding weekly for five times. The sensitized mice were challenged intraperitoneally (i.p.) with 5 mg of CPE or OVA 2 weeks post the last oral dosing. Changes in the animals' core body temperature and anaphylactic clinical scores were monitored for the ensuing 60 mins (26, 27).

Measurement of Ag-specific Igs

Peanut- or OVA-specific IgG1, IgG2a, and IgE were measured by ELISA described (28) with modification. Rat anti-mouse IgE (2.5 µg/ml) was used for coating. Following blocking, the diluted sera were incubated for 2 h. Subsequently, a digoxigenin (DIG)-coupled CPE (or OVA) conjugates (2 µg/ml) were incubated for 2 h, followed by incubation with alkaline phosphatase-conjugated anti-DIG antibody. The developed color was measured at 650 nm. The Ig titers were determined by comparing a reference serum with arbitrary titer set as 160, 320 000, and 320 000 units/ml for IgE, IgG1, and IgG2a, respectively.

Cell preparation, culture, cytokine production, and cell subpopulation analysis

Mononuclear cells from the spleen, inguinal lymph node (ILN), and mesenteric lymph node (MLN) were prepared 2 weeks after the last oral sensitization for cellular and cytokine analysis. CPE and control protein OVA (or vice versa) were added at 100 µg/ml as the stimulators for cytokine production. Following 120-h culture, supernatants were harvested and stored at –80°C for later cytokine measurement using mouse TH1/TH2/Th17 Cytometry Beads Array assays (BD Bioscience, San Diego, CA, USA). For regulatory T-cell analysis, the single-cell suspension from the draining ILN was analyzed using a Treg detection kit (Biolegend) by FACS. For dendritic cells (DCs) analysis, the cells were stained with anti-CD8α, anti-CD11c, anti-MHC-II, anti-CD40, and anti-CD207 antibodies and subjected to FACS analysis.

Data analysis

Data are expressed as mean ± SEM, unless indicated in the figure legend. ANOVA was used to determine the statistical significance. The statistically significant difference was defined as $P < 0.05$, which was indicated with one asterisk, and very significant difference as $P < 0.01$, which was indicated with double asterisks in the Figures.

Results

CPE exposure on the intact skin fails to induce allergic sensitization to peanut

To define whether cutaneous exposure with peanut would drive peanut sensitization, we repeatedly applied differing doses of endotoxin-free CPE to the intact skin (Fig. 1A). This treatment itself did not induce peanut-specific IgE and/or IgG1 (Fig. 1B). CPE challenge did not induce clinical pea-

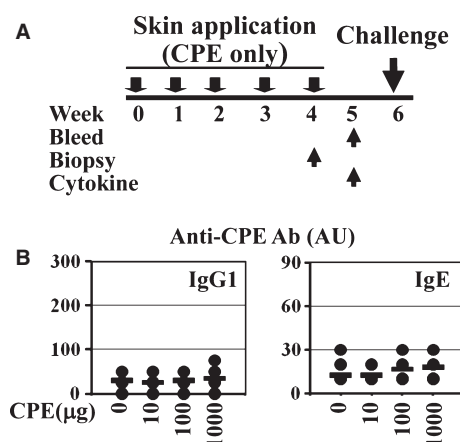


Figure 1 Effects of complete peanut extract (CPE) skin application on immune responses. (A) Diagram of the experimental schedules of bleeding, biopsy, tissue collection, and CPE challenge. (B) Anti-CPE-specific IgG1 and IgE levels in the mice with various doses of CPE skin application.

nut reactivity as the allergic clinical index and core body temperature changes were not different among all groups of mice including control animals (data not shown). Thus, we concluded that skin CPE application for five times was not sufficient to sensitize animals.

