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Research Article **Immunology**

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Skin exposure promotes a Th2-dependent sensitization to peanut allergens

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Sensitization to foods often occurs in infancy, without a known prior oral exposure, suggesting that alternative exposure routes contribute to food allergy. Here, we tested the hypothesis that peanut proteins activate innate immune pathways in the skin that promote sensitization. We exposed mice to peanut protein extract on undamaged areas of skin and observed that repeated topical exposure to peanut allergens led to sensitization and anaphylaxis upon rechallenge. In mice, this epicutaneous peanut exposure induced sensitization to the peanut components Ara h 1 and Ara h 2, which is also observed in human peanut allergy. Both crude peanut extract and Ara h 2 alone served as adjuvants, as both induced a bystander sensitization that was similar to that induced by the atopic dermatitis-associated staphylococcal enterotoxin B. In cultured human keratinocytes and in murine skin, peanut extract directly induced cytokine expression. Moreover, topical peanut extract application induced an alteration dependent on the IL-33 receptor ST2 in skin-draining DCs, resulting in Th2 cytokine production from T cells. Together, our data support the hypothesis that peanuts are allergenic due to inherent adjuvant activity and suggest that skin exposure to food allergens contributes to sensitization to foods in early life.

Introduction

IgE-mediated food allergy affects 6%–8% of children, and although allergies to foods such as milk or egg are frequently outgrown, allergies to peanut are commonly lifelong and affect 1%–2% of the population (1). Prevalence of peanut allergy is increasing (2), and reactions to peanut can be severe and occur at a very low dose. It is difficult to avoid peanut due to many factors, including cross-contamination during food processing. What makes peanut such a potent allergen is not understood, although in human in vitro systems it was described that Ara h 1 from peanut could bind to DC-specific ICAM3-grabbing nonintegrin (DC-SIGN) in a glycosylation-dependent manner, resulting in a modified DC phenotype and increased capacity for Th2 skewing (3, 4). Innate recognition of allergens has been described for other clinically relevant allergens, including dust mite allergens that activate epithelial cells and DCs through TLR4 and dectin-1 (5, 6).

The vast majority of first reactions to peanut and tree nuts occur on the first known ingestion (7), suggesting that exposure resulting in primary sensitization likely occurred through another route. There is mixed evidence about a potential role of exposure to peanut in utero or through breast milk (8, 9). House-hold use of peanut, independent of maternal or infant ingestion of peanut, is a risk factor for development of peanut allergy (10). Furthermore, peanut antigen is readily detectable in household dust (11, 12), indicating that environmental exposure to peanut is likely. Studies on peripheral blood from patients with peanut allergy demonstrate that peanut-responsive T cells are found within the fraction bearing the skin-homing receptor CLA (13), providing support for the hypothesis that initial priming may have occurred through the skin. Ara h 1–MHC II tetramers identify CD4^{+} T cells in peanut-allergic subjects that express high levels of CCR4 (14), another skin-homing receptor, suggesting that skin exposure may be a highly relevant route of exposure for peanut sensitization. It is not clear if this is unique to peanut or may be common to all foods.
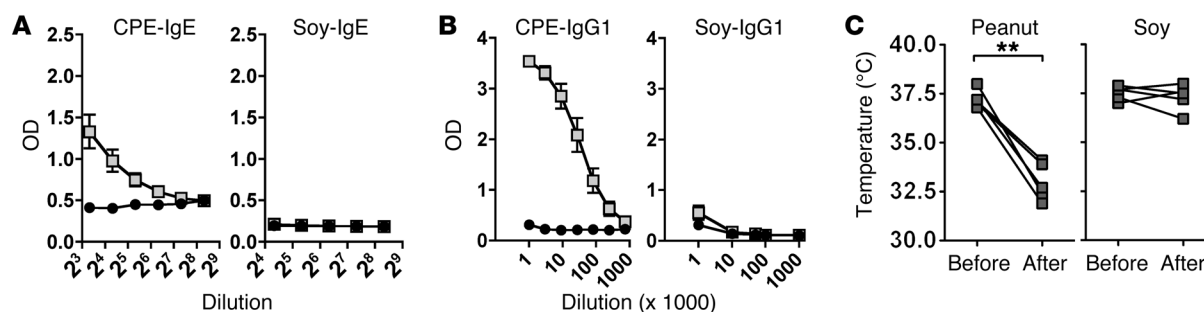
Previous studies have shown that epicutaneous exposure of mice to allergens results in sensitization when skin has been lightly damaged through tape stripping and that this is downstream of an altered immune milieu in the skin, including elevated TSLP and IL-21 (15–17). Tape stripping is a model of excoriation of the skin analogous to that observed in atopic dermatitis, a condition that is a risk factor for the development of food allergy. Furthermore, filaggrin mutations that are associated with defective skin barrier also convey increased risk for peanut allergy (18). Although food allergy is a common feature in children with atopic skin inflammation, children with healthy skin also become sensitized to peanut. We have shown previously that exposure of mice to milk allergens by the epicutaneous route can induce sensitization, but only in the

Figure 1. Adjuvant-independent sensitization to peanut through epicutaneous exposure. Mice were topically exposed to 1 mg CPE or soy extract weekly for 6 weeks. Antigen-specific (**A**) IgE and (**B**) IgG1 were measured prior to allergen challenge. Black circles represent values in naive mice, and gray rectangles represent mice exposed to the extract. Mice were challenged with 0.1 mg CPE by intraperitoneal challenge. Body temperature prior to challenge and 30 minutes after the last challenge are shown in **C**. **P < 0.01.

presence of adjuvant, which was needed to drive dermal DCs to the draining lymph node (19). Our previous studies indicate that skin exposure is not inherently sensitizing and that some form of adjuvant activity is needed to drive sensitization through the skin. In this study, we show that peanut can induce sensitization when applied to healthy intact skin and that it does so by activating innate immunity in the skin, altering the DC phenotype through mechanisms mediated by the IL-33 receptor ST2, and driving a Th2-polarized T cell response. Our results with peanut allergen support the hypothesis that environmental exposure to peanut is an important risk factor for sensitization.
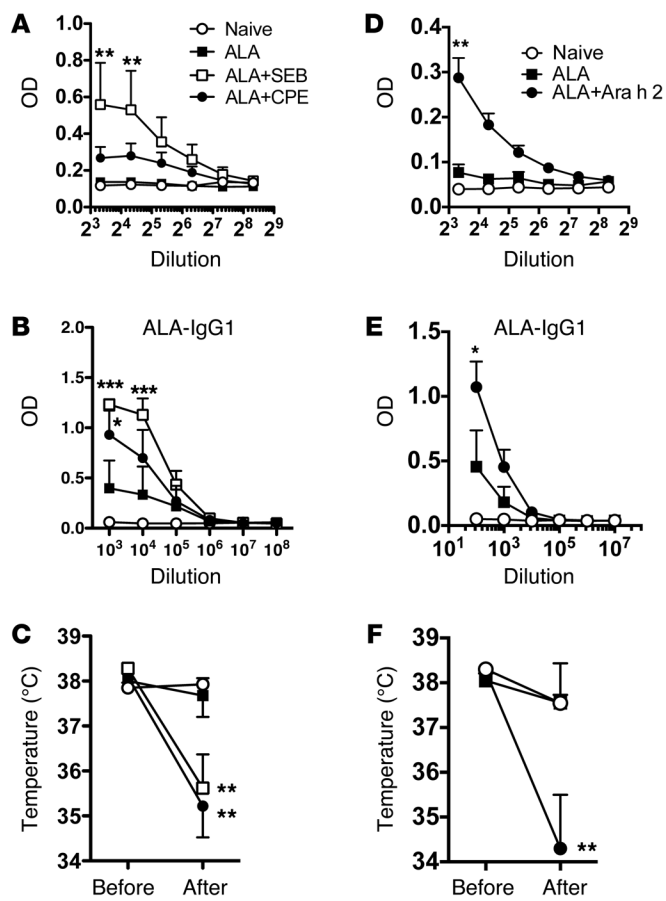
Results

