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Epicutaneous Immunotherapy Results in Rapid Allergen Uptake by Dendritic Cells through Intact Skin and Downregulates the Allergen-Specific Response in Sensitized Mice

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Epicutaneous immunotherapy onto intact skin has proved to be an efficient and safe alternative treatment of allergy in an animal model with various allergens and in children for cow’s milk allergy. The aim of this study was to analyze the different steps of the immunological handling of the allergen when deposited on intact skin using an epicutaneous delivery system and its immune consequences in sensitized BALB/c mice. As expected, when applied on intact skin, OVA exhibits neither a passive passage through the skin nor any detectable systemic delivery. The current study demonstrates that, after a prolonged application on intact skin, OVA is taken up by dendritic cells in the superficial layers of the stratum corneum and transported, after internalization, to the draining lymph nodes, with variations according to the previous level of sensitization of the mice. When OVA is applied with the epicutaneous delivery system repeatedly, specific local and systemic responses are down-modulated in association with the induction of regulatory T cells. Besides providing new insights into skin function in the presence of allergens, this study indicates that the skin might have a tolerogenic role, at least when kept intact. The Journal of Immunology, 2011, 186: 5629–5637.

The demonstration of the long-term benefits of subcutaneous immunotherapy for the treatment of allergy (1) has led to the evaluation of alternative noninvasive methods. These include nasal, oral, anal, and sublingual routes of administration (2). Senti et al. (3) recently suggested using the epicutaneous route for patients sensitized to pollen as a safe and effective approach to activate the immune system without the risk of massive passage of allergen into the bloodstream (2).

In parallel, we reported encouraging results of epicutaneous immunotherapy (EPIT) for children allergic to cow’s milk (4) and published EPIT preclinical data from mice using Viaskin (DBV Technologies, Paris, France), a new epicutaneous delivery system (EDS) that allows application of various allergens to the skin (5, 6). When applied onto intact skin, the EDS preserves its architecture, including the more superficial layers. Experiments with labeled Ags in in vitro transdermal models have shown that this EDS allows native protein to concentrate inside the stratum corneum within the vicinity of immunological cells, without passage through the skin (7).

Skin dendritic cells (DCs), for example, dermal DCs and Langerhans cells (LCs), have been implicated in Ag trafficking from skin to lymph node (LN) in steady state (8–10). An allergen may trigger sensitization when applied onto the disrupted skin of naive mice (11, 12). In contrast, when applied onto intact skin, a protein or a peptide may induce suppressor cells in animal models of autoimmunity (13–15). Both of these actions are likely to involve dermal DCs and LCs (16–19). However the precise outcome of a protein deposited onto intact skin in vivo and its potential interaction with the local immune system has not been investigated. Similarly, the influence of the previous sensitization stage on allergen capture and subsequent immune response has also not been studied.

The aim of this study was to analyze comprehensively the different steps of the immunological handling of the allergen deposited on the skin by the EDS as well as the potential local immune consequences of this. We showed that OVA applied onto intact skin, in contrast with stripped skin, does not lead to passive passage and systemic delivery in vivo. Instead, the allergen is captured by skin DCs, which migrate to afferent LNs to activate immune responses and OVA-specific cytokine production. Furthermore, repeated applications down-modulate the local eosinophil recruitment after OVA exposition and decrease systemic allergen-specific immune responses while increasing regulatory T cells (Tregs).

Materials and Methods

Animals and treatments

Five-week-old female BALB/c mice (Charles River Laboratories, Lyon, France) were housed under standard animal husbandry conditions. Experiments were performed according to the European Community rules for animal care and with permission 92-305 of the French Veterinary Services. Mice were acclimated during 1 wk before sensitization. Mice were sensitized on days 0 and 7 with 200 μl of a homogenous suspension of 10 μg OVA (grade V; Sigma, St. Quentin Fallavier, France) and 1.6 mg
aluminum hydroxide (Sigma) by s.c. administration on the back of the neck. Mice were then challenged intranasally on day 14 with OVA (10 μg). For EPIT, EDSs with 100μg OVA (OVA-EDS) were applied for 48 h to the backs of mice once a week for 8 wk. Twenty-four hours before application, the skin was shaved with an electric clipper, and depilatory cream was applied. As previously demonstrated, this technique does not modify the barrier properties of the skin (5).