Skin CPE application preferentially inhibits the subsequent CPE oral sensitization-induced IgE responses

We next tested how the skin CPE application modified the subsequent peanut oral sensitization. The CPE-pretreated mice underwent a peanut oral sensitization protocol (Fig. 2A). The kinetics of the peanut-specific IgE, IgG1, and IgG2a response were monitored. As shown in Fig. 1B, the CPE skin application alone did not induce CPE-specific IgE and a weak IgG1 and IgG2a response (Fig. 2C, upper pan-

els). In control mice without CPE pretreatment, oral sensitization leads to increasing CPE-specific IgE, IgG1, and IgG2a over time from week 8 to 12, indicating that the oral sensitization protocol induced robust CPE-specific response ($n = 10$, Fig. 2B). The mice undergone cutaneous CPE treatment showed significantly lower CPE-specific IgE in response to oral sensitization (Fig. 2B,C). The effects on IgE suppression were CPE dose-dependent. At the pretreated CPE doses at 100 and 1000 μg , IgE was inhibited ($P < 0.01$, Fig. 2B,C), whereas IgG1 and IgG2a inhibition was only seen at 1000 μg of CPE ($P < 0.05$, Fig. 2B,C). These results demonstrated that CPE pretreatment on intact skin did not potentiate, but rather inhibited subsequent oral sensitization-induced antibody responses in a dose-dependent manner.

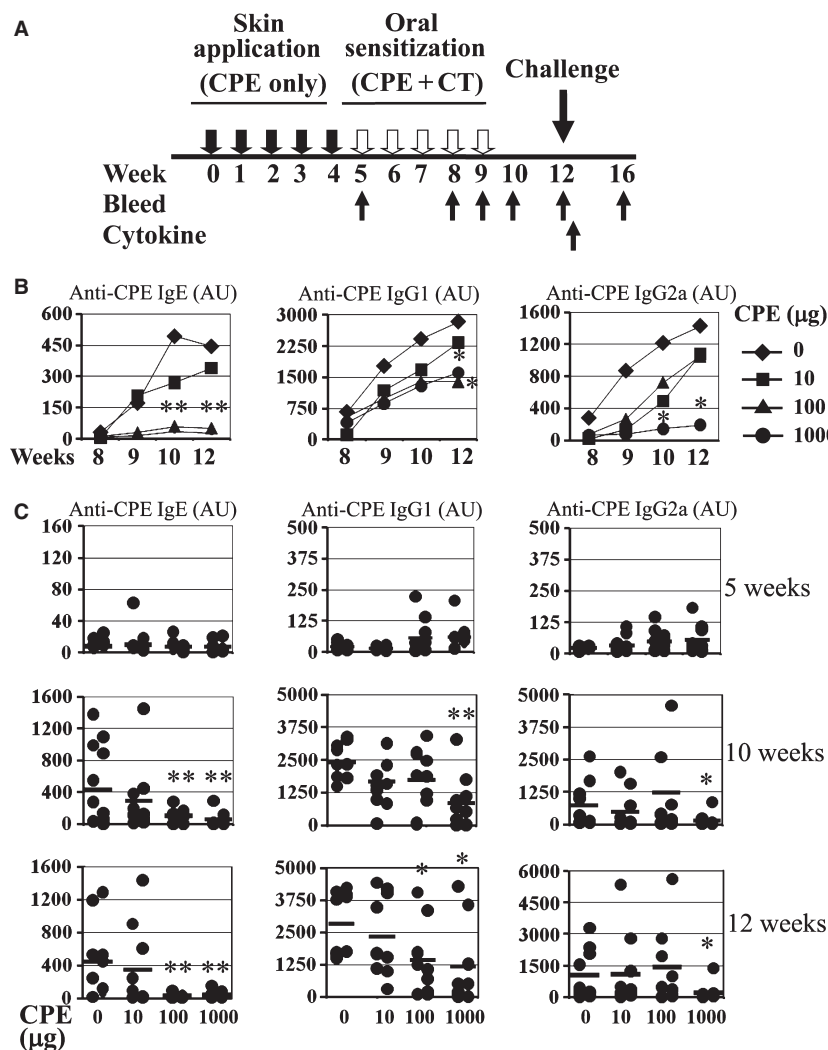


Figure 2 Effects of complete peanut extract (CPE) skin application on the antibody responses of subsequent oral sensitization. (A) Diagram of the experimental schedules. (B) Kinetic serum IgE, IgG1, and IgG2a levels in skin CPE-treated and orally sensitized

mice. (C) Distribution of the Ig levels at the specified time points (weeks 5, 10, and 12). The statistical differences between groups were indicated with asterisks (* $P < 0.05$; ** $P < 0.01$).

