

Epicutaneous Immunotherapy Using a New Epicutaneous Delivery System in Mice Sensitized to Peanuts

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Key Words

Food allergy • Epicutaneous immunotherapy • Peanut • Plethysmography • Resistance-compliance • Viaskin[®]

Abstract

Background: Peanut allergy is a life-threatening condition for which new efficient and safe treatment is expected. We evaluated epicutaneous immunotherapy (EPIT) as a new alternative treatment for peanut allergy in sensitized mice. **Methods:** Sixty BALB/c mice were sensitized by gavages with peanut protein extract (PPE) mixed with cholera toxin. An epicutaneous delivery system, coated with 100 µg PPE (Viaskin[®], DBV Technologies, Paris, France), was applied to intact skin every week during 48 h (EPIT; n = 20). This group was compared with sensitized mice treated with subcutaneous immunotherapy (SCIT; n = 20), untreated sensitized mice (sham, n = 20), and naive mice (naive; n = 20). After the 8-week treatment, a histamine release test, airway hyperreactivity measurement by plethysmography, and a resistance-compliance measurement after the challenge were performed. Blood and bronchoalveolar lavage were sampled for serology, cytokines, and cytology. **Results:** Specific IgE (sIgE) increased after sensitization in the EPIT (0.26 µg/ml) and SCIT (0.21 µg/ml) groups and decreased after treatment (0.09 µg/ml, p < 0.001 and 0.06 µg/ml, p < 0.001, respectively). The

IgG1/IgG2a ratio decreased in the EPIT and SCIT groups versus the sham group (3.7; p < 0.001 and 2.7; p < 0.01 and 15.1, respectively). At the higher metacholine concentration, enhanced pause values were lower in the EPIT and SCIT groups than in the sham group (7.29, 6.74, and 10.99, p < 0.01, respectively), and did not differ from that of the naive group (5.06). Resistance-compliance was reversed in the treated groups versus the sham group (p < 0.001). IL-4, IL-5, IL-13, eotaxin, and eosinophils were reduced in the BAL of the EPIT and SCIT groups versus the sham group (p < 0.001). **Conclusion:** In peanut-sensitized mice, based on biological and physiological responses, EPIT is as efficient as subcutaneous treatment which is the reference method in immunotherapy.

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Introduction

Allergy has become a major burden in developed countries during the 20th century. Among allergic manifestations, food allergy accounts for a substantial part of the morbidity rate. Patients with food allergy are handicapped by a strict avoidance diet and a potentially life-long-lasting disease. Furthermore, they may be at a daily risk of fatal reactions [1].

Among food allergies, peanut allergy is common and severe [2]. It is estimated to affect 1.8% of young infants in the UK [2, 3]. A national survey in the USA indicated that about 1.1% of the general American population, i.e. more than 3 million people, are allergic to peanuts and/or tree nuts [2].

Desensitization is currently recognized by the World Health Organization as the gold standard of treatment for allergy with long-term benefits. It may be carried out via the skin, i.e. subcutaneously, considered as the reference method [4] or, alternatively, via the oral mucosa, i.e. the sublingual route, already largely investigated for aeroallergens [5]. Further developments will probably improve the tolerance and safety of these methods [6].

Concerning food allergies, especially to peanuts, the subcutaneous route is not available due to practical and safety-related concerns. Some attempts have been made via the oral mucosa and encouraging results have already been reported in children [7], even in case of very severe allergy [8]. However, this method might be hampered by the potentiality of local and systemic adverse effects in certain patients. Alternative ways must, then, be evaluated.

In that respect, a new method of immunotherapy via the epicutaneous route has been developed thanks to a new epicutaneous delivery system (EDS) (Viaskin®, DBV Technologies, Paris, France). This method has already been described with various allergens in mice [9]. In the present work, dedicated to peanut epicutaneous immunotherapy (EPIT), our goal was to conduct a thorough investigation of the 2 major health risks for patients allergic to peanuts, i.e. anaphylactic shock and lung reactions [10], since the protracted bronchospasm developed after accidental ingestion in severe peanut allergy might be linked to pulmonary inflammation [11].

Materials and Methods

Study Design

After a phase of sensitization validated by an increase in the specific IgE (sIgE), mice were divided into 3 groups of 20 animals each and received EPIT, subcutaneous immunotherapy (SCIT), or a sham treatment during 8 weeks. Twenty naive mice were also included.

Ten days after the end of the treatment, the groups were split in half. The first-half groups were submitted to the measurement of histamine release in blood after a peanut oral challenge, and then to the evaluation of airway hyperresponsiveness (AHR) by plethysmography after 3 days of a peanut aerosol challenge. The second-half groups underwent an evaluation of AHR with the resistance-compliance technique (flexiVent®, SCIREQ, Montreal,

Que., Canada) after 3 days of aerosol challenge. In all animals, a bronchoalveolar lavage (BAL) was finally performed for cytological and immunological analyses. A scheme of the study design is shown in figure 1.

Animals

Three-week-old ($n = 80$) female BALB/c mice (Charles River, Lyon, France) were purchased and housed under standard animal husbandry conditions. All experiments were performed according to the European Community rules on animal care, with permit 92-305 from the French Veterinary Services and with a positive evaluation of the Ethical Committee of Université Paris Descartes (Paris, France; P2.LM.059.08). The mice were acclimated for 1 week before immunization.

Skin preparations before EDS application were all performed under general anesthesia with ketamine (Imalgene 1000, Merial) (100 mg/kg body weight) and xylazine (Rompun®, Bayer) (10 mg/kg body weight); BAL and respiratory tests were performed under anesthesia with pentobarbital (Nembutal®, Sanofi Santé Animale, CEVA) (50 mg/kg body weight) administered intraperitoneally, and venipuncture was performed under isoflurane.

Immunization

Mice ($n = 60$) were sensitized to peanuts by means of 6 intragastric gavages, once a week during 6 weeks, with 200 μ l of a phosphate-buffered saline (PBS) solution containing 1 mg of homogenized peanut protein extract (PPE) mixed with 10 μ g of cholera toxin (CT; Servibio, USA). Sensitization was controlled by the production of specific IgE in blood 10 days after the last injection.

Naive mice received 200 μ l of PBS by oral administrations using the same scheme as for sensitized mice.

Treatment

Epicutaneous Immunotherapy

EPIT was performed using an original EDS (Viaskin®, DBV Technologies) consisting of a central transparent plastic membrane (11 mm in diameter) of polyethylene electrically charged with electrostatic forces (fig. 2). Dry PPE powder is maintained on the backing by electrostatic forces. An occlusive chamber is created on the skin that rapidly generates moisture and releases the allergen from its support. The allergen is then absorbed by the skin where it interacts with epidermal immune cells. The use of this technology for diagnostic purposes (Diallertest®) was previously published [12].