Adjuvant-independent sensitization to peanut through epicutaneous exposure. We demonstrated previously that mice can be sensitized to cow's milk allergens through the epicutaneous route, but only in the presence of exogenous adjuvant (19). To determine whether this adjuvant dependence also applies to the potent food allergen peanut, we exposed mice to crude protein extracts from peanut. Soy extract was used as a comparison extract. The major peanut allergens, Ara h 1, Ara h 2, and Ara h 3, are highly homologous to proteins found within soy, yet soy allergy is rarely persistent or severe. Extracts were applied epicutaneously once per week for 6 weeks. Briefly, hair was removed with depilatory cream, but no tape stripping was performed and antigen was added topically in PBS without occlusive bandages. Histology of the skin after hair removal confirmed that the stratum corneum was intact (data not shown). Mice were then rechallenged and monitored for a drop in body temperature indicative of anaphylaxis. Mice exposed to peanut extract developed robust peanut-specific IgE and IgG1 responses and, when challenged by the intraperitoneal route with 100 μg peanut extract, developed severe anaphylaxis (Figure 1). In contrast, mice exposed to soy extract generated no soy-specific antibodies and did not develop anaphylaxis on soy challenge. However, when mice were exposed to soy in the presence of the superantigen staphylococcal enterotoxin B (SEB), they developed IgE and IgG1 antibody responses to soy (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI75660DS1). SEB did not further amplify the IgE and IgG1 response to peanut but increased the low IgG2a response observed with peanut alone. In the same exper-

iment, milk allergen α-lactalbumin (ALA) exposure was used as a negative control group, and mice exposed to ALA alone did not develop ALA-specific IgE and IgG1 responses (data not shown). To further explore the relationship between known allergenicity and responses in this mouse model, mice were epicutaneously exposed to extract from cashew, a tree nut with high allergenicity, and green bean, a legume with low allergenicity in humans (Supplemental Figure 2). Mice exposed to cashew without exogenous adjuvant developed cashew-specific IgE and IgG1 responses, while mice exposed to green bean extract developed IgE and IgG1 responses only in the presence of exogenous adjuvant (cholera toxin). These results suggest that the response of mice to epicutaneous food allergen exposure is consistent with the allergenicity of these foods in humans.

To determine whether the process of hair removal contributed to the sensitization response to peanut, perhaps through emptying of the hair follicle that is densely surrounded by DCs (20), the experiment was repeated by applying peanut extract to the ear, without any pretreatment of the skin. Exposure of mice to peanut extract topically on the ear induced peanut-specific IgE and IgG1 responses, basophil activation after in vitro allergen stimulation, and anaphylaxis in mice when rechallenged (Supplemental Figure 3). Altogether, these results show that peanut, similar to cashew and unlike weak allergens such as soy, green bean, or milk ALA, is capable of inducing adjuvant-independent sensitization by topical exposure on healthy skin.

We next examined which allergens were recognized in mice sensitized by the epicutaneous route (Supplemental Figure 4B) and compared this to the sensitization profile of peanut-allergic subjects (Supplemental Figure 4A). Immunoblotting of peanut extract performed with human sera identified 3 bands in the 14- to 20-kDa range, consistent with Ara h 2 (approximately 17 kDa) and Ara h 6 (approximately 14 kDa). These 3 bands were also detectable in mice epicutaneously exposed to crude peanut extract (CPE). A higher molecular weight band was also detectable in both humans and mice, consistent with Ara h 1 (63–68 kDa) or Ara h 3 (57 kDa). Using purified Ara h 1 and Ara h 2, we examined the contribution of these allergens in mice sensitized to CPE. Mice exposed to peanut developed detectable IgE antibodies against both Ara h 1 and Ara h 2 (Supplemental Figure 4C), and basophil activation tests performed with whole blood from peanut-sensitized mice showed a similar threshold of reactivity to the 2 dom-

Figure 2. Peanut and Ara h 2 induce bystander sensitization, demonstrating adjuvant activity. (**A**–**C**) Mice were exposed to 0.1 mg ALA on the ear, alone, or in the presence of 10 μg SEB or 1 mg CPE once a week for 6 weeks. ALA-specific (**A**) IgE and (**B**) IgG1 were measured in serum prior to oral challenge with ALA. (**C**) Body temperature was measured at baseline and 30 minutes after challenge. $n = 5$ mice per group. (**D**–**F**) The experiment was repeated with exposure to ALA in the presence or absence of 0.1 mg purified Ara h 2. $n = 4$ per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, vs. ALA alone.
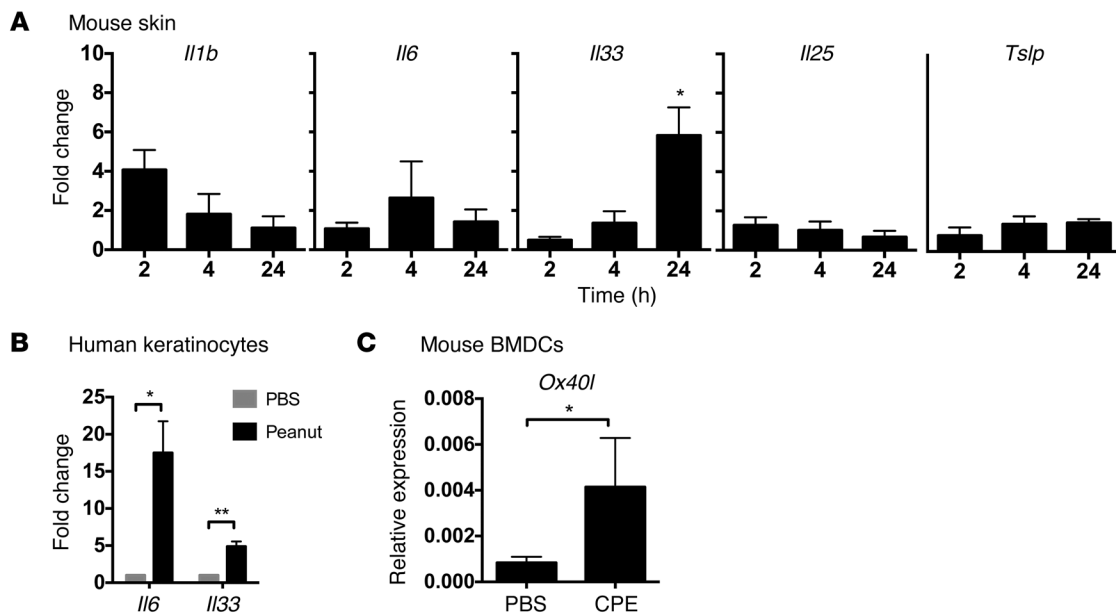
inant allergens (Supplemental Figure 4D). A titrated intraperitoneal challenge of peanut-sensitized mice demonstrated that both Ara h 1 and Ara h 2 could trigger anaphylaxis, beginning at a dose of 10 μg, but Ara h 2 induced a stronger anaphylactic reaction than Ara h 1 (Supplemental Figure 4E).