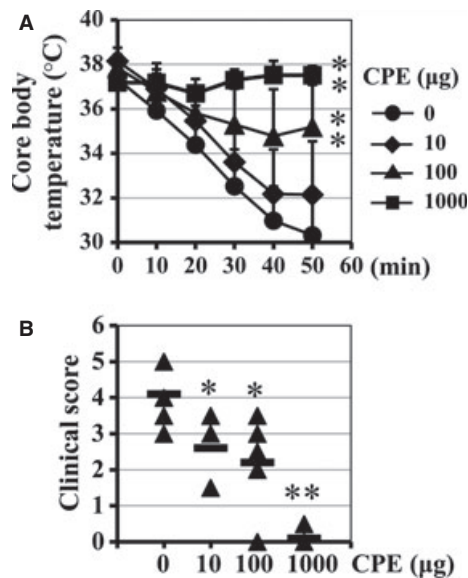


Figure 3 Effects of complete peanut extract (CPE) skin application on the subsequent CPE oral sensitization-induced peanut-allergic anaphylaxis. (A) The core body temperature changes within the first hour upon the CPE challenge. Data are expressed as mean $\pm$ SD. (B) The clinical index of the allergic anaphylaxis upon the CPE challenge.

CPE skin application prevents oral sensitization-induced allergic anaphylaxis

The effect of skin CPE application on oral sensitization-induced allergic clinical reactivity was determined. Mice undergone the CPE skin application and subsequent oral sensitization protocol were challenged with CPE at week 12, and clinical manifestations reflecting the systemic anaphylaxis were assessed (Fig. 2A). Upon CPE challenge, all the control mice without skin CPE application developed anaphylaxis as judged by the core body temperature decrease (Fig. 3A) and their allergic clinical index (Fig. 3B). In contrast, mice pre-treated with 1000 μ g CPE had neither a significant temperature fall nor an increased allergic clinical score upon CPE challenge. Mice with skin CPE application of 100 and 10 μ g showed relatively mild peanut anaphylaxis (Fig. 3A,B). These results indicated that oral sensitization-induced anaphylaxis was blocked by skin CPE application in a dose-dependent manner with profound inhibition being seen at higher dose.

Skin CPE application inhibits oral CPE sensitization-induced cytokine responses

To examine the effects of skin CPE application on T-cell-mediated cytokine production following oral sensitization, we examined the cytokine expression profiles from various lymphoid tissues. Cells were prepared at week 12 and were cultured for 5 days in the presence (CPE) or absence (using OVA as control) of the specific allergens. Cytokine production from splenocytes, and to a lesser extent from MLNs, from the control mice without skin CPE application was quite pronounced with IL-2, IL-4, IL-6, TNF- α , IFN- γ , and IL-10

levels being markedly increased (Fig. 4), showing that both Th1 and Th2 type T-cell responses were systemically induced by the CPE oral sensitization. Skin CPE application led to a significant and dose-dependent reduction in cytokine production from splenocytes and MLN (Fig. 4), indicating that skin CPE application has a broad ranging impact on the oral CPE sensitization-induced cytokine production that included cytokines involved in both Th1- and Th2-like responses.