**Epicutaneous delivery system**

Viaskin is a new, patented EDS developed by DBV Technologies. Viaskin is made of a central translucent polyethylene membrane (11 mm in diameter) surrounded by an adhesive polymer nonwoven crown as contact support to maintain the chamber on the skin. A dry layer of allergenic materials (100 μg OVA) was deposited on this backing. The delivery system creates an occlusive chamber on the skin that rapidly generates moisture and releases the allergens from the plastic support onto the skin, allowing adequate diffusion of the proteins toward the epidermal immune-competent cells.

**Study design**

The analysis (Fig. 1A) concerned the different steps of the sensitization/immunotherapy processes: day 21 corresponds with the end of sensitization and beginning of EPIT, day 70 is the end of EPIT, and day 84 corresponds with 2 wk after the end of EPIT. The investigation focused on the transcutaneous passage and immune uptake of OVA applied by the EDS to the mice. The transcutaneous passage was monitored by Alexa 488 conjugated to OVA (OVA*) in the LN cells 2 h after application. This method has demonstrated the passive passage of the protein across the skin into lymphatics (20). Thereafter, the cell-mediated passage was studied by migration of DCs that had captured the allergen in the skin at different time points, through the follow-up of OVA*-positive CD11c+ cells. The phenotype of migrating DCs was characterized at the migration peak (Fig. 1A). To characterize the effect of repeated applications on allergen uptake by skin immune cells, the kinetics of capture and migration of DCs were also analyzed at day 70, after the last OVA-EDS application (Fig. 1B).

Finally, the effect of EPIT on the local and systemic allergen-specific immune responses was evaluated at day 84 (2 wk after the end of treatment) after application of an atopy patch test and comparing EPIT treated, untreated (sham), and naive mice (Fig. 1C): the evaluation comprised the eosinophilic infiltration of the skin, blood OVA specific Ig, and the in vitro restimulation of spleen cells with OVA.

**Passive passage through the skin**

Two-hour application: comparison of intact and stripped skin. The transcutaneous passage of OVA was measured using an EDS with 100 μg OVA* (OVA-EDS) (Invitrogen, Cergy Pontoise, France) applied onto intact skin (n = 5) and on skin stripped by 10-fold application and removal of cellulose tape (Scotch; 3M, Cergy Pontoise, France) (n = 5). Controls received OVA* s.c. (n = 4) or an empty EDS (n = 5). Inguinal LNs were harvested after 2 h for cell isolation and flow cytometry.

Forty-eight-hour application on intact skin: comparison of OVA-conjugated and free Alexa 488. The 48-h application of OVA*-EDS onto intact or stripped skin was compared with application of OVA-EDS on skin painted with 100 μg Alexa 488 hydrazide (Invitrogen). The comparison involved OVA-sensitized (n = 10) and naive (n = 10) mice. Controls (n = 10) received neither fluorochrome nor allergen. After application, the skin below the EDS was harvested: one half was used for fluorescence microscopy, and the other half was used for cell isolation and flow cytometry.

**Allergen capture, migration of DCs, and demonstration of stimulation of LN cells**

**Identification of skin cells that captured allergen**. Cells that captured the allergen in the skin were identified 48 h after OVA*-EDS application on the backs of OVA-sensitized (n = 10) or naive (n = 10) mice by skin cell isolation and flow cytometry.

**Kinetics of allergen capture and migration of DCs**. The capture of allergen and migration of DCs was analyzed 6 h (n = 5), 24 h (n = 5), 48 h (n = 5), and 24 h after stopping a 48-h application of OVA*-EDS on the backs of OVA-sensitized (n = 20) and naive (n = 20) mice, with additional controls receiving no EDS (n = 5 in each group). Skin and inguinal LNs were harvested immediately after euthanasia for flow cytometry. All experiments were repeated at least three times.

**Phenotype of migrating DCs in sensitized mice**. The expression of myeloid lineage marker CD11b and F4/80, of activation markers CD80, CD86 (costimulatory molecules), and MHC class II (MHC II), and of DC maturation markers CD205 (DC-205), CD103, and CD83 was analyzed on Alexa 488-positive DCs (CD11c+) in LN cells by flow cytometry 24 h after application of OVA*-EDS onto the backs of sensitized mice (n = 16). Inguinal LNs were harvested and pooled from all sensitized mice. Stimulation of LN cells: cytokine production by draining LN cells. Stimulation of LN cells was measured by in vitro production of cytokine after restimulation with OVA. The OVA-EDSs were applied in sensitized (n = 15) and naive (n = 15) mice for 48 h, and inguinal LNs were harvested 24 h later, with additional controls receiving no EDS (n = 15 in each group).