To determine whether sensitization could also be established using purified allergens on exposure, we exposed mice to peanut extract or purified Ara h 1 or Ara h 2 epicutaneously without adjuvant (Supplemental Figure 5). Mice developed marked antibody responses to the peanut components (Supplemental Figure 5A), indicating that other components in crude extract are not necessary for sensitization. Despite the induction of antibody responses to Ara h 1 and Ara h 2, only mice exposed to Ara h 2 experienced significant anaphylactic reactions following intraperitoneal challenge (Supplemental Figure 5B). Ara h 2 is the component of peanut linked most closely to clinical reactivity in humans (21).

Peanut has adjuvant activity on the skin. Sensitization to antigens after epicutaneous exposure has been shown previously to be dependent on additional factors, including skin damage (17), presence of inflammation (22), or use of exogenous adjuvants such as SEB (23) or cholera toxin (19). We hypothesized that peanut could be inducing "adjuvant-free" sensitization by providing inherent adjuvant activity. To test this, we examined the capacity of peanut to induce bystander sensitization to the milk allergen ALA that we have previously shown is adjuvant dependent (19). Mice were exposed to ALA alone or in the presence of peanut or SEB as positive control once a week for 6 weeks. Mice exposed to ALA alone

did not have detectable levels of ALA-specific IgE and had low levels of ALA-specific IgG1 (Figure 2, A and B). Mice exposed to ALA in the presence of either SEB as adjuvant or peanut developed a significant ALA-specific antibody response, including IgE and IgG1 antibodies (Figure 2, A and B), and to a lesser extent IgG2a (data not shown). To test the functional consequence of bystander sensitization, mice were challenged with ALA by oral gavage (Figure 2C). Mice exposed to ALA alone did not undergo anaphylaxis when they were orally rechallenged with ALA, but when mice exposed to ALA together with either SEB or peanut were orally rechallenged with ALA alone, they developed anaphylaxis, as measured by a significant drop in body temperature. We then tested whether Ara h 2, the dominant allergen in peanut, could induce bystander sensitization. Similar to the results with CPE, topical exposure to Ara h 2 resulted in bystander sensitization to ALA (Figure 2, D and E) and anaphylaxis in mice orally challenged with ALA alone (Figure 2F). These data conclusively demonstrate that peanut, and its major allergen Ara h 2, have adjuvant activity when applied to the skin.

Peanut induces an innate cytokine response. To determine whether peanut can induce innate responses in vivo, peanut extract was applied on the ear, followed by harvesting of tissue RNA over time (2–24 hours) and assessment of cytokine expression by RT-PCR (Figure 3A). We examined expression of the cytokines *Il1b*, *Il6*, *Il33*, *Il25*, and *Tslp*. These cytokines were chosen since they can alter the phenotype of resident DCs and drive the adaptive immune response away from one of tolerance. We observed significant upregulation of *Il33* expression and a trend of increased expression of *Il1b* but did not observe upregulation of other Th2-promoting cytokines (*Il25*, *Tslp*) or proinflammatory cytokines. To test whether keratinocytes could directly recognize and respond to peanut exposure, we used primary neonatal human keratinocytes (Figure 3B). We examined expression of the cytokines *IL1A*, *IL1B*, *TNFA*, *IL6*, *GMCSF*, *TSLP*, *IL25*, and *IL33* after stimulation with CPE that had been cleaned of endotoxin. Of these cytokines, *IL6* and *IL33* were significantly upregulated by exposure of keratinocytes to peanut extract. IL-6 was confirmed at the protein level to be released in response to peanut in a dose-dependent manner (data not shown). Peanut has been shown previously to have direct effects on mouse and human DCs (3, 24). We stimulated bone marrow–derived DCs with peanut extract and assessed phenotype by flow cytometry and real-time PCR. Stimulation of cells with peanut extract did not alter the expression of *MHC II*, *Cd80*, or *Cd86* or significantly change the expression of cytokines, including *Il1b*, *Il6*, or *Il10*. However, stimulation with peanut extract did induce a significant upregulation of *Ox40l* (Figure 3C), a costimulatory molecule that we and others have previously shown is involved in the induction of Th2 responses after oral sensitization using cholera toxin adjuvant (25, 26).

Figure 3. Innate response to epicutaneous peanut exposure. (**A**) Mice were exposed to peanut or PBS as control on the ear, followed by RNA isolation and RT-PCR for cytokines. (**B**) Human keratinocytes or (**C**) mouse bone marrow DCs were exposed in vitro to CPE or PBS as control. Fold change is shown with respect to PBS. Relative expression refers to data normalized to the housekeeping gene. Data are mean ± SEM of a least 3 different experiments. *$P < 0.05$, **$P < 0.01$.
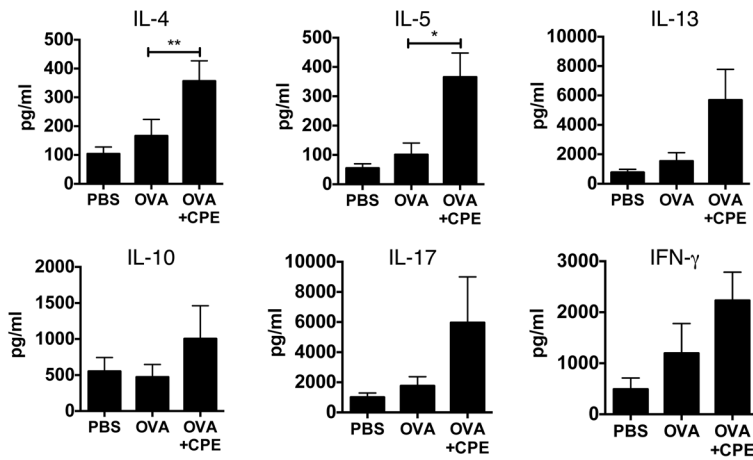
DCs from peanut-exposed mice are sufficient to drive Th2 priming. To determine whether epicutaneous peanut exposure modifies the phenotype of DCs in vivo, resulting in functional consequences on T cell priming, we purified DCs from the skin-draining lymph nodes of mice 24 hours after epicutaneous exposure to ovalbumin (OVA) in the presence or absence of peanut. Purified DCs were cocultured with OVA-specific CD4+ T cells from DO11.10 mice at a 1:5 ratio for 3 days, followed by restimulation with anti-CD3/CD28 to maximize cytokine output. Cytokine secretion was measured by ELISA (Figure 4). DCs harvested from mice epicutaneously exposed to OVA in the presence of peanut induced significantly greater Th2 (IL-4 and IL-5) responses compared with those from mice exposed to OVA alone, while IL-10 was unchanged. IL-13, IFN-γ, and IL-17 secretion was elevated but did not reach the level of statistical significance. CPE alone was used as a negative control (without OVA to activate the OVA-specific T cells), without significant differences with respect to PBS or OVA alone (data not shown). These results demonstrate that topical peanut exposure alters the phenotype of skin-draining DCs in vivo, resulting in an amplification of adaptive immunity, including an elevated Th2 immune response. Interestingly, the DC-driven increase in Th2 priming was not observed when we used SEB rather than CPE as an adjuvant in this model (data not shown), indicating that the mechanism mediating the induction of Th2 responses is different depending on the nature of the adjuvant factor.