EDS with 100 μ g of PPE were applied for 48 h to the backs of the mice once a week during 8 weeks. Twenty-four hours before application, their skin was shaved with an electric clipper and depilatory cream was applied. This technique does not modify the barrier properties of the skin. This was demonstrated in a previous experiment by the absence of change in transepithelial water loss as compared with hairless mice (6.45 ± 1.22 vs. 6.63 ± 1.49 g/h/m², not significant).

Subcutaneous Immunotherapy

Once a week during 8 weeks, the mice of the SCIT group received 200 μ l of a homogeneous suspension of 100 μ g of PPE injected subcutaneously between the shoulders, as previously described [13].

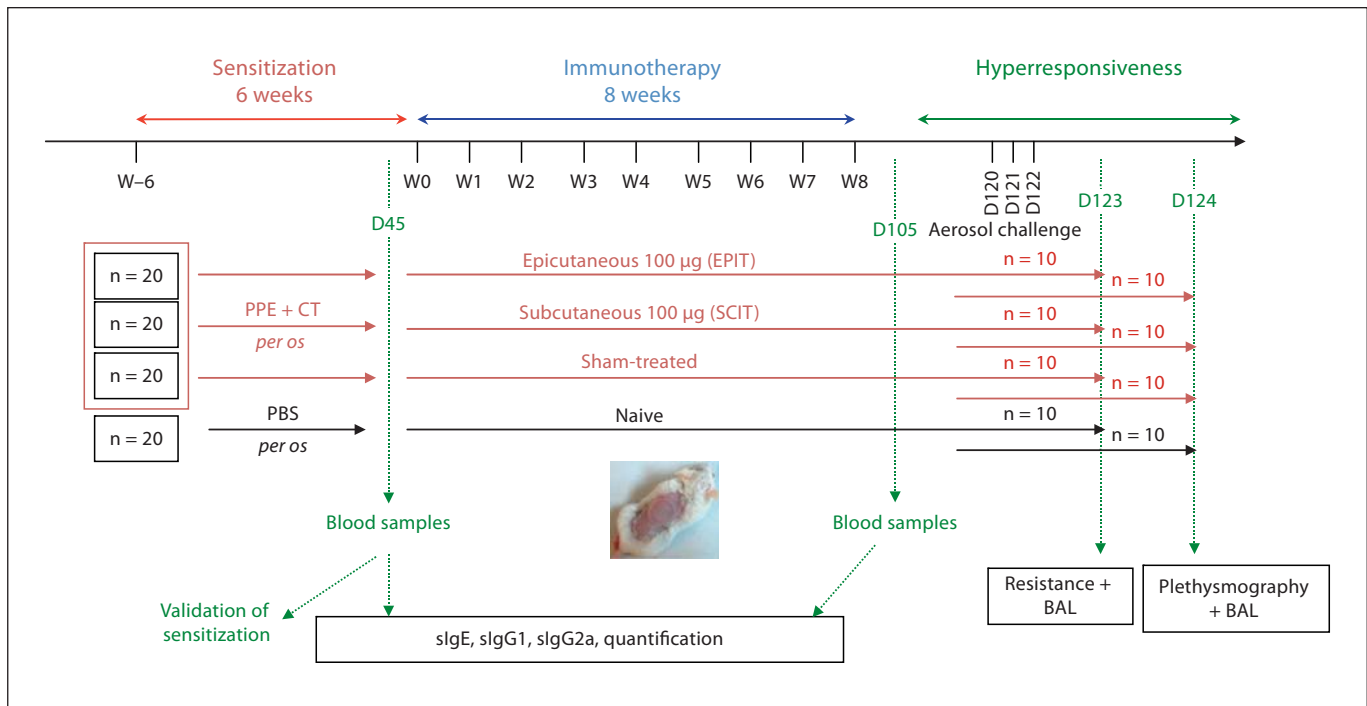
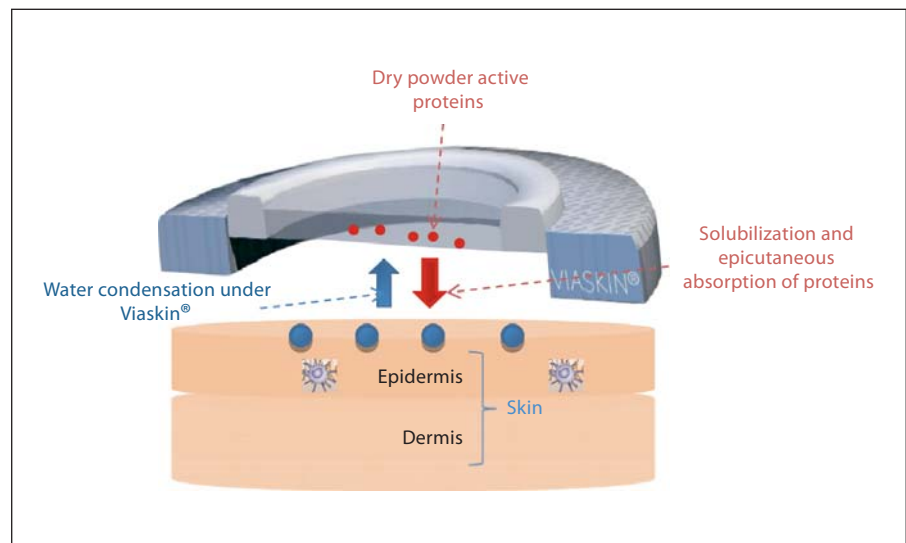


Fig. 1. Study design for peanut immunotherapy. The first phase was the sensitization of mice to peanut proteins from weeks –6 to 0 (from W–6 to W0). After validation of the sensitization, immunotherapy was performed by epicutaneous (EPIT) or subcutaneous (SCIT) routes during 8 weeks (from W0 to W8). Blood sam-

ples were taken on days 45 (D45) and 105 (D105). An aerosol challenge was performed on 3 consecutive days (D120, D121, and D122). Resistance was analyzed on day 123 (D123) and plethysmography on day 124 (D124).

Fig. 2. Viaskin® technology. Proteins are loaded into the central polyethylene membrane charged with electrostatic forces. The delivery system creates an occlusive chamber on the skin that generates moisture and releases the proteins from the membrane. The proteins are then absorbed through the skin where they interact with epidermal immune cells.



Sham and Naive Groups

The sham group received an empty EDS during immunotherapy with the same design as for EPIT. No treatment was administered to the naive group.

Specific IgE, IgG1, and IgG2a in Blood

Blood was collected from the retro-orbital venous plexus 10 days after sensitization (D45) and 10 days after treatment (D105).