Skin application of OVA suppresses subsequent oral OVA-induced sensitization

We also used OVA as another experimental allergen to test the effect of skin OVA application on subsequent oral sensitization. Mice were skin-painted with various amounts of OVA, followed by oral OVA plus CT sensitization as shown in Fig. 2A. At week 5, OVA skin application failed to induce OVA-specific IgE and induced low levels of OVA-specific IgG1 and IgG2a (data not shown), indicating that OVA skin application itself was insufficient to induce IgE antibody responses. Following oral sensitization, in control mice without skin OVA application, the serum IgE, IgG1, and IgG2a levels at week 10 (data not shown) and week 12 were elevated. This induction of antibody and particularly IgE to OVA was inhibited by cutaneous OVA application in a dose-dependent fashion (Fig. 5A).

Cytokines, including IL-2, IL-4, IL-6, IL-10, TNF- α , and IFN- γ , were induced from splenocytes and/or MLN from the mice given oral sensitization without skin OVA application. Higher (1000 μ g), but not the lower (10 and 100 μ g), doses of OVA skin application significantly blocked the following oral sensitization-induced cytokine production (Fig. 5B). These results showed that just as with peanut, OVA application to intact skin could block the subsequent oral sensitization-induced IgE, IgG1, and IgG2a production and suppressed the polarized cytokine production to OVA in a dose-dependent fashion.

Cutaneous CPE application does not induce local inflammation but promotes cytokine responses in local draining lymph node

As cutaneous allergen application would induce allergic tolerance that suppresses the subsequent oral sensitization, we examined the pathological change in the local skin where CPE was repeatedly administrated, and the cytokine responses in the local draining lymph nodes as a step toward understanding the mechanisms involved. As shown in Fig. 6A, the repeated application of CPE onto the carefully shaved mouse dorsal skin in all the doses tested (0, 10, 100, and 1000 μ g) induces no histological evidence of inflammation. To assess the effects of the skin application on local T-cell responses, the cytokine response profiles from skin local draining ILN were examined, with the gut draining lymph node MLN serving as controls. The control (CPE = 0) and the lower dose (e.g. 10 and 100 μ g) of CPE application did not induce significant amounts of cytokine production in these lymph node, whereas the highest dose of CPE

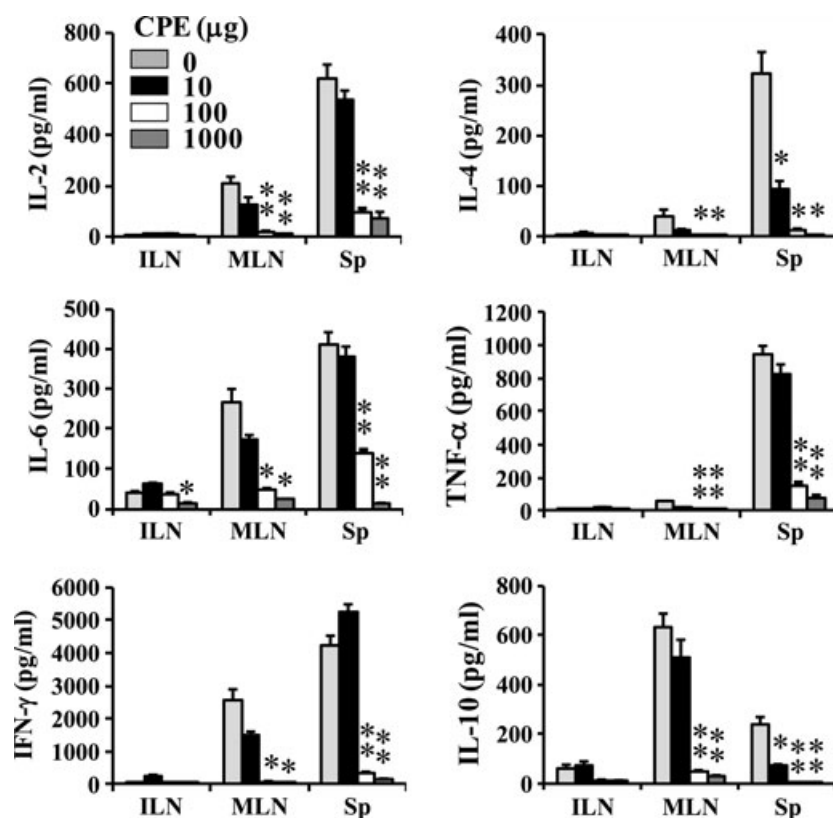


Figure 4 Effects of complete peanut extract (CPE) skin application on the subsequent CPE oral sensitization-induced cytokine responses. The polarized cytokine production profiles from the inguinal lymph node (ILN), mesenteric lymph node (MLN), and spleen