**The effect of EPIT on allergen capture, migration of DCs, and immune response**

**Kinetics of allergen capture and migration of DCs**. The allergen uptake by DCs in skin and LNs of EPIT-treated mice were followed after different durations of OVA*-EDS application as described for naive and sensitized mice.

**Local immune response and stimulation of LN cells**, and **systemic cellular response**. Skin eosinophil recruitment, allergen-specific cellular responses, and the proportion of Tregs in the spleen were analyzed 24 h after a 48-h application of OVA-EDS in sensitized mice treated with EPIT. These were compared with sensitized untreated (sham) mice and naive mice (n = 21 for each group). To evaluate desensitization, specific IgE and IgG were measured before (day 24), and after treatment (day 81).

**Ancillary methods**

**Fluorescence microscopy**. Skin tissue was harvested in Shandon cryomatrix (Thermo Fisher Scientific, Courtabeuf, France) and frozen in liquid nitrogen immediately after removing the EDS. The localization of both fluorochrome and allergen was done on cryostat sections (7 μm) mounted with Vectashield DAPI (Vector Laboratories, Orton Southgate, UK) using a DMR microscope (Leica Microsystem, Rueil-Malmaison, France) and IM 1000 software.

**Cell isolation**. Skins were incubated for 90 min at 37°C on 0.5% trypsin (Sigma) in PBS. The epidermis was physically separated from the dermis, washed in 10% heat-inactivated FCS in RPMI 1640 medium, disrupted by pipetting, and filtered on a 100-μm cell strainer (BD Biosciences, Le Pont de Claira, France). Dermis samples as well as LNs were incubated for 45 min in collagenase D (200 Mandl U/ml; Roche, Meylan, France) and DNase I (25 μg/ml; Roche). EDTA 100 mM was added, and samples were squeezed through 100-μm cell strainer and washed in 10% heat-inactivated FCS in RPMI 1640 medium.

**Flow cytometry**. Cell suspensions pooled from five mice were incubated with anti-mouse CD16/CD32 Fc Block (BD Biosciences) and then stained for cell-specific markers. The following fluorochrome-labeled Abs and the corresponding isotype control were used: CD11c, CD11b, CD3, CD80, CD86, CD83, MHC II, CD86, Gr-1, and B220 (BD Biosciences), F4/80 (AbD Bio-Sciences, Düsseldorf, Germany), CD205 and CD103 (eBioscience, Hatfield, UK). For Treg analysis, spleen cells were stained with anti-mouse CD4–FITC and CD25–allophycocyanin before fixation and permeabilization, using Cytofix/Cytoperm kit following the manufacturer’s instructions, and staining with Foxp3–PE or control isotype (BD Biosciences). Flow cytometry was performed on FACSCalibur and analyzed using CellQuest software.

**Blood-specific IgE, IgG1, and IgG2a**. Blood was collected from the retroorbital venous plexus after anesthetization of the mouse. Specific Abs were quantified using a quantitative ELISA developed in-house according to the 2001 Food and Drug Administration guidelines (5).

**Stimulation of LN cells**. LN cells pooled from three to five mice were cultured at 2 × 10^6 cells/ml in the presence of 100 μg/ml OVA or culture medium alone (10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin in RPMI 1640 with glutamine). Supernatants were collected after 72 h and stored at –20°C for later cytokine measurement with the Bio-Plex System (Bio-Rad, Marnes-la-Coquette, France) or specific ELISAs (BD Biosciences). Measurements involved IL-13, IL-5, and IL-4 (Th2 response); IL-17 (Th17); IL-10 (Treg); and IFN-γ, TNF-α, and IL-12p70 (Th1).

**Systemic immune response**. Spleens were squeezed through a 100-μm cell strainer and washed in 10% heat-inactivated FCS in RPMI 1640. After RBC lysis, splenocytes (2 × 10^6/ml) were cultured with 100 μg/ml OVA or culture medium alone. Supernatants were collected after 72 h and stored at –20°C for later cytokine measurement. For proliferation assays, 10^6 spleen cells were cultured in 96-well plates with 10 or 100 μg/ml OVA or culture medium alone. After 7 d, cell proliferation was determined using the WST-1 kit (Roche).