Specific antibodies were quantified using a quantitative ELISA developed in-house according to 2001 FDA guidelines. Briefly, microtiter plates were coated with 100 μ l per well of 5- μ g/ml peanut solution. Serial dilutions of 50 μ l of each serum were dispensed per well and incubated for 24 h at 4°C. A polyclonal anti-mouse IgE (STAR110A), IgG1 (STAR132A), or IgG2a (STAR82A) antibody labeled with alkaline phosphatase (Serotec, Oxford, UK) was used as a tracer. Reagent (pNPP, Sigma, France) was used as an enzyme substrate. Specific IgE, IgG1, and IgG2a were quantified by comparison with concentration-response curves obtained with a total IgE, IgG2a, or IgG1 assay performed under identical conditions using a solid phase coated with a monoclonal anti-mouse IgE (clone LO-ME-3) or IgG2a (clone LO-MG2a-9) antibody or a polyclonal anti-mouse IgG (STAR8B) antibody (Serotec). The cross-reactivity of secondary antibodies with immunoglobulin was less than 4% for all the antibodies and less than 0.1% for anti-IgG1 and anti-IgG2a antibodies against purified IgE.

Histamine Release in Blood

A histamine increase in blood reflects the percentage of mast cell degranulation [14]. It was assayed in plasma samples 30 min after the peanut oral challenge as a marker of anaphylactic reaction. Ten mice from each group were challenged at 30-min intervals by 2 oral administrations of 10 mg PPE diluted in 200 μ l of PBS. Histamine was assayed using a competitive enzyme immunoassay kit (SPI-BIO, Montigny-le Bretonneux, France) in blood collected 30 min after the second oral challenge.

Airway Hyperresponsiveness

Whole-Body Plethysmography

Whole-body plethysmography was performed by placing mice in a closed chamber allowing the recording of the pressure fluctuations during the breathing cycle. 'Enhanced pause' (Penh) was calculated as previously described [13, 15], from the box pressure recorded during inspiration and expiration and the timing comparison of early and late expiration. Penh corresponds to the ratio of peak expiratory pressure to peak inspiratory pressure. Mice were challenged with peanuts via 30 min of aerosol (10 ml of 1% PPE in 0.9% NaCl) during 3 consecutive days. Pressures were measured 24 h after the challenge and Penh values were calculated prior to and 10 min after the administration via aerosol of various doses of methacholine (Sigma-Aldrich, Steinheim, Germany). For each mouse, Penh values were plotted against methacholine concentration (0–40 mg/ml) and the area under the curve (AUC) was calculated [16]. Due to technical reasons, 8 mice per group were analyzed by plethysmography.

Resistance-Compliance

Bronchial resistance and dynamic compliance were measured using the flexiVent system (SCIREQ) after exposure to increasing doses of methacholine. Mice were anesthetized with intraperito-

neal injections of pentobarbital (70 mg/kg). The trachea was exposed and a 19-gauge metal needle was inserted into it. The needle was then connected to a computer-controlled small animal ventilator for quasi-sinusoidal ventilation of the mice with a tidal volume of 10 ml/kg at a frequency of 150 per minute and a positive end-expiratory pressure of 2 cm H₂O to achieve a mean lung volume close to that observed during spontaneous breathing. After measurement of baseline resistance and compliance, the mice were challenged with increasing concentrations (0, 0.625, 1.25, 2.5, 5, and 10 mg/ml) of methacholine aerosol generated with an in-line nebulizer and administered directly through the ventilator for 5 s. Resistance and compliance were measured using a 'snapshot' protocol every 20 s for 2 min. The mean of these 6 values was determined for each methacholine concentration, unless the coefficient of the determination of a measurement was smaller than 0.95. For each mouse, resistance values were plotted against the methacholine concentration (from 0 to 10 mg/ml) and the AUC was calculated [17].

Cytokine Levels and Cell Composition in Blood and BAL Fluid

Blood samples were collected for cytokine analyses in anesthetized mice the day after AHR measurement.

Eotaxin, interleukin (IL)-4, IL-5, IL-10, IL-13, IL-17, interferon (IFN)- γ and tumor necrosis factor (TNF)- α were assayed with a Bio-Plex cytokine assay according to the manufacturer's recommendations (Bio-Rad, Marnes-La-Coquette, France). Tumor growth factor (TGF)- β was analyzed using an ELISA kit (R&D Systems, Minneapolis, Minn., USA).

In our assay, quantification thresholds were defined by the manufacturer as 5.9 pg/ml (IL-4), 2.9 pg/ml (IL-5), 1.1 pg/ml (IL-10), 1.1 pg/ml (IL-13), 2.1 pg/ml (IL-17), 1.8 pg/ml (IFN- γ), 3.0 pg/ml (TNF- α), 5.8 pg/ml (eotaxin), and 12 pg/ml (TGF- β).

Cytokines and cells were measured in BAL fluids 24 and 48 h after the last aerosol challenge for all the mice. After AHR measurement, the mice were anaesthetized, the trachea was cannulated, and the BAL fluids were collected in sterile saline (1.4 ml) and kept in ice until of cellular influx was determined. Total cell counts were performed on Malassez cells.

Differential cell counts were performed after cytocentrifugation and staining with Diff-Quick (Baxter Dade AG, Düringen, Switzerland).

Statistical Analysis

GraphPad Prism Software 5.0 (San Diego, Calif., USA) was used for statistical analysis ($n = 10$ –20 mice per group). Results are expressed as means $\pm$ standard deviations (SD). Antibody responses as well as cell and cytokine data were analyzed using analysis of variance (ANOVA) and Tukey's test for intergroup comparisons. The raw data of the Penh values were analyzed using the nonparametric Mann-Whitney U test. Penh and resistance data were also analyzed using the complete methacholine dose-response curve. For each mouse, Penh or resistance was plotted against methacholine concentration (from 0 to 40 or from 0 to 10 mg/ml) and the AUC was calculated. Then, the data were analyzed using ANOVA and Dunnett's test when comparing treated mice with the controls, and using ANOVA and Tukey's test when comparing all the groups with each other.