were determined, and the statistical differences between the various CPE-treated groups with the control were indicated with asterisks (* $P < 0.05$; ** $P < 0.01$).

(1000 µg) application leads to the production of multiple cytokines, including IL-2, IL-10, and IFN- γ , in the local draining ILN, but not in the distal MLN (Fig. 6B). However, IL-6 and TNF- α levels were not significantly changed (Fig. 6B); notably, IL-4 was not induced, and there was no difference in the level of TGF- β among all groups (data not shown).

Cutaneous OVA application modulates Treg and DC responses in local draining lymph node

Regulatory T cells (Treg) and DCs, including migratory Langerhans cells, in the draining ILN of mice with skin-applied OVA were also investigated. The Treg population (Fig. 6C) and the migratory Langerhans cells (Fig. 6D), which are identified as CD8 α -CD207⁺ CD11c^{med} (29), were increased compared with the control, in the ILN of 1000 µg OVA-treated mice. However, the DC activation markers, MHC-II and CD40, in CD11c-positive DC population showed no difference between OVA and control mice (data not shown).

Discussion

In this study, we investigated how cutaneous application of allergens may function as a novel approach to induce food

allergy tolerance. The cutaneous application of higher doses of CPE or OVA to the structurally intact skin was not sufficient to induce allergy sensitization but instead substantially suppress the subsequent induction of the oral allergen sensitization. Thus, our results mirrored those findings of Mondoulet et al. (20–22) that it is possible to induce a state of allergic unresponsiveness to subsequent oral sensitization to the skin preadministered allergens. As an alternate to the oral tolerance induction, skin allergen application may provide a simple and novel approach for the induction of food allergy tolerance.

Skin application of antigens/allergens to 'disrupted skin' can induce local dermatitis-like inflammation and promote immunization, allergic sensitization, and even systemic immune tolerance (6–16). For the induction of allergic sensitization, structural disruption of the normal skin via various approaches, such as repeated patching or tape stripping, was a prerequisite as sensitization was not achieved with undisturbed skin (6). Our results showed that the defined exposure of food allergens to intact skin suppresses the subsequent food allergy oral sensitization, suggesting that such an approach has the potential as an alternate for peanut and/or other food allergy tolerance induction.

The successful use of food and inhalant allergen application to intact skin as a therapeutic approach for allergic dis-