Induction of Th2 immunity can be driven by peanut or adjuvant, but only adjuvant induces T follicular helper cells. The generation of IgE antibodies resulting in clinical food allergy could potentially be driven by 2 T helper subsets: Th2 cells producing IL-4 and IL-13 and T follicular helper (Tfh) cells. Tfh cells are a subset of CD4+ T cells, which induce immunoglobulin class switching and promote germinal center reactions (27). These cells also secrete low levels of IL-4, which is required for somatic hypermutation and

affinity maturation. We hypothesized that peanut or SEB promote bystander sensitization or self-sensitization by inducing Th2 and/or Tfh responses. To test whether there was an induction of Th2 immunity after sensitization, 4get mice (which have GFP expression in IL-4–expressing cells) (28) were epicutaneously exposed to PBS, SEB, or peanut. One week after exposure, the draining lymph nodes were harvested and assessed for GFP. We observed a significant increase of GFP-positive CD4+ T cells in the skin-draining lymph nodes (Figure 5A) in response to either peanut or SEB. Induction of reporter activity was restricted to the draining lymph nodes and was not observed at distal lymph nodes (the mesenteric lymph nodes). GFP reporter activity was observed primarily within the memory T cell population (CD44hiCD4+ cells, data not shown). We verified the peanut-dependent induction of Th2 responses by using IL-13 reporter mice (IL-13eGFP mice) (29). An increase in IL-13 reporter activity was also observed in the skin-draining lymph nodes after epicutaneous exposure to peanut in total CD4+ and CD44hiCD4+ cells (Supplemental Figure 6 and data not shown). Induction of memory Th2 cells is therefore a common response to exposure to peanut and SEB.

We next wanted to determine whether there was a local induction of Tfh cells after epicutaneous exposure to peanut or SEB. CXCR5+ICOS+ cells in the CD4+ population were defined as the Tfh cell population. Mice exposed to SEB via skin showed an increased population of CXCR5+ICOS+ cells in skin-draining lymph nodes within both total CD4+ and CD44hi memory populations (Figure 5B and data not shown). In contrast, mice epicutaneously exposed to peanut did not have an expansion of local Tfh cells, indicating that SEB induces a broader immune response than that generated by peanut. IgE class switching has been reported to occur outside of germinal centers (30); however, we do not have direct evidence to suggest non-germinal center localization of the peanut-specific IgE response.
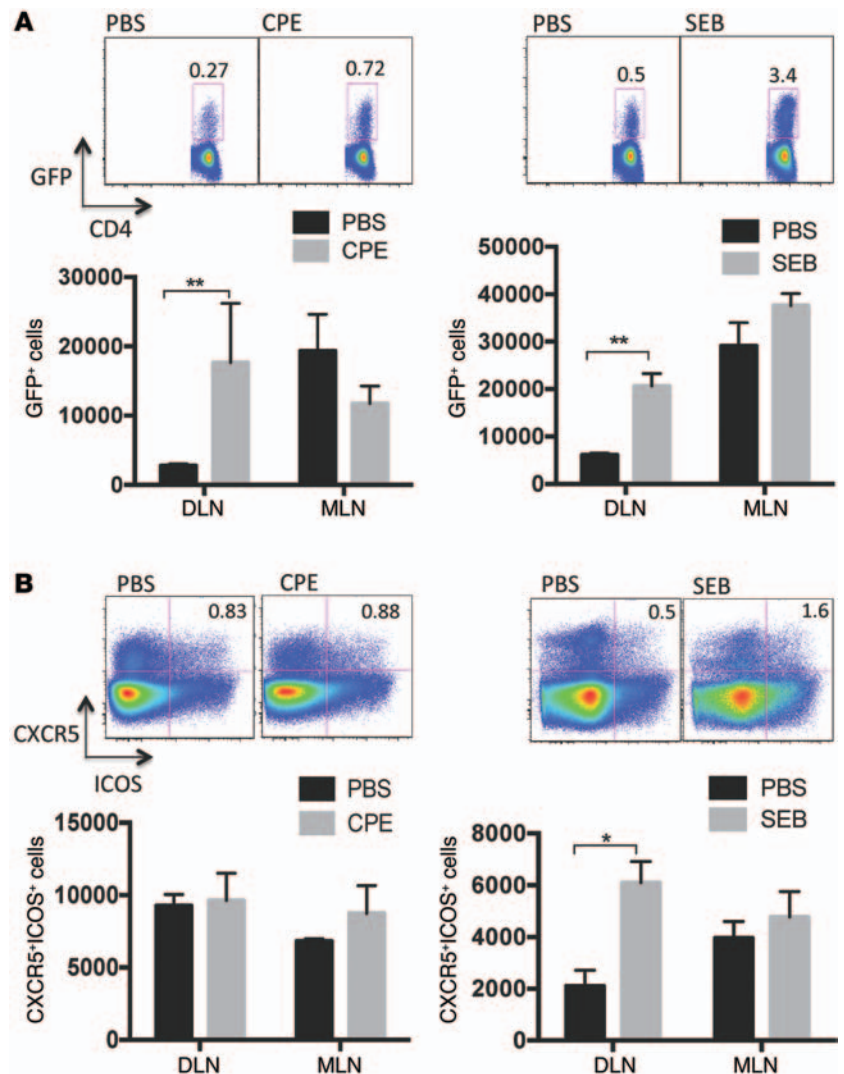
Figure 4. Effect of in vivo exposure to CPE on priming by DCs. DCs were purified from the skin-draining lymph nodes of BALB/c mice epicutaneously exposed to PBS, OVA, or OVA and CPE and cocultured with DO11.10 CD4+ T cells for 3 days, followed by restimulation with CD3/CD28. Data are mean ± SEM of 7 independent experiments, with each experiment consisting of 3 pooled mice per group. *$P < 0.05$, **$P < 0.01$.
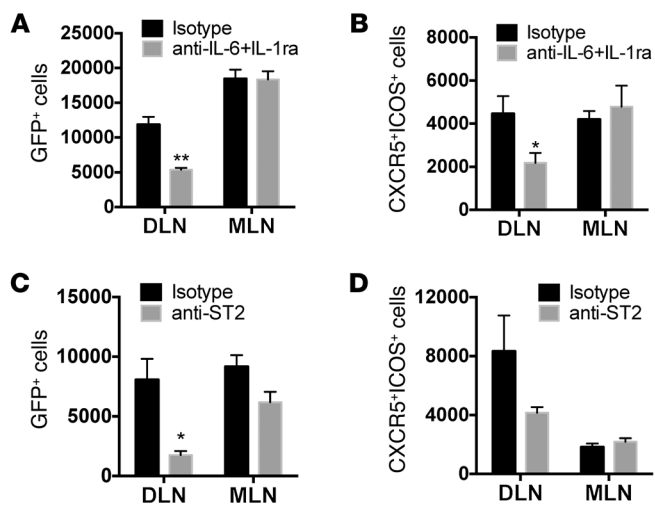
IL-1, IL-6, and IL-33 link innate and adaptive immunity in the skin. We observed that peanut induces an innate cytokine response from keratinocytes and alters the phenotype of DCs, contributing to altered T cell priming. We sought to establish a link between the innate immune response and the adaptive immune response in vivo. We first blocked cytokines, specifically IL-6 and IL-1, that we had observed were upregulated in keratinocytes or skin by peanut exposure. IL-6 was blocked with neutralizing anti–IL-6 antibody, while IL-1 signaling was blocked with IL-1 receptor antagonist (anakinra). Blockade of IL-6 and IL-1 signaling prior to SEB exposure resulted in a significant suppression of IL-4 reporter activity in the skin-draining lymph nodes but did not influence constitutive IL-4 reporter activity in a distal lymph node (Figure 6A). We also observed attenuated numbers of CXCR5+ICOS+ Tfh cells in response to SEB when IL-1 and IL-6 signaling was blocked (Figure 6B). Blockade of ST2, the receptor for IL-33, also led to a significant reduction in IL-4 reporter activity in vivo (Figure 6C) and a trend toward a reduced number of Tfh cells (Figure 6D).