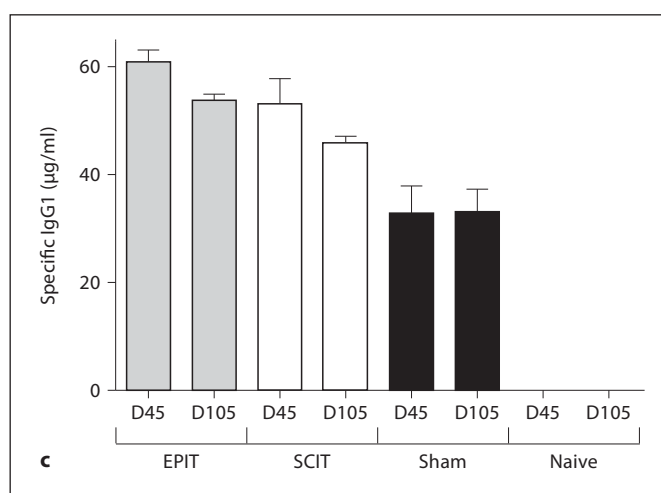
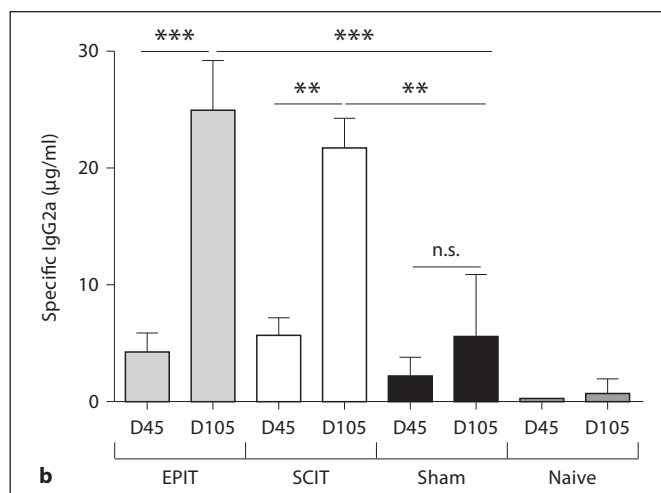
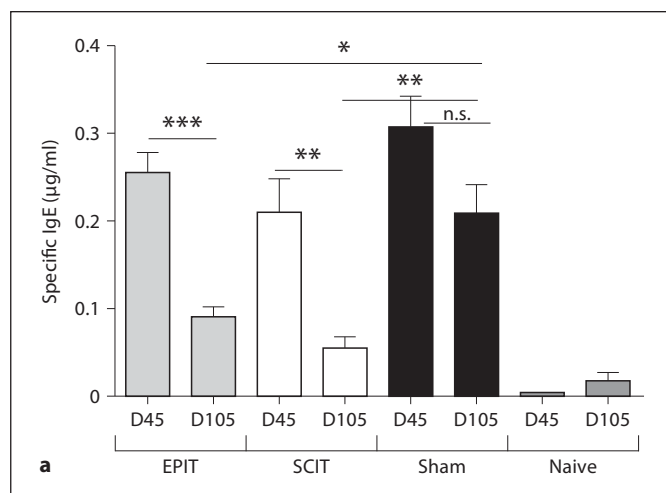


Fig. 3. Quantity of sIgE (a), sIgG2a (b), and sIgG1 (c). D45 = Values obtained at the end of the sensitization phase (before immunotherapy); D105 = values obtained at the end of the immunotherapy. The groups were EPIT (epicutaneously treated mice), SCIT (subcutaneously treated mice), sham (sham-treated mice), and naive (naive mice). Data are presented as means $\pm$ SEM for each group of 20 mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Results

Serological Response to Sensitization and Immunotherapy

Specific sensitization against the peanut protein was confirmed on day 45 by increased sIgE in the sera of EPIT, SCIT, and sham mice as compared with naive mice (fig. 3). Sensitization was not significantly different between groups (EPIT, SCIT, and sham). Ten days after the end of the treatment (D105), the EPIT and SCIT groups had decreased sIgE ($p < 0.001$) and increased sIgG2a ($p < 0.001$ and $p < 0.01$ vs. the sham group, respectively). The treatment did not modify sIgG1, but dramatically decreased the sIgG1/sIgG2a ratio ($p < 0.001$ and $p < 0.01$ vs. the sham group, respectively) (data not shown).

General Reactivity: Histamine Release after Oral Challenge

The levels of histamine released in plasma sampled 30 min after the oral challenge were high in sham mice (142 ± 34 nM) and very low in naive mice (1.5 ± 0.2 nM). They were significantly reduced in the EPIT and SCIT groups ($p < 0.05$ vs. the sham group) (fig. 4).

Airway Hyperresponsiveness Plethysmography

Dose-response curves for methacholine were significantly different between the treated (EPIT, SCIT) and sham groups at methacholine dosages from 10 to 40 mg/ml. At the higher methacholine concentration (i.e. 40 mg/ml), the sham mice responded to the peanut challenge with a marked AHR (fig. 5a). Compared to the sham

Fig. 4. Effect of immunotherapy (EPIT and SCIT) on plasma histamine levels. Blood was collected and plasma was obtained 30 min after the challenge. Histamine levels were measured using an enzyme immunoassay kit. Data are presented as means $\pm$ SEM for each group of 10 mice. * $p < 0.05$.

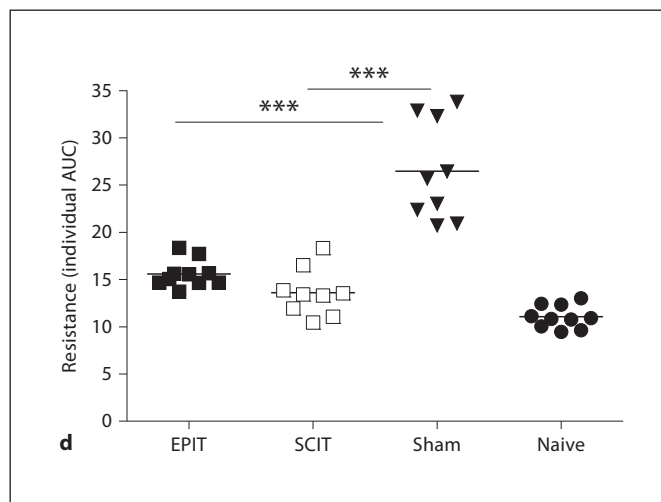
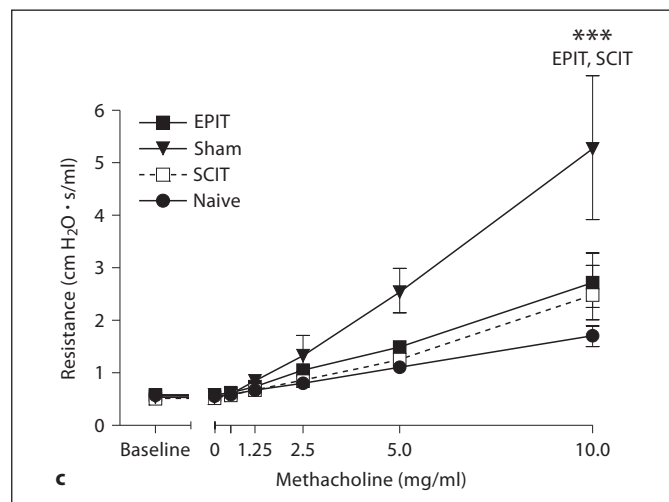
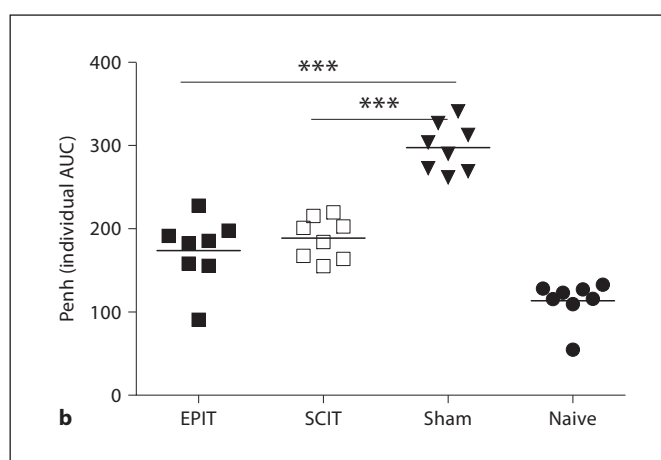
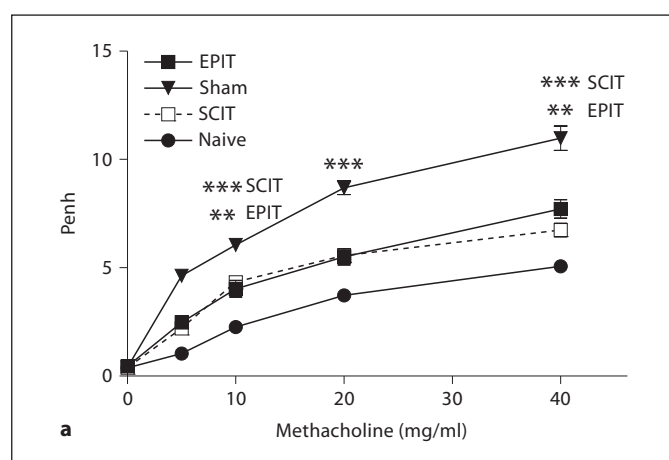
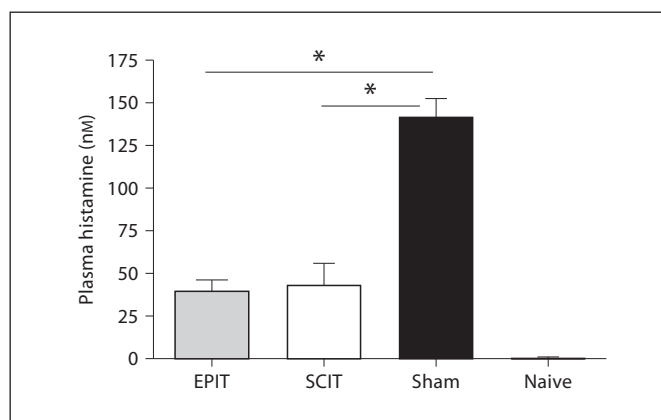