**Statistical analysis**

GraphPad Prism Software 5.0 (GraphPad, San Diego, CA) was used for statistical analysis. Results are expressed as mean ± SEM. Comparison of DC migration between groups was analyzed using unpaired *t* test. Ab responses were analyzed using the non-parametric Mann–Whitney *U* test. Cytokine data were analyzed using ANOVA with Bonferroni post hoc correction.

**Results**

**Passive passage of Ag through the skin**

The capacity of the intact skin to behave as an efficient barrier against the passive passage of the allergen and its delivery to lymphatics was tested by the measurement of OVA* in LN cells 2 h after application of EDS onto the skin (Fig. 1A); the proportion of OVA*-positive B cells and DCs in LNs was not different from the background fluorescence in control mice (Fig. 2). To obtain a positive control of allergen passive passage through the skin, the OVA*-EDS was applied onto stripped skin: the proportion of OVA*-positive B cells and DCs in LNs increased markedly compared with that of intact skin (*p*, 0.01). This was also seen when OVA* was administered s.c. (*p*, 0.05). When the OVA*-EDS was applied onto intact skin for 48 h to evaluate the free passage of allergens through intact skin after a long-lasting application, fluorescence was mainly observed in epidermis and in some cells of the dermis (Fig. 3A). OVA*-positive cells in epidermis represented 62.9 ± 10.2% of total cells in sensitized mice and 54.2 ± 7.2% in naive mice (Fig. 3E). In the dermis, respectively 24.9 ± 2.5% and 28.3 ± 7.5% of cells were positive for OVA* (Fig. 3F). In contrast, the application of OVA*-EDS on stripped skin resulted in a passage of allergen into epidermis and dermis (Fig. 3B, 3E, 3F). A control application of free fluorochrome, small enough to pass through the skin, induced a diffuse cell staining in epidermis (87.5 ± 3.25%) and dermis (81.1 ± 3.6%) (Fig. 3D, 3E, 3F). Control mice receiving neither allergen nor fluorochrome displayed no fluorescence in skin (Fig. 3C, 3E, 3F). Subsequent experiments were carried out on intact skin.

**Allergen capture, migration of DCs, and demonstration of stimulation of LN cells**

**Identification of skin cells capturing the allergen.** After 48-h application of the OVA*-EDS on intact skin, fluorescence was detected in cells from all skin layers in both sensitized and naive mice (Fig. 3F, 3G). In the epidermis of sensitized mice, the proportion of OVA*-positive cells was 13.3 ± 1.6% for CD3+ T cells, 44.3 ± 8.6% for B220+ B cells, 77.9 ± 7.2% for CD11b+ cells, and 81.8 ± 5.9% for CD11c+ DCs. In the dermis of naive mice, these proportions of OVA*-positive cells were respectively 12.0 ± 4.2%, 17.8 ± 4.8%, 35.7 ± 9.6%, and 46.1 ± 11.5%. When gating on the population of OVA*-positive cells after 48 h of OVA*-EDS application, only 9.0 ± 5.37% of these cells in the epidermis and 0.45 ± 0.45% in the dermis were negative for specific markers of APCs (CD11c, CD11b, and B220). These results, confirmed by immunohistological analysis with MHC II staining of frozen skin sections (data not shown), indicate that when applied on intact skin, allergen is specifically captured by APCs.
Kinetics of allergen capture by DCs in skin of naive and sensitized mice. DCs appear to dominate the Ag trafficking from skin to LN, and the evaluation thus focused on the kinetics of allergen capture by DCs and their migration after application of the OVA*-EDS (Fig. 1A).

The OVA*-EDS was applied on sensitized and naive mice for different durations, and the percentages of OVA*-positive cells among CD11c<sup>+</sup> cells was measured. DCs captured the allergen significantly faster and more efficiently in sensitized versus naive mice; in the epidermis, 77.0 ± 4.0% versus 42 ± 4% of DCs were OVA*-positive after 6 h (p < 0.05), and these percentages were 83.7 ± 5.9% versus 65.3 ± 4.2% after 24 h (Fig. 4A). Similarly, in the dermis, the peak capture in sensitized mice was 60.1 ± 7.7% of OVA*-positive DCs, which occurred at 24 h, compared with a peak of 33.5 ± 4.9% in naive mice, which occurred at 48 h (Fig. 4B). These differences between naive and sensitized mice were not due to an increased passive passage of allergen, as the lack of passive passage through intact skin was comparable in naive and sensitized mice (data not shown).