In contrast to the findings with SEB, blocking IL-6 and IL-1 had no effect on peanut-induced IL-4 reporter activity in draining lymph nodes (Supplemental Figure 7). However, similar to that with SEB-induced IL-4 production, neutralizing anti-ST2 antibody administered before epicutaneous exposure to peanut significantly suppressed IL-4 reporter activity in skin-drain-

ing lymph nodes but not distal lymph nodes (Figure 7A). ST2 is expressed on a number of different cell types, including DCs, T cells, and innate lymphoid cells. We hypothesized that IL-33 from keratinocytes was modulating the function of DCs. Mice were treated with isotype or anti-ST2 antibody prior to epicutaneous exposure to OVA with peanut as adjuvant. DCs were then purified

Figure 5. Effect of CPE or SEB on Th2 and Tfh cells. 4get mice were epicutaneously exposed to PBS, peanut (CPE), or SEB. One week later, skin-draining lymph nodes (DLN) and mesenteric lymph nodes (MLN) were assessed for (**A**) IL-4 reporter expression or (**B**) Tfh markers CXCR5 and ICOS. Representative plots of IL-4+ or CXCR5+ICOS+ cells of total CD4+ cells in skin-draining lymph nodes are shown above summary plots of the mean ± SEM number of cells normalized per million CD4+ T cells for 3 mice per condition. *$P < 0.05$, **$P < 0.01$.

Figure 6. Contribution of IL-1, IL-6, and IL-33 to Th2 and Tfh induction by SEB. 4get mice were pretreated with either isotype control or (**A** and **B**) neutralizing IL-6 antibodies plus IL-1 receptor antagonist or (**C** and **D**) neutralizing anti-ST2 antibody before epicutaneous exposure to SEB. Draining lymph nodes and mesenteric lymph nodes were harvested 1 week later. Summary plots of (**A** and **C**) IL-4+ cells or (**B** and **D**) CXCR5+ICOS+ cells per million CD4+ T cells are shown. Data are mean ± SEM of 3 mice per condition. *$P < 0.05$, **$P < 0.01$.

from the skin-draining lymph nodes and cocultured with DO11.10 T cells. DCs from peanut-exposed mice induced Th2 priming that was significantly reduced in mice treated with anti-ST2 antibody (Figure 7B), indicating that ST2 was acting at or above the level of the DC, resulting in an altered DC phenotype. We and others have shown that cholera toxin–driven sensitization by gastrointestinal DCs is dependent on OX40L expression by DCs (25, 26); however, blockade of OX40L in the skin DC–T cell cocultures resulted only in a minor reduction in Th2 cytokine production (Figure 7C). The lack of significant effect of OX40L neutralization in response to CPE was also observed using 4get mice in vivo (data not shown). Together, these data suggest that a diverse set of sensitizing stimuli at the level of the skin can induce Th2-skewed immune responses through a ST2-dependent modulation of skin DCs.
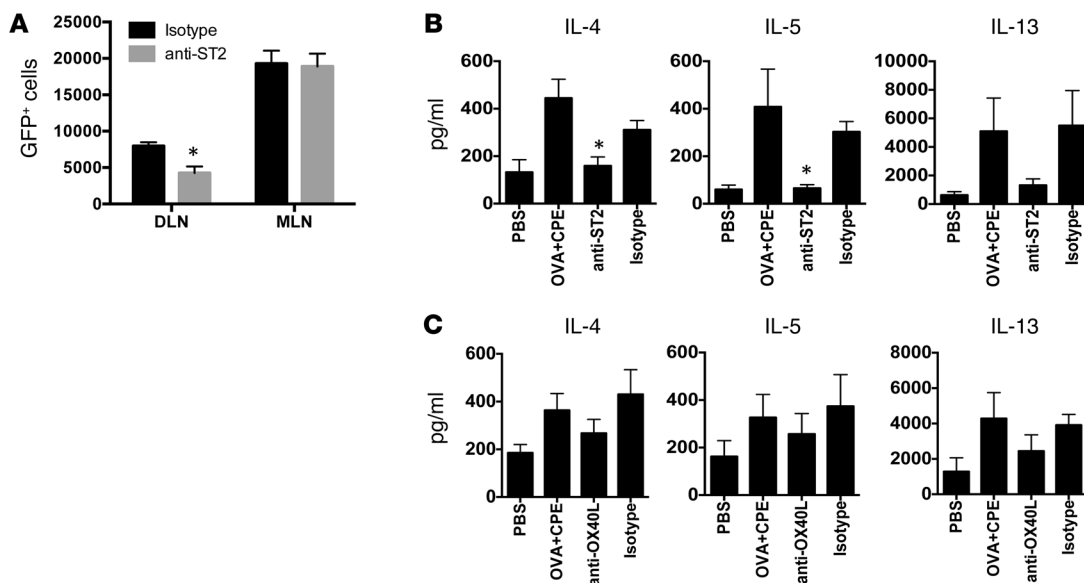
Discussion