Fig. 5. AHR measured by whole-body plethysmography 24 h after the last aerosol challenge to peanuts. Dose-response curves obtained for each group (a) and individual AUC calculated from these data (b). Penh values were recorded after increasing doses of methacholine (from 0 to 40 mg/ml). EPIT group: $n = 8$, SCIT group: $n = 8$, sham group: $n = 8$, naive group: $n = 8$. ** $p < 0.01$

and *** $p < 0.001$. AHR measured by dynamic resistance 24 h after the aerosol challenge to peanuts. Dose-response curves obtained for each group (c) and individual AUC calculated from these data (d). EPIT group: $n = 10$, SCIT group: $n = 9$, sham group: $n = 9$, naive group: $n = 10$. *** $p < 0.001$.

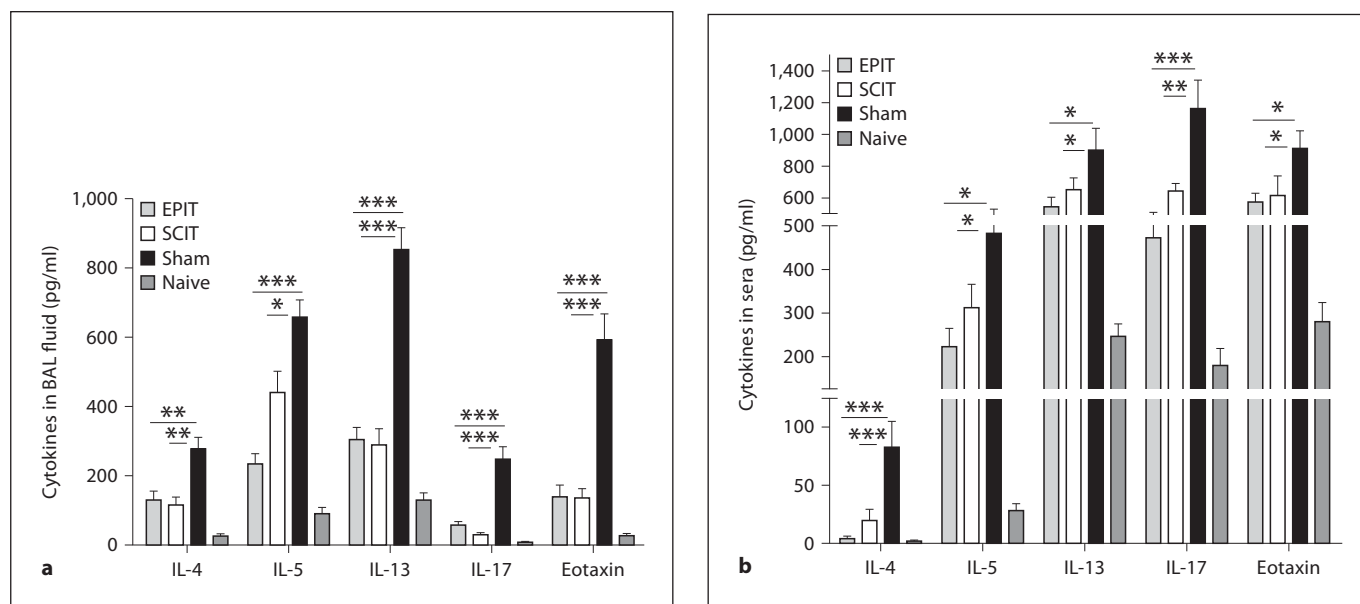


Fig. 6. Inflammatory cytokine levels in the BAL fluid (**a**) and serum (**b**) of mice challenged to peanuts via aerosol. The groups were EPIT, SCIT, sham, and naive. Data are presented as means $\pm$ SEM for each group of 10 mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

group, the EPIT and SCIT groups showed significantly decreased Penh values ($p < 0.01$ and $p < 0.001$, respectively), which did not differ significantly from those of the naive group. The AUC values for Penh were also significantly lower with EPIT and SCIT than with sham ($p < 0.001$), and did not differ from those of naive mice (fig. 5b).

Resistance-Compliance

For SCIT and sham 9 dots were shown and for EPIT and naive 10 dots. In the resistance-compliance analysis, we encountered difficulties intubating 1 SCIT mouse and 1 sham mouse, resulting in an experimental failure. Their baseline values actually happened to largely differ from those of mice in the same group. We thus decided not to include them in the resistance-compliance results.

At the higher methacholine concentration (i.e. 10 mg/ml), bronchial resistance was dramatically increased in the sham group as compared to the control ($p < 0.001$), EPIT ($p < 0.001$), and SCIT ($p < 0.001$) groups (fig. 5c). The AUC for resistance was significantly lower in the EPIT and SCIT groups than in the sham group, and did not differ from that in the naive group (fig. 5d).