Migration of DCs from skin to LN. The proportion of OVA*-positive CD11c<sup>+</sup> cells in the epidermis of sensitized mice rapidly decreased from 2.02 ± 0.65% at 6 h to 0.05 ± 0.02% at 24 h (p < 0.05 versus naive) (Fig. 4C), suggesting a rapid migration of OVA-bearing epidermal DCs. In the dermis, the proportion of OVA*-positive CD11c<sup>+</sup> cells increased to 2.22 ± 0.62% after 24 h in sensitized mice, before rapidly decreasing to 0.23 ± 0.17% at 48 h (p < 0.05 versus 24-h time point) (Fig. 4D), implying that skin DCs were migrating through the dermis. In naive mice, this proportion remained stable.

In sensitized mice, the migration of DCs from the skin was confirmed by the appearance of OVA*-positive DCs in LN from 6 h onward. These composed 0.15 ± 0.07% of the total cell population (5.8 ± 2.6% of DCs), and this level increased to 0.24 ± 0.05% (9.9 ± 1.6% of DCs) after 24 h (p < 0.05 versus naive) corresponding with 3.3 ± 1.0 × 10<sup>3</sup> OVA*-positive DCs/LN, and slowly decreased thereafter. In naive mice, OVA*-positive DCs remained low or undetectable (Fig. 4E).

Phenotype of migrating DCs. To characterize the phenotype of migrating DCs in LN, the OVA*-EDS was applied on sensitized...
mice for 24 h (i.e., the migration peak) and LN cells then harvested for flow cytometry. The analysis focused on markers of DC activation and maturation, such as costimulatory molecules CD80 and CD86, the maturation marker CD83, and the member of the macrophage mannose receptor family of C-type lectin. Migrating OVA*-positive DCs in the LNs of sensitized mice expressed high levels of the myeloid markers F4/80 and CD11b (Fig. 5), as well as high levels of MHC II molecules, which are crucial for Ag presentation. In contrast, they expressed only relatively moderate levels of the costimulatory molecule CD80. Migrating DCs consisted of two populations according to CD205 level: CD205low DCs, which expressed low levels of CD86 and CD83 (suggestive of immature DCs), represented 55 ± 2% of migrating DCs; the other population consisted of CD205high, CD83high, and CD86high and represented 45 ± 2% of migrating DCs. CD103 was not expressed in migrating DCs (data not shown).

**LN cell stimulation.** To evaluate the effect of migration of DCs from skin on activation of LN cells and orientation of this activation, the OVA-EDS was applied for 48 h on sensitized and naive mice, with no EDS in controls, and LN cells were harvested 24 h later to measure cytokine production after in vitro restimulation with OVA. In the absence of OVA-EDS application, LN cells from sensitized and naive mice did not secrete any cytokine in response to in vitro restimulation, suggesting undetectable levels of OVA-specific cells in inguinal LN. After epicutaneous application of OVA-EDS, restimulated LN cells from sensitized mice responded to OVA by secreting significant levels of the Th2 cytokines IL-13 and IL-5 (p < 0.01) and the regulatory oriented cytokine IL-10 (p < 0.05), with low levels of Th1 cytokines (Fig. 6).

**The effect of EPIT on allergen capture, migration of DCs, and immune response**

**Kinetics of allergen capture and migration of DCs.** To evaluate the ability of EPIT to influence the kinetics of allergen uptake by the

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**FIGURE 5.** Phenotype of DCs that captured allergen in skin and migrated into LNs. Cells, isolated from inguinal LNs of sensitized mice that received OVA*-EDS for 24 h, were gated on Alexa 488-positive CD11c-positive (A488+CD11c+) cells, and expression of the different markers was analyzed. For CD83 and CD86 expression, cells were further gated according to CD205 expression.