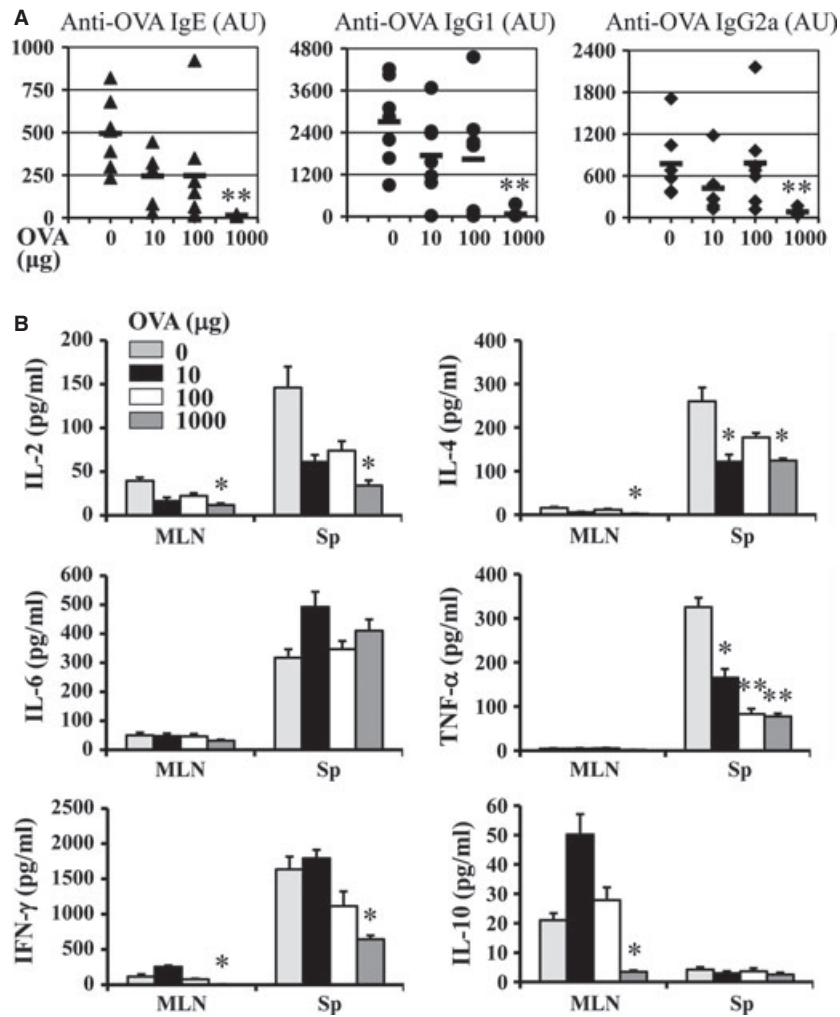


Figure 5 The skin-induced tolerance to ovalbumin (OVA). (A) The anti-OVA IgE, IgG1, and IgG2a responses from OVA oral-sensitized mice that had been pretreated with cutaneous OVA. (B) The OVA-specific cytokine production profiles *in vitro* from the inguinal lymph

node, mesenteric lymph node, and spleen cells from the various OVA-treated groups vs the control animals. The statistical differences between the various OVA-treated groups with the control were indicated with asterisks (* $P < 0.05$; ** $P < 0.01$).

orders has recently been reported (20–22). Two significant distinctions should be noted. In that study, a device was employed to deliver allergens to the skin via tightly skin contact, which led to the induction of a localized inflammatory response, whereas our approach simply painted the CPE on the intact skin through air-dry without local inflammation induction. Mondoulet et al. (20–22) were aimed to treat the allergic disorders via allergen skin application, whereas our current protocol was optimized for the prevention of sensitization but has not been optimized as a treatment approach.

The mechanisms underlie the skin allergen application for the prevention of the subsequent oral sensitization remain to be fully explored. Our analysis of the cytokine expression profiles suggested that a complex set of processes are involved. Following the oral sensitization, animals that received high-dose CPE pretreatment displayed a broad

suppressive effect on antigen-specific stimulation of multiple cytokines from the MLN and spleen. The cytokines inhibited included those involved in Th1 (IFN- γ), Th2 (IL-4), and regulatory T-cell responses (IL-2, IL-6, IL-10, and TNF- α). The correlation of this broad inhibition of the oral sensitization-induced cytokine production, and increase in the Treg, particularly the migratory Langerhans cells, with the suppression of multiple Ig isotypes (IgE, IgG1, and IgG2a), which relate to both Th1 and Th2 responses, supports the idea that the tolerance effects are not simply that of a more Th1-polarized response being driven by the high-dose CPE.