Peanut is one of the most common foods causing allergic reactions, and peanut allergy is increasing in prevalence in the US (2) and the UK (31). Peanut allergy, unlike milk or egg allergy, is commonly lifelong and is one of the causes of potentially life-threatening anaphylactic reactions (32, 33). In this report, we identify novel innate responses to epicutaneous peanut exposure in vivo that contribute to the generation of Th2-biased adaptive responses to peanut. Our results point to inherent adjuvant factors in peanut combined with a nonoral exposure route as being critical to the development of peanut allergy.

Environmental exposure to peanut is a risk factor for the development of peanut allergy while early dietary exposure of infants to peanut is associated with low prevalence of peanut allergy (10, 34). The skin is of particular interest as a route of allergen exposure, since infant exposure to peanut oil–containing ointments was found to be a risk factor for peanut allergy in the UK (35). Exposure to wheat hydrolysates in facial soap has also been reported to be associated with self-reported wheat allergy (36). We have previously demonstrated that sensitization to milk allergens can be induced via multiple physiological routes in mice, including the epicutaneous route, but only in the presence of an exogenous adjuvant (19). These previous results suggest that skin exposure is not inherently allergenic but, given the appropriate immune environment, can result in sen-

sitization. For example, the presence of filaggrin mutations, which confer susceptibility to atopic dermatitis, is a risk factor for peanut allergy independent of atopic dermatitis (18, 37). This is supported by preclinical data showing increased susceptibility to sensitization in mice with filaggrin mutations (38). In addition, models of skin sensitization to food allergens commonly use tape stripping that causes mechanical injury to promote skin inflammation and the induction of TSLP and Th2 cytokines that lead to sensitization (16, 17, 39, 40). Vitamin D analogs, which upregulate TSLP expression in the skin, can also induce sensitization to food allergens via the skin (22). However, here we show evidence that mice can be sensitized to peanut or cashew via intact skin in an adjuvant-independent fashion, while soy or green bean extracts, used as control legume extracts, do not sensitize. This suggests that while the skin is not inherently sensitizing upon antigen exposure, it is permissive to sensitization to peanut in the absence of adjuvants, unlike oral exposure, which leads to tolerance. This finding supports the clinical observations that exposure to peanut through nonoral routes promotes sensitization (10), while early oral exposure is associated with lower risk of peanut sensitization (34). Our findings also indicate that this paradigm may not fit all allergens, as it does not apply to milk (19). The mechanism by which skin, but not gut, exposure to peanut alone can induce sensitization is not clear but may relate to features of the resident DCs within each site, such as differential expression of innate receptors.

Using a series of in vivo approaches, we demonstrated that peanut elicits an innate response after topical application to intact skin, followed by an induction of the Th2-priming potential of DCs in the draining lymph node, leading to the induction of IL-4– and IL-13–expressing CD4+ T cells. Although there was an induction of IL-1β and IL-6 from skin and keratinocytes, respectively, and these cytokines were required for the induction of Th2 and Tfh responses using the adjuvant SEB, we did not find evidence for a role of IL-1β and IL-6 signaling in the induction of Th2 responses to peanut. In both intact mouse skin and human keratinocytes, there was an upregulation of IL-33 in response to peanut exposure. In keratinocytes this was a late response (24 hours), occurring after the early peak of proinflammatory cytokines (2 hours), suggesting that IL-33 could be upregulated in response to autocrine signals. Blockade of ST2, the IL-33 receptor, led to a significant reduction in Th2 cells in response to epicutaneous peanut exposure. There is growing evidence for a central role of the IL-1 family member IL-33 or ST2 in the induction of sensitization at multiple tissue sites. Sensitization to dust mite through airway exposure is dependent on ST2 (26, 41), downstream of the innate recognition of dust mite through a TLR4-dependent mechanism (41). Oral sensitization to peanut (requiring cholera toxin adjuvant) is also ST2 dependent (26). ST2-expressing resident eosinophils in the gastrointestinal tract were

Figure 7. Peanut induces Th2 polarization through ST2-mediated effects on skin-draining DCs. 4get mice were pretreated with either isotype control or neutralizing ST2 antibodies before epicutaneous exposure to CPE. One week after exposure, draining lymph nodes and mesenteric lymph nodes were harvested, and (**A**) IL-4 reporter activity was quantified. Summary data (mean ± SEM) showing number of cells per million CD4+ T cells are shown. *n* = 3 mice per condition. *P < 0.05. (**B**) BALB/c mice were pretreated with isotype control or anti-ST2 antibody prior to exposure to PBS or OVA and CPE. DCs from skin-draining lymph nodes were cocultured with DO11.10 T cells, and cytokine output was measured. Data are mean ± SEM of 3 independent experiments. *P < 0.05, compared with isotype control. (**C**) DCs from skin-draining lymph nodes were isolated after exposure to OVA and CPE and cocultured with DO11.10 T cells in the presence of anti-OX40L or isotype control antibody. Data are mean ± SEM of 4 independent experiments.

recently reported to be essential for this oral sensitization to peanut by driving CD103+ DCs expressing elevated levels of CD86 and OX40L from the lamina propria to the mesenteric lymph node (42). Kobayashi et al. recently showed that airway administration of exogenous IL-1β or IL-33 together with OVA or ragweed extract could induce IgE production against the allergens (43). Interestingly, IL-1β induced IL-17 production in addition to Th2 cytokines, while IL-33 induced a more restricted Th2 cytokine induction. Similarly, we observed a broader immunologic impact when mice were exposed to SEB, which induced Th2 and Tfh cells via IL-1–, IL-6–, and ST2-dependent pathways, compared with that when mice were exposed to peanut that induced Th2 cells solely through an ST2-dependent pathway. ST2 is found on a number of different cell types, including innate lymphoid cells (44), allergic effector cells (45, 46), and DCs (47). We did not observe any increase in IL-4 or IL-13 reporter activity in non-CD4+ T cells after peanut exposure in the draining lymph nodes. Furthermore, purified DCs from peanut-exposed mice were able to induce Th2 responses from naive T cells in the absence of any other accessory cells, suggesting that DCs are sufficient for the induction of Th2-skewed immune responses to epicutaneous peanut exposure, although the finding does not necessarily rule out contribution from innate sources of Th2 cytokines in vivo. Although TSLP can promote sensitization to allergens via the skin (17, 22, 39), neither mouse skin nor human keratinocytes upregulated TSLP upon peanut exposure, and sensitization of mice to peanut through the skin was normal in TSLP receptor–deficient mice (data not shown). This is consistent with our previous findings and those of other groups that primary sensitization and generation of IgE in response to oral sensitization can occur through TSLP-independent pathways (26, 48).