**FIGURE 6.** Cytokine production by OVA-restimulated lymphocytes from draining LNs. Thirty mice were sensitized and compared with 30 naive mice. One week after sensitization, 15 mice of each group (sensitized or naive) received OVA-EDS, and 15 of each group were left untreated (control). Twenty-four hours after stopping application, cells were isolated from inguinal LNs. Cells from five mice were pooled and cultured at 2 × 10^6 cells/ml in the presence of 100 μg/ml OVA or medium alone. Supernatants were collected after 72 h and stored at −20°C. Level of Th2 [IL-13 (A), IL-5 (B), IL-4 (C)], Th17 [IL-17 (D)], Treg [IL-10 (E)], and Th1 [IFN-γ (F), TNF-α (G), and IL-12p70 (H)] cytokines were measured and analyzed in supernatant with the Bio-Plex system. Data represent the mean ± SEM. *p < 0.05.
DCs, EPIT was initiated 1 wk after sensitization of mice. At the end of EPIT, the OVA*-EDS was applied and allergen uptake by DCs evaluated (Fig. 1B). Mice exhibited a response similar to that of sensitized mice before EPIT. In the epidermis, 73.3 ± 6.6%, 76.1 ± 6.45%, and 79.0 ± 5.8% of DCs were OVA*-positive at 6, 24, and 48 h, respectively (Fig. 7A). The migration of DCs to LNs peaked at 24 h with OVA*-positive DCs representing 0.21 ± 0.06% of total cells (8.6 ± 3.4% of DCs) (p < 0.05 versus t = 0 h) (Fig. 7B). The phenotype of migrating DCs at 24 h was also similar to that of sensitized mice with high expression of MHC II and both populations CD205high CD86high and CD205low CD86low OVA*-positive DCs (Fig. 7C).

**Stimulation of LN cells.** Activation of LN cells after DC migration was measured 24 h after a 48-h application of OVA-EDS by cytokine production after OVA in vitro restimulation such as for sensitized mice. The migration of DCs in the draining LNs from sensitized untreated mice stimulated the LN cell production of high OVA-specific and nonspecific levels of IL-5 and IL-10 (p < 0.05 and p < 0.01 versus control, respectively) (Fig. 7D and data not shown). Although the OVA-specific production of IL-4 and IFN-γ was undetectable (data not shown), the nonspecific production of IL-4, but not of IFN-γ, was significantly higher than that in naive mice (Fig. 7E). For EPIT mice, the OVA-specific IL-5 and IL-10 responses, as well as the nonspecific IL-4 production by afferent LN cells, decreased significantly after application of the OVA-EDS (p < 0.01 versus sham), whereas IFN-γ remained comparable with sham (Fig. 7D, 7E).

**Effect of EPIT on OVA-specific Ab response.** As already reported (5), specific IgE strongly increased in sensitized mice. After treatment, EPIT induced a significant decrease of specific IgE and a significant increase in specific IgG2a (p < 0.001 versus sham) (Fig. 8A).

**Effect of EPIT on the local immune responses to application of allergen.** The local response to the allergen was measured by the skin eosinophilic infiltration. The OVA-EDS was applied for 48 h onto intact skin; epidermis and dermis were isolated 24 h later to measure eosinophils by flow cytometry. In sensitized mice, there was a massive recruitment of eosinophils in the epidermis and dermis compared with that in naive mice (4.49 ± 1.10% versus 0.89 ± 0.28% in the epidermis, and 4.99 ± 0.4% versus 0.93 ± 0.3% in the dermis, p < 0.05) (Fig. 8B). After EPIT, the recruitment of eosinophils to the epidermis and dermis remained similar to that of control and significantly decreased compared with that of sensitized sham mice (epidermis, 0.72 ± 0.17%, p < 0.01, and dermis, 1.14 ± 0.30%, p = 0.05).

**Systemic cellular immune response.** The systemic allergen-specific response was measured using splenocytes harvested from sham, EPIT, or control mice and cultured in vitro with OVA, Con A, or medium alone (control) to measure proliferation and cytokine production (Fig. 1C). After EPIT, OVA-specific proliferation decreased significantly compared with that of sham after restimulation with 10 μg/ml OVA (p < 0.05), but not after restimulation with 100 μg/ml OVA (Fig. 8C). OVA-specific production of cytokines with EPIT was significantly lower than that of sham: for IL-4, 631 ± 133 versus 1290 ± 431 pg/ml; for IL-5, 2509 ± 572 versus 4898 ± 1249 pg/ml; for IL-13, 397 ± 100 versus 1413 ± 625 pg/ml; for IL-10, 213 ± 83 versus 672 ± 258 pg/ml; and for IFN-γ, 84.4 ± 32.9 versus 234 ± 91 pg/ml (p < 0.05) (Fig. 8D). At the same time point, the presence of Tregs in the spleens of EPIT, sham, and control mice was analyzed by flow cytometry. With EPIT, the percentages of CD25+Foxp3+ CD4 T cells in the spleen were significantly higher than those of sham (0.93 ± 0.09% versus 0.50 ± 0.08%, p < 0.05) and of control (0.43 ± 0.05%, p < 0.01) (Fig. 8E).