Cytokine Levels and Cell Composition in Blood and BAL Fluid

The results for IL-4, IL-5, IL-13, IL-17, and eotaxin are presented in figure 6a (BAL fluid) and b (serum) taken 24 h after the last aerosol challenge. Similar trends were obtained at 24 and 48 h. The higher levels were seen at 24 h for cytokines, with the maximal significance between groups at 24 h.

The BAL fluid and serum of the sham mice showed high levels of eotaxin, IL-4, IL-5, IL-13, and IL-17. EPIT and SCIT induced a significant decrease in eotaxin, IL-4, IL-5, IL-13, and IL-17 in both the BAL fluid and the serum.

IFN- γ , IL-10, and TNF- α levels in BAL fluid were very low for the 4 groups. EPIT and SCIT did not modify the serum levels of IFN- γ and IL-10 (data not shown) but significantly decreased the TNF- α levels in serum (380.2 ± 84.16 pg/ml, $p < 0.01$ and 219.9 ± 21.9 pg/ml, $p < 0.05$, respectively), as compared to sham (881.9 ± 153.6 pg/ml). TGF- β was measurable in BAL and in serum but without any significant difference between the treated (EPIT and SCIT) and sham groups. The values were: 270.0 ± 10.5 , 292.7 ± 19.2 , and 260 ± 17.3 pg/ml in BAL, respectively, and 147.2 ± 13.1 , 198.8 ± 27.2 , and 133.8 ± 17.5 pg/ml in serum, respectively. The TGF- β

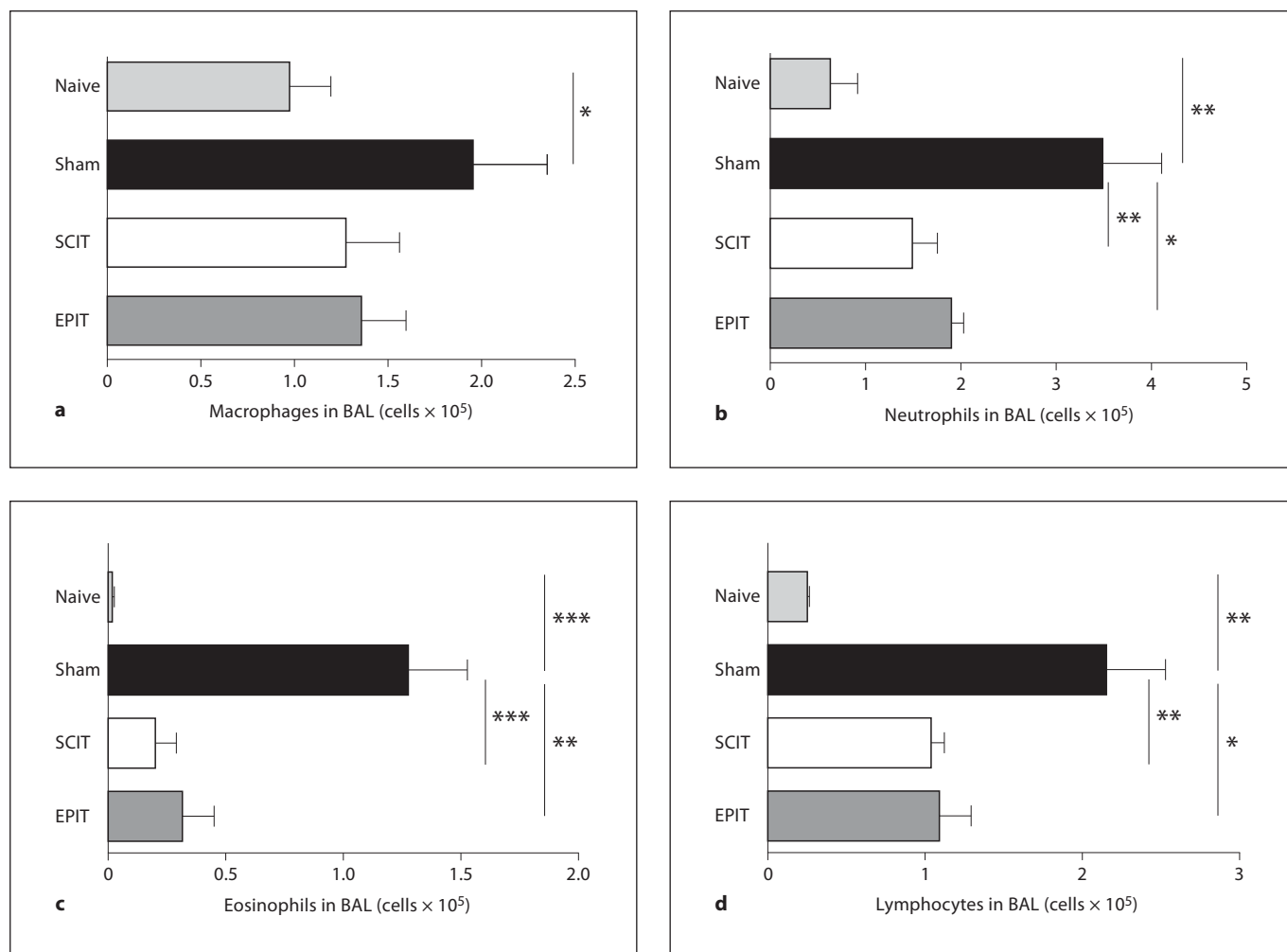


Fig. 7. Number of inflammatory cells in BAL fluid 48 h after the last aerosol challenge to peanuts: macrophages (a), neutrophils (b), eosinophils (c), and lymphocytes (d). The groups were EPIT, SCIT, sham, and naive. Data are presented as means $\pm$ SEM for each group of 10 mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Table 1. Total cell count and percentage of the BAL cell composition after the aerosol challenge

Groups	Total cell count, /ml	Macro-phages, %	Neutro-phils, %	Eosino-phils, %	Lympho-cytes, %
Naive	188,515	52	34	1	14
Sham	870,086	23	40	13	25
EPIT	466,000	29	41	7	23
SCIT	400,222	32	37	5	26

Results are expressed as means.

values measured in the BAL fluid and serum of the treated groups were not different from those of naive mice (129.2 ± 10.2 and 136.0 ± 13.1 pg/ml, respectively).

Forty-eight hours after the last challenge, sensitized mice showed eosinophilia with a concomitant increase in neutrophils, lymphocytes, and macrophages (fig. 7; table 1). In agreement with the decreased eotaxin levels in the BAL fluid, EPIT and SCIT decreased the number of eosinophils significantly. EPIT and SCIT also decreased the recruitment of neutrophils and lymphocytes but did not decrease BAL macrophages. Similar trends were obtained at 24 h but the higher levels and the maximal significant results between groups were seen at 48 h.

Discussion

SCIT, the reference method in desensitization [4], could not be applied for food allergies due to the high risk of anaphylactic reactions. An alternative route, such as EPIT, should be considered. The development of EPIT needs reliable safety and efficacy data, especially in food allergy. For obvious ethical reasons, animal models are first needed to evaluate its potential efficacy. Here, we used a model of mice orally sensitized to peanuts and explored a new immunotherapy method consisting of the use of an original EDS for repeated and prolonged applications of the allergen to nonstripped skin. We observed a shift in serum antibody isotypes and a reduction in AHR and lung eosinophilia.