Our results with intact skin induction of allergic tolerance are theoretically consistent with the current understanding of cutaneous immunobiology. It is well appreciated that the skin Langerhans cells constantly monitor the epidermal compartment for foreign antigens/pathogens, inhibit T-cell activation, and maintain a state of unresponsiveness or tolerance to

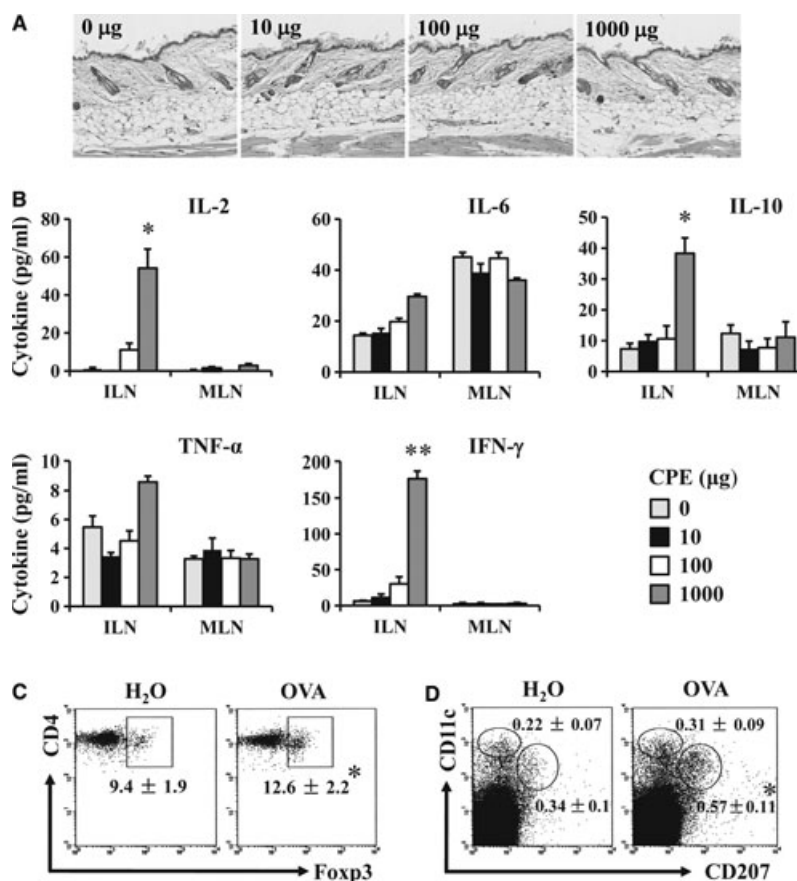


Figure 6 Dissection of the potential mechanisms involved in allergen tolerance induced by allergen skin application. (A) Skin histopathology of the sites painted with various doses of complete peanut extract (CPE). (B) Cytokine responses in the local and distal draining lymph nodes by CPE skin application. The statistical differences between different groups were

indicated with asterisks (* P < 0.05; ** P < 0.01). (C) Treg induction from the local draining inguinal lymph node (ILN) upon allergen skin application (* P < 0.05). (D) Migratory Langerhans cells induction from the local ILN upon allergen skin application (* P < 0.05). The shown results are the representative of four similar experiments.

environmental antigens. In contrast, in disease, for example, atopic dermatitis and/or the structurally disrupted skin, pro-inflammatory cells such as inflammatory dendritic epidermal cells are already recruited into the epidermis, directing the immune response toward a biased T-cell/cytokine response and exacerbating the allergic inflammation (30–34). Thus, our results showed that biopsies from the allergen skin application sites did not display any evidence of inflammatory changes as expected. In contrast, the cytokine expression profiles and the increased Treg and migratory Langerhans cells from the local draining ILNs showed that local DC- and T-cell-mediated responses were indeed induced by the topical allergen application (21). These local effects are likely the result of cutaneous dendritic cells, Langerhans cells migrating to the local lymph nodes. The increased IL-2, IL-10, and IFN- γ and lack of IL-4 induced by the higher CPE dose (Fig. 6B) suggest that the high-dose cutaneous CPE application is capable of promoting a local regulatory T-cell response.

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Author contributions

W. Li performed and designed the experiments; Z. Zhang performed the Treg and DC experiments; A. Saxon coordinated the research and wrote the manuscript; K. Zhang designed the experiments and wrote the manuscript.

Conflict of interest

None.

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