**Discussion**

We analyzed the different steps of immunological handling of allergens deposited by a novel, needle-free and painless mode of administration through intact skin using a new EDS (Viaskin). We showed that, when applied with this EDS onto intact skin, OVA exhibits neither a passive passage through the skin nor any detectable systemic delivery. The allergen is captured by skin DCs that migrate to the afferent LNs and activate immune responses and OVA-specific cytokine production. The current immunological status plays an important part in this uptake, which appears to be enhanced in sensitized animals. Moreover, EPIT, consisting of repeated applications, down-modulates recruitment of eosinophils...
to the skin after OVA exposure, and decreases the systemic allergen-specific immune responses and induces Tregs.

As a potentially new noninvasive method of immunotherapy (3, 4), EPIT requires further insights into its mode of action. After application onto disrupted skin of naive mice, various allergens induced sensitization (11, 12) whereas proteins or peptides seem to induce suppressor cells after application onto intact skin (13–15). LCs and dermal DCs were differently implicated in Ag trafficking from skin to LN in steady state (8–10) and during sensitization with haptens (19).

We investigated the various steps of the skin immune response after allergen delivery using an EDS. To evaluate a possible passive passage of the allergen through intact skin, we measured the level of fluorescence in LN cells 2 h after OVA*-EDS application onto skin, which was intact or severely stripped. This was compared with s.c. injection of OVA* in BALB/c mice. After the s.c. injection of a protein, it has been shown that the protein is detected in LN DCs within 2–3 h, whereas after acquiring the protein Ag at the injection site, the DCs appear in the draining LN more than 18 h later (20). Our study confirms the presence of OVA* in LN cells 2 h after s.c. injection. Similarly, 2 h after application of OVA*-EDS onto stripped skin, LN B-cells and DCs were positive for OVA*, whereas no fluorochrome appeared in LN cells if the skin was not stripped. This concentration of labeled protein in LN cells 1–3 h after exposure strongly implicates the overwhelming role of immune capture and drainage by local lymphatics and argues against a passive passage with intact skin.

To confirm the absence of free passage of allergens through the skin after a long-lasting application, skin fluorescence was analyzed by either immunohistology or flow cytometry of cells isolated from epidermis and dermis after application of OVA* or free fluorochrome. This clearly showed that free fluorochrome diffused passively throughout the skin, as expected, whereas OVA* appeared mainly in epidermis and was confined to only a few cells in the dermis. The phenotype of cells that captured the allergen in the epidermis and dermis was clearly characterized as APCs, particularly DCs, suggesting that the passage is only mediated by these specialized immune cells.

Ag outcome also depended on the sensitization status of the mice. DCs from epidermis of naive mice captured the allergen delivered by the EDS but did not migrate to the draining LNs. Stoitzner et al. (21) showed that epicutaneous immunization of mice required a barrier disruption to activate OVA-specific CD8 T cells in the draining LNs. This suggests that the Ag application on intact skin in naive mice does not have much effect on DC migration. In sensitized mice, we observed that DCs, having captured the allergen from the epidermis, migrate through the dermis into the draining LNs. This is consistent with a two-step model of LC migration (22). We cannot exclude the involvement of dermal DCs in the migration to LNs. Indeed, in our model, skin DC migration seems less delayed than that in the study of Kissenpfennig et al., where LC migration occurred only after 48–72 h (18). However, the absence of free passage of allergen into the dermis suggested a role of LCs; the faster migration in our model probably due to the immunological status of the sensitized mice.

DCs migrating from the skin into the LN were myeloid DCs and could be divided into at least two subpopulations: CD205high, CD86high, and CD83high mature DCs and a less mature population...
of CD205low, CD86low, and CD83low. Both express comparably high levels of MHC II, which is crucial for Ag presentation, as well as moderate levels of CD80. CD205 is upregulated during activation of DC, plays a role in Ag uptake, processing, and presentation, and has been implicated in induction of tolerance (23, 24). This suggests that both cell subpopulations can present the allergen and modulate immune responses toward a different profile of response, although further studies are required to clarify their precise role. However, the migration of these DCs was associated with the activation of OVAspecific cytokine responses in the LNs of sensitized mice, suggesting both presentation of allergen and activation of lymphocytes.