The protocol of sensitization, already validated by Adel-Patient et al. [18], was based on the weekly oral administration of PPE combined with cholera toxin to 3-week-old BALB/c mice during 6 weeks. As observed in the current study, this protocol allows optimal sIgE induction [19]. In opposition to previous experiments where skin had been previously stripped [20–22], EDS was here applied to intact skin. The preparation of the skin was such as to avoid any alteration of its barrier properties.

EPIT induced a major decrease in sIgE together with an increase in sIgG2a; sIgG1 remained unchanged. SCIT achieved similar results. In a comparable study with ovalbumin-sensitized mice, SCIT administered twice a week for 8 weeks increased the serum levels of ovalbumin-specific IgE, IgG1, and IgG2a until the 5th week of treatment [23]. Then, sIgE levels decreased sharply, while sIgG2a and sIgG1 continued to increase for 2 additional weeks before starting to decrease. Thus, the antibody pattern of the present study is very close to the immunological changes observed after 8 weeks of SCIT in this OVA experiment, further confirming that the epicutaneous route induces the same level of immunological changes as the subcutaneous one.

Poulsen et al. [24] demonstrated very early on that intestinal anaphylaxis after an oral challenge is associated with the release of histamine and other mediators leading to acute systemic symptoms. We thus performed a histamine provocation test, as already described by Li et al. [25–27]. The absence of a significant increase in histamine in the treated group after the oral challenge with PPE confirms the ability of EPIT to prevent mastocyte degranulation.

To date, the *in vivo* measurement of respiratory function in mice is based on both noninvasive and invasive approaches. Whole-body plethysmography is noninva-

sive and has been used in various mouse models of allergy [13, 28]. It allows the recording of the pressure fluctuations that occur during the breathing cycle of mice and measures a single parameter, *i.e.* Penh. However, Penh may be strongly influenced by events unrelated to lung mechanics such as hyperoxia, the timing of ventilation and humidification, and the warming of inspired gas [29]. The compliance-resistance measurement technique is invasive but offers the key advantage of combining orotracheal intubation via direct laryngoscopy and the local administration of allergenic extracts directly into the lung. Bronchoconstriction is assessed by the 'gold standard' parameters, *i.e.* airway resistance and dynamic compliance, in response to aerosolized methacholine or allergens in anesthetized mice [30]. This method could also be performed using increasing doses of methacholine. It has been validated in several groups of BALB/c mice and is considered reproducible without causing an alteration in the BAL fluid [31].

Actually, a combination of invasive and noninvasive techniques is most often required to fully understand the physiological significance of a respiratory phenotype [31]. This option was retained in the present study. Treatment dramatically improved the respiratory condition of the sensitized animals and the results appeared to be highly concordant with all techniques.

In line with these results, EPIT and SCIT decreased the IgG1/IgG2a ratio, serum eotaxins, and Th2-related cytokines (IL-4, IL-5, and IL-13). Overall, these changes reflect the switch of the immune response from a Th2 profile to a balanced Th2/Th1 profile. The switch to a Th1 profile accompanying an increased tolerance to peanuts has also been established in humans. Indeed, when comparing the cytokine-producing phenotypes of peanut-specific lymphocytes from children who had outgrown peanut allergy and from children who had always tolerated peanuts, Turcanu et al. [32] showed that peanut antigens induced a Th2-biased response in children with peanut allergy while nonallergic ones exhibited a Th1-skewed response. Moreover, the resolution of the peanut allergy was accompanied by a shift to the Th1 cytokine profile [32].

Another finding is the dramatic decrease in cell recruitment, in particular eosinophils, and the decrease in eotaxin, IL-5, and IL-13 in the lungs of treated mice. Eotaxin induces a rapid recruitment of eosinophils in allergen-stimulated tissues [33]. Actually, treatment seems to influence all the steps of eosinophilic recruitment, from the IL-5-induced release of eosinophils from the bone marrow [34] to the IL-13-favored eosinophil infiltration

of the lung, which is responsible for the overexpression of eotaxin by lung epithelial cells in allergic mice after a challenge [35].

As suggested in studies by Strid et al. [20–22], this process is obviously the first step of an immune reaction. Further studies are needed to determine which exact processes are set up to finally influence the induction of an immune tolerance profile.

Recently, Senti et al. [36] described the epicutaneous administration of specific grass pollen allergens in patients with seasonal rhinitis in a pilot study suggesting that immunotherapy could occur successfully via the skin route with repeated and prolonged applications of the allergen to the skin. A pilot study was also conducted in children with IgE-mediated cow's milk allergy using an EDS quite similar to that used in the present mouse study [37]. The method used in that clinical study does not need any skin preparation, in contrast to the procedure described by Senti et al. [36] and the epicuta-

neous treatment applied in the milk EPIT pilot trial in children [37], further supporting the good tolerance of the EDS.

In conclusion, these data suggest that the epicutaneous route might be as potent as the subcutaneous route during immunotherapy in an animal model. The immune changes, especially at the skin level, need further investigation. These data also suggest that this new approach for immunotherapy could be of value in clinical situations where injection or oral consumption might put the patient at risk.