Keratinocytes are presumed to be a major source of Th2-inducing cytokines, including IL-33, IL-25, and TSLP, and, consistent with a keratinocyte source of IL-33 in our model, we observed that *IL33* mRNA expression was regulated by peanut exposure in human keratinocytes in vitro. The receptor for IL-33 is expressed by DCs, among many other cell types, and treatment of DCs with IL-33 has been shown to upregulate OX40L expression (49). ST2 may modulate DC function upstream through an intermediate cell. As an example, gastrointestinal eosinophils express ST2 and are required for oral sensitization to peanut through eosinophil peroxidase-dependent modulation of DC phenotype (42). Recently, two reports identified a skin-draining DC subset as being critical for the induction of Th2 immunity (50, 51). The markers CD301b and PD-L2 were identified on these Th2-inducing DCs, and this DC subset expressed high levels of the IL-33 receptor ST2 (50). Furthermore, these DCs were required for induction of Th2 responses but not Tfh responses. In addition to acting on DCs, IL-33 can also be produced by DCs and act directly on T cells. IL-33 was recently shown to be regulated by IRF-4 in DCs, and DC-derived IL-33 was necessary for the induction of Th2 immunity (52). In a model of bee venom–induced Th2 immunity, IL-33 was shown to be central to the induction of Th2 immunity but via direct actions on T cells rather than through actions on a DC subset (53). Our data suggest that ST2 is acting at the level of the DC or higher, rather than directly on the T cells or through innate lymphoid cells, since neutralization of ST2 in vivo suppressed the ability of DCs to directly induce Th2 priming after peanut exposure. ST2 also negatively regulates TLR2 signaling (54), suggesting that ST2 blockade may have effects beyond neutralization of IL-33 signaling.

We and others have shown that OX40L on DCs is responsible for DC-mediated Th2 skewing and sensitization in the gastrointestinal tract (25, 26, 55). In the current studies, OX40L neutralization had only a minor inhibitory effect on DC-driven Th2 cytokine production in vitro and in vivo, indicating that other mechanisms for DC-driven Th2 priming must predominate in the skin.

Our data contributes to the growing body of literature showing that allergens activate innate immunity. Allergy to house dust mite is one of the most common allergic responses worldwide (56). In the case of house dust mite, there are several allergens that have protease activity (Der p 1, Der p 3, Der p 6, Der p 9) and a capacity to bind to lipids (Der p 2) (6, 57, 58). Der p 2 activates the TLR4 signaling pathway by acting as a MD2 mimetic and induces sensitization in a TLR4-dependent manner that is dependent on presence of LPS within the preparation (5). In pollens, the presence of pollen-associated lipid mediators that promote Th2 responses has been described (59). Lectins derived from legumes are able to activate basophils to release IL-4 and IL-13 (60). Peanut has innate activity on human DCs, in part through Ara h 1 binding to DC-SIGN (3). Peanut can also activate complement that may influence adaptive immune responses (61). We found that purified Ara h 2 was sufficient to induce IgE production in mice, leading to anaphylaxis upon challenge, and could induce bystander sensitization, demonstrating inherent adjuvant activity. Ara h 2 is the dominant allergen in peanut, and component testing has indicated that IgE against Ara h 2 is predictive of clinical reactivity in patients (21). Interestingly, we did not observe innate effects of Ara h 2 on human keratinocytes (data not shown), and, unlike Ara h 1, Ara h 2 is not significantly glycosylated. Innate effects of peanut extract on human DCs were previously shown to be glycosylation dependent, and purified Ara h 2 did not induce a Th2-skewing phenotype in human DCs (3). Thus, the molecular mechanism by which Ara h 2 may induce bystander sensitization is unclear. It was shown, using timothy grass extract, that Th2-inducing activity could be discriminated from IgE binding, and several novel proteins were identified that were capable of Th2-inducing activity but were not recognized by IgE (62). Similar approaches are needed to identify all potential T cell modulatory fractions of peanut that may be distinct from the dominant allergens.

In summary, we have shown that the skin is permissive to allergic sensitization on exposure to peanut without additional adjuvants or tissue damage and that peanut induces Th2 skewing through a DC-autonomous pathway that is ST2 dependent. We contrast this with sensitization induced by the superantigen SEB, which induces both Th2 skewing and Tfh induction through IL-1– and IL-6–dependent as well as ST2-dependent pathways. Our data provide a mechanistic explanation for the epidemiologic observations that nonoral household exposure to peanut is a risk factor for peanut allergy. Furthermore, this model of adjuvant-independent sensitization to peanut provides a clinically relevant model of peanut allergy that can be used to dissect the molecular basis of peanut allergenicity.

Methods

Antigens and adjuvants. Defatted CPE was prepared as previously described (24). Briefly, roasted peanuts were shelled, ground to a paste, defatted with acetone, dried overnight, and then extracted with PBS with complete protease inhibitor (Roche). Soy extract was prepared from soy flour (Arrowhead Mills). Green bean and cashew extracts were prepared as described above. ALA and OVA (grade V) were purchased from Sigma-Aldrich. Purified Ara h 1 and Ara h 2 for in vivo studies and basophil activation tests were prepared as previously described (63, 64). Extracts used in cell culture were purified from endotoxin using Detoxi-Gel columns (Pierce). SEB was from Toxin Technologies. Cholera toxin was from List Biological Laboratories.

Mice. C3H/HeJ and BALB/c mice were obtained from National Cancer Institute, and C3H/HeOuJ mice were obtained from Jackson Laboratories. 4get (28) and DO11.10 mice (Jackson Laboratories) were maintained as breeding colonies at Mount Sinai. IL-13eGFP (29) mice were originally developed by Andrew McKenzie (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom) and provided by Paul Bryce (Northwestern University, Chicago, Illinois, USA).

Epicutaneous sensitization of mice. Six- to eight-week-old female mice were anesthetized, and abdominal fur was removed with depilatory cream (Veet; Reckitt Benckiser), immediately followed by exposure to antigens (1 mg crude extract or 0.1 mg purified antigens) in 50 to 100 μl PBS spread thinly on the abdominal skin to dry. SEB or cholera toxin were applied at a dose of 10 μg. In some experiments, antigens were applied to the ear pinnae. Mice were exposed weekly for a total of 6 exposures and challenged a minimum of 1 week after the last exposure.

Allergen challenge and assessment of anaphylaxis. Mice underwent an oral challenge to allergen (50 mg ALA or OVA) and were observed for 30 minutes following challenge. Body temperature was measured before and 30 minutes after challenge. For extracts, mice were challenged by intraperitoneal route with 100 μg antigen and followed for a further 30 minutes prior to measuring temperature with a rectal thermometer (WPI Instruments).