The significant differences between sensitized and naive mice with respect to migration of DCs underline the importance of the immunological status during EPIT. Sensitization has different effects on the various steps of allergen passage from the EDS to the LNs. The increased number of OVA*-positive DCs in LNs of sensitized mice suggested that sensitization increases skin DC migration in response to allergen. Although we cannot totally exclude that a difference in migration was the consequence of a higher capture of allergen, the absence of observed migration of skin DCs in naive mice, while they captured allergen to a not-negligible level (even if less effectively than in sensitized ones), suggests that skin DCs of sensitized mice are more prone to migrate in response to allergen stimulation. This could be explained by induction of local innate response to allergen in sensitized mice. The stronger capture and, more specifically, the higher migration observed in sensitized mice was not due to higher passive passage. Different mechanisms could be involved. The presence of OVA-specific IgE could induce a higher migration of LCs (25). A high level of OVA-specific IgG1 could influence the capture of allergen by DCs and their subsequent migration. Moreover, histamine is able to modulate endocytosis and crosspresentation mediated by immature DCs, and stimulation of LCs with histamine increases their migration from epidermis (26, 27). At last, the difference in allergen uptake in sensitized mice could be attributed to differences in activation of keratinocytes, which could modulate LC activation (28). In contrast, although EPIT even clearly affected sensitization status and specific Ab concentrations, it did not significantly modify the allergen uptake and migration. This suggests a complex regulation of migration of skin DCs, possibly via subtle modulations of cytokine release, Ab and histamine receptor expression, or other unknown mechanisms. Furthermore, if skin DCs exhibit a similar migration, the activation of LN cells is clearly modified by EPIT, which could be partially explained by the presence of Tregs. However, more studies are required to explain fully these phenomena.

Our data give further support to the clinical efficacy of EPIT. In addition to its action on specific IgE, specific IgG2a, and organ responses to allergenic stimulation, EPIT appears to act on the allergen-specific cellular response. Tissue eosinophilia is a typical feature of atopic dermatitis, the numbers of eosinophils in the skin usually being modest and correlated to disease severity (29). Indeed, in our model, application of allergen onto the skin of sensitized untreated mice induced recruitment of eosinophils. This recruitment significantly decreased after repeated applications of allergen using Viaskin. This regulation of allergen responsiveness is not limited locally and extends to the spleen, where allergen-specific proliferation and cytokine production significantly decreased with EPIT compared with sham. In mice sensitized to aeroallergens (pollen and house dust mite) or food allergens (OVA and peanut), we previously demonstrated that in addition to its action on specific Abs, EPIT was beneficial through decreasing airway hyperresponsiveness in sensitized mice (5). Concomitant with the decrease of allergen reactivity, we observed a significant increase of CD25Foxp3CD4+ T cells in the spleens of treated animals, suggesting that the general downregulation of allergen-specific responses could be mediated by Tregs.

The effects of EPIT appear to depend on the initial immunological status of the animal, the timing of the skin applications, and the preparation of the skin. In naive mice, three 1-wk cutaneous applications of OVA every other week, with physical removal of the stratum corneum, followed by a single exposure to aerosolized OVA increases total and OVA-specific serum IgE (11). Moreover, 3 d of cutaneous applications of peanut onto disrupted skin may convert established Ag-specific Th1 to Th2 responses (12). As evidenced here, the disruption of the stratum corneum allowed free passive passage of the allergen into the circulation. This considerably differs from the specialized capture and migration of skin DCs observed after application onto intact skin. In different animal models of autoimmune disease, epicutaneous application of protein or peptide onto intact skin has been proposed to induce suppressor cells (13–15).

Our data provide further support to the clinical safety of EPIT. The allergen delivery through EPIT is restricted to the active immune cells of the epidermis. The epidermis does not contain blood vessels, so a massive release of allergens into the bloodstream is unlikely to occur.

In summary, in the current study, we demonstrated that epicutaneous application of an allergen onto intact skin leads to its transport via DCs to the draining LNs, with variations according to the previous immunological status toward this allergen and the timing of the application. A prolonged and repeated application of an allergen by EDS onto intact skin of sensitized animals induced a down-modulation of allergen-specific local and systemic responses possibly through the induction of Tregs. Besides providing new insights into the skin function in front of allergens, this study indicates that the skin might have a tolerogenic role, at least if kept intact.

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Disclosures

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