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References

- 1 Eigenmann PA: Future therapeutic options in food allergy. *Allergy* 2003;58:1217–1223.
- 2 Sicherer SH, Sampson HA: Peanut allergy: emerging concepts and approaches for an apparent epidemic. *J Allergy Clin Immunol* 2007;120:491–503.
- 3 Du Toit G, Katz Y, Sasieni P, Mesher D, Maleki SJ, Fisher HR, Fox AT, Turcanu V, Amir T, Zadik-Mnuhin G, Cohen A, Livne I, Lack G: Early consumption of peanuts in infancy is associated with a low prevalence of peanut allergy. *J Allergy Clin Immunol* 2008;122:984–991.
- 4 Greenberger PA, Ballou M, Casale TB, Platts-Mills TA, Sampson HA: Sublingual immunotherapy and subcutaneous immunotherapy: issues in the United States. *J Allergy Clin Immunol* 2007;120:1466–1468.
- 5 Bousquet J: Sublingual immunotherapy: validated. *Allergy* 2006;61:5–6.
- 6 Valenta R: The future of antigen-specific immunotherapy of allergy. *Nat Rev Immunol* 2002;2:446–453.
- 7 de Boissieu D, Dupont C: Sublingual immunotherapy for cow's milk protein allergy: a preliminary report. *Allergy* 2006;61:1238–1239.
- 8 Longo G, Barbi B, Berti I, Meneghetti R, Pittalis A, Ronfani L, Ventura A: Specific oral tolerance induction in children with very severe cow's milk-induced reactions. *J Allergy Clin Immunol* 2008;121:343–347.
- 9 Mondoulet L, Dioszeghy V, Ligouis M, Dhelt V, Dupont C, Benhamou PH: Epicutaneous immunotherapy on intact skin using a new delivery system in a murine model of allergy. *Clin Exp Allergy* 2010;40:659–667.
- 10 Sampson HA, Mendelson L, Rosen JP: Fatal and near-fatal anaphylactic reactions to food in children and adolescents. *N Engl J Med* 1992;327:380–384.
- 11 Bock SA, Munoz-Furlong A, Sampson HA: Fatalities due to anaphylactic reactions to foods. *J Allergy Clin Immunol* 2001;107:191–193.
- 12 Kalach N, Soulaïnes P, de Boissieu D, Dupont C: A pilot study of the usefulness and safety of a ready-to-use atopy patch test (Dialler-test) versus a comparator (Finn Chamber) during cow's milk allergy in children. *J Allergy Clin Immunol* 2005;116:1321–1326.
- 13 Lagranderie M, Abolhassani M, Vanoirbeek J, Lefort J, Nahori MA, Lapé E, Silva JR, Huerre M, Vargaftig B, Marchal G: *Mycobacterium bovis* BCG killed by extended freeze-drying reduces airway hyperresponsiveness in 2 animal models. *J Allergy Clin Immunol* 2008;121:471–478.
- 14 Li XM, Serebrisky D, Lee SY, Huang CK, Bardina L, Schofield BH, Stanley JS, Burks AW, Bannon GA, Sampson HA: A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. *J Allergy Clin Immunol* 2000;106:150–158.
- 15 Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG, Gelfand EW: Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am J Respir Crit Care Med* 1997;156:766–775.
- 16 Vanoirbeek JA, Vanhooren HM, Nawrot TS, Nemery B, Hoet PH: How long do the systemic and ventilatory responses to toluene diisocyanate persist in dermally sensitized mice? *J Allergy Clin Immunol* 2008;121:456–463.
- 17 Vanoirbeek JA, Rinaldi M, De Vooght V, Haenen S, Bobic S, Gayan-Ramirez G, Hoet PH, Verbeke E, Decramer M, Nemery B, Janssens W: Noninvasive and invasive pulmonary function in mouse models of obstructive and restrictive respiratory diseases. *Am J Respir Cell Mol Biol* 2010;42:96–104.
- 18 Adel-Patient K, Bernard H, Ah-Leung S, Creminon C, Wal JM: Peanut- and cow's milk-specific IgE, Th2 cells and local anaphylactic reaction are induced in Balb/c mice orally sensitized with cholera toxin. *Allergy* 2005;60:658–664.
- 19 von der Weid T, Bulliard C, Fritzsche R: Suppression of specific and bystander IgE responses in a mouse model of oral sensitization to beta-lactoglobulin. *Int Arch Allergy Immunol* 2001;125:307–315.

- 20 Strid J, Callard R, Strobel S: Epicutaneous immunization converts subsequent and established antigen-specific T helper type 1 (Th1) to Th2-type responses. *Immunology* 2006;119:27–35.
- 21 Strid J, Hourihane J, Kimber I, Callard R, Strobel S: Epicutaneous exposure to peanut protein prevents oral tolerance and enhances allergic sensitization. *Clin Exp Allergy* 2005; 35:757–766.
- 22 Strid J, Thomson M, Hourihane J, Kimber I, Strobel S: A novel model of sensitization and oral tolerance to peanut protein. *Immunology* 2004;113:293–303.
- 23 Van Oosterhout AJ, Van Esch B, Hofman G, Hofstra CL, Van Ark I, Nijkamp FP, Kapsenberg ML, Savelkoul HF, Weller FR: Allergen immunotherapy inhibits airway eosinophilia and hyperresponsiveness associated with decreased IL-4 production by lymphocytes in a murine model of allergic asthma. *Am J Respir Cell Mol Biol* 1998;19:622–628.
- 24 Poulsen OM, Nielsen BR, Basse A, Hau J: Comparison of intestinal anaphylactic reactions in sensitized mice challenged with untreated bovine milk and homogenized bovine milk. *Allergy* 1990;45:321–326.
- 25 Li XM, Serebrisky D, Lee SY, Huang CK, Bardina L, Schofield BH, Stanley JS, Burks AW, Bannon GA, Sampson HA: A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. *J Allergy Clin Immunol* 2000;106:150–158.
- 26 Li XM, Kleiner G, Huang CK, Lee SY, Schofield B, Soter NA, Sampson HA: Murine model of atopic dermatitis associated with food hypersensitivity. *J Allergy Clin Immunol* 2001;107:693–702.
- 27 Li XM, Schofield BH, Huang CK, Kleiner GI, Sampson HA: A murine model of IgE-mediated cow's milk hypersensitivity. *J Allergy Clin Immunol* 1999;103:206–214.
- 28 Spergel J, Mizoguchi E, Brewer JP, Martin TR, Bahn AK, Geha RS: Epicutaneous sensitization with protein antigen induces localized allergic dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice. *J Clin Invest* 1998;101:1614–1622.
- 29 Lundblad LK, Irvin CG, Adler A, Bates JH: A reevaluation of the validity of unrestrained plethysmography in mice. *J Appl Physiol* 2002;93:1198–1207.
- 30 Hoymann HG: New developments in lung function measurements in rodents. *Exp Toxicol Pathol* 2006;57(suppl 2):5–11.
- 31 Glaab T, Mitzner W, Braun A, Ernst H, Korolewitz R, Hohlfeld JM, Krug N, Hoymann HG: Repetitive measurements of pulmonary mechanics to inhaled cholinergic challenge in spontaneously breathing mice. *J Appl Physiol* 2004;97:1104–1111.
- 32 Turcanu V, Maleki SJ, Lack G: Characterization of lymphocyte responses to peanuts in normal children, peanut-allergic children, and allergic children who acquired tolerance to peanuts. *J Clin Invest* 2003;111:1065–1072.
- 33 Fulkerson PC, Rothenberg ME: Origin, regulation and physiological function of intestinal eosinophils. *Best Pract Res Clin Gastroenterol* 2008;22:411–423.
- 34 Collins PD, Marleau S, Griffiths-Johnson DA, Jose PJ, Williams TJ: Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. *J Exp Med* 1995;82:1169–1174.
- 35 Wang Y, McCusker CT: Interleukin-13-dependent bronchial hyper-responsiveness following isolated upper-airway allergen challenge in a murine model of allergic rhinitis and asthma. *Clin Exp Allergy* 2005;35:1104–1111.
- 36 Senti G, Graf N, Haug S, Ruedi N, von Moos S, Sonderegger T, Johansen P, Kundig TM: Epicutaneous allergen administration as a novel method of allergen-specific immunotherapy. *J Allergy Clin Immunol* 2009;124: 997–1002.
- 37 Dupont C, Kalach N, Soulaïnes P, Legoué-Morillon S, Benhamou PH: Cow's milk epicutaneous immunotherapy in children: a pilot trial of safety, acceptability and impact on allergic reactivity. *J Allergy Clin Immunol* 2010;125:1165–1167.