Cytokine neutralization. During neutralization experiments, mice were pretreated with neutralizing anti–IL-6 (0.5 mg) or isotype antibody (BioXCell) together with the IL-1 receptor antagonist anakinra (0.1 mg per mouse, provided by Raphaela Goldbach-Mansky, NIH, Bethesda, Maryland, USA). IL-33 receptor (ST2) was blocked with neutralizing anti-ST2 (20 μg) or isotype control antibody (R&D Systems). Mice were then epicutaneously exposed to OVA and SEB or CPE. Mice undergoing IL-6/IL-1 or ST2 neutralization received half of the initial antibody amount administered every other day for 7 days. OX40L was blocked with anti-OX40L (0.2 mg per mouse) or isotype control antibody (R&D Systems) twice in 1 week.

Flow cytometry. One week after epicutaneous exposure mice were sacrificed, and cells from skin-draining lymph nodes and mesenteric lymph nodes were harvested, washed, and stained with anti-mouse CXCR5 (BD Biosciences), anti-mouse CD44 (Biolegend), anti-mouse CD4 (eBioscience), and anti-mouse ICOS (eBioscience), in the presence of FcBlock (eBioscience). Cells were acquired on a BD LSR Fortessa cytometer (BD Biosciences). Data were analyzed using FlowJo software (TreeStar Inc.).

Skin gene expression. CPE (1 mg) applied on the left ears and right ears of mice was used as negative control. Ears were harvested 2, 4, and 24 hours after the CPE application. Total RNA was isolated with Trizol (Invitrogen, Life Technologies), followed by RNA clean up with the RNeasy Mini Kit (Qiagen).

Human keratinocytes. Human epidermal keratinocytes (neonatal cells, Invitrogen, Life Technologies) were cultured using EpiLife medium with growth supplements (all from Gibco, Life Technologies).

Cells were seeded in 24-well plates and stimulated with CPE or soy extract. Cells were recovered at several time points for RNA isolation with the RNeasy Mini Kit (Qiagen).

Bone marrow–derived DCs. Bone marrow cells were collected from femurs and cultured for 10 days in RPMI 1640 with L-glutamine (Gibco, Life Technologies), supplemented with 10% fetal bovine serum (Gemini Bio-products), penicillin/streptomycin (Gibco, Life Technologies), 50 μM 2-Mercaptoethanol (Sigma-Aldrich), and 20 ng/ml GM-CSF (Peprotech). At day 10, bone marrow–derived DCs were collected and resuspended in complete RPMI at a concentration of $5 × 10^5$ cells per ml. Cells were stimulated for 3 hours with CPE (50 μg/ml), followed by RNA isolation with the RNeasy Mini Kit (Qiagen).

Real-time PCR. RT-PCR was performed, starting from 1 μg total RNA, using SuperScript II Reverse Transcriptase (Invitrogen, Life Technologies). cDNA was amplified using the Power SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies) and run on an Applied Biosystems 7300 Real-Time Detection System, using the following primers: mouse *Il6*, F, TTCCATCCAGTTGCCTTCTTG; R, GGGAGTGGTATCCTCTGTGAAGTC; mouse *Il1b* (65), F, TTGACGGACCCCAAAAGAT; R, GAGCGCTCACGAACAGTTG; mouse *Il10* (25), F, TGCTATGCTGCCTGCTCTTA; R, TCATTTCCGATAAGGCTTGG; mouse *Ox40l* (25), F, CCCTCCAATCCAAAGACTCA; R, ATCCTTCGACCATCGTTCAG; mouse *Il33*, F, ATCGGGTACCAAGCATGAAG; R, GACTTGCAGGACAGGGAGAC; mouse *Tslp* (48), F, GAGAGAAATGACGGTACTCA; R, CTACAGTTAGGTTTGCCCTA; mouse *Il25*, F, TGTTGCATTCTTGGCAATGATC; R, GACTGCAGCCCTCCTGGAT; human *IL6*, F, AAAGAGGCACTGGCAGAAAA; R, CAGGGGTGGTTATTGCATCT; human *IL33*, F, GAGCTAAGGCCACTGAGGAA; R, TGGGCCTTTGAAGTTCCATA.

GADPH and β-actin were used as the housekeeping genes. Relative quantification was performed using the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$). The changes in gene expression were calculated with respect to the untreated cells. All amplifications were carried out in duplicates.

Antigen presentation assay. Antigen presentation assay was performed as previously described (19). BALB/c mice were exposed to 10 mg OVA in the presence or absence of 1 mg CPE. After 24 hours, DCs were purified from inguinal lymph nodes by using CD11c microbeads (Miltenyi Biotec). DCs were cultured at a ratio of 1:5 with DO11.10 CD4+ T cells. After 72 hours, cells were restimulated with anti-CD3/CD28, supernatants were harvested, and cytokines were measured by ELISA according to manufacturer's instructions (all from eBioscience).

In neutralization experiments, anti-ST2 was injected prior to exposure with OVA and CPE, as described above. For the OX40L neutralization, DCs were cocultured with T cells in the presence of anti-OX40L (10 μg/ml) or isotype control antibody (R&D Systems).

Immunoblotting. CPE (15 μg/cm) was separated on SDS-PAGE (NuPage MES, 4% to 12% ZOOM gels, Invitrogen). The protein was transferred onto nitrocellulose membranes (Bio-Rad). After blocking, membranes were incubated with diluted human sera (1:50), followed by detection with mouse Anti-Human IgE-Alkaline Phosphatase (Sigma-Aldrich), alkaline phosphatase chemiluminescent substrate (CDP-Star, Sigma-Aldrich), and exposure to film (30–90 minutes). Alternatively, membranes were incubated with diluted mouse sera (1:20), followed by detection with sheep anti-mouse IgE (Binding Site), donkey anti-sheep HRP (Binding Site), and HRP substrate (Millipore). The blot was exposed for 1 to 5 minutes.

Basophil activation tests. Basophil activation tests were performed as previously described (66). Blood was diluted in RPMI and incubated at 37°C for 90 minutes with extracts or purified allergen at 50 μg/ml except where indicated. Red blood cells were lysed, and cells were stained with CD49b and IgE to detect the population of basophils, after gating out T and B cells with CD3/CD19 staining. CD200R was used as basophil activation marker (all antibodies were from eBioscience).

Statistics. Statistical analyses were performed using GraphPad Prism software, version 6.0e (GraphPad). Two-tailed Student's *t* test or 2-way ANOVA followed by Bonferroni analysis were used for determining statistical significance ($P < 0.05$). Results are expressed as mean ± SEM.

Study approval. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai. Clinical specimens were provided by the Jaffe Food Allergy Resource Initiative. Inform consent was obtained for all the studied subjects. All the procedures were approved by the Institutional Review Board at Mount Sinai School of Medicine.

Acknowledgments

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