Specific epicutaneous immunotherapy prevents sensitization to new allergens in a murine model

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Background: Allergy to cow’s milk increases the risk of sensitization to other foods in young children. Objectives: We sought to evaluate the effect of early epicutaneous immunotherapy (EPIT) on further sensitization to peanut or house dust mite (HDM) in a murine model of sensitization to cow’s milk.

Methods: BALB/c mice orally sensitized to milk were epicutaneously treated with a Viaskin patch (DBV Technologies) loaded with milk proteins for 6 weeks. Mice were then sensitized to peanut or HDM. After sensitization to peanut, mice were exposed to a peanut regimen known to induce eosinophilic esophageal inflammation. After sensitization to HDM, mice were challenged with aerosols to HDM, and airway hyperresponsiveness was evaluated by using plethysmography. Humoral response was also analyzed. The role of regulatory T (Treg) cells was evaluated by adoptively transferring Treg cells from milk EPIT–treated mice to naive mice before sensitization to peanut. Protection against anaphylaxis was also investigated. Methylation of the promoter region of transcription factors was analyzed by using PCR assays.

Results: In milk-sensitized mice specific EPIT prevented further sensitization to peanut or HDM. EPIT significantly modified the humoral response, reduced TH2 cytokine levels, decreased eosinophilic esophageal infiltration, and suppressed airway hyperresponsiveness. The protective effect was sustained over 2 months. Moreover, the adoptive transfer of milk EPIT Treg cells completely prevented sensitization to peanut and peanut-induced anaphylaxis. Milk EPIT enhanced methylation of the GATA-3 promoter region.

Conclusions: Our results showed that EPIT influences the natural history of allergy and reduces the risk of further sensitization through a Treg cell–dependent mechanism.

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Key words: Allergy, treatment, food allergy, epicutaneous immunotherapy, peanut, milk, prevention

Cow’s milk allergy (CMA) affects 2% to 3% of infants and is one of the most frequent food allergies in childhood. 1,2 CMA is usually transient, and most children tolerate cow’s milk at approximately 5 years of age or later. 3 However, children allergic to milk in the first years of life have a higher risk of allergic airways diseases and sensitization to other food products. 4-7 As a consequence, an early intervention able to reduce this “allergic march” or “allergic cosensitization,” which is also referred to as “collateral priming,” has become an aim in these children. 8-10

Such an early intervention can be obtained by using allergen-specific immunotherapy. For instance, subcutaneous immunotherapy and sublingual immunotherapy initiated at the age of 6 years in patients with respiratory allergy displays a preventive effect on both the occurrence of new sensitizations 11-15 and asthma onset. 14,16,17 Epicutaneous immunotherapy (EPIT) is an allergen-specific immunotherapy that represents a safe and promising treatment for food and respiratory allergies. It has been shown to be efficient in children with CMA 18 and in adults with grass pollen–induced rhinoconjunctivitis. 19,20

In a previous article we showed that EPIT is as efficient as subcutaneous immunotherapy in a murine model of allergic sensitization (pollen, ovalbumin [OVA], house dust mite [HDM], or peanut). 21-24 Although increasing the quantity of regulatory T (Treg) cells, EPIT decreased the systemic allergen-specific immune responses. 25

To date, no evidence had been presented in allergic subjects regarding the long-term effect of EPIT on the development of additional allergies. The present data show that EPIT alters the allergic march and prevents the development of new sensitizations in cow’s milk– or HDM-sensitized mice. This effect is notably due to a reduction of the TH2 inflammatory pathway and decreased IL-4 production.

METHODS
Animals
Three-week-old female BALB/c mice (Charles River, Chatillon-sur-Chalaronne, France) were housed under standard animal husbandry conditions. All experiments were performed according to the European Community rules on animal care, with permission 92-305 from the French Veterinary Services and approval of the Ethical Committee number 26 (authorization 2012-091).

Sensitization
Mouse sensitization was performed according to previously described procedures. 21,22,26,27 In brief, milk-sensitized mice were orally sensitized to milk proteins for 6 consecutive weeks by means of weekly gavage with 5 mg of cow’s milk protein (CMP) from commercial whole cow’s milk.
sensitized to milk and bled on day 42 to assay milk-specific IgE (sIgE), IgG1, and T-bet (T-box transcription factor) expression. Experimental schemes

**Milk EPIT prevention of peanut sensitization.** Mice were sensitized to milk and bled on day 42 to assay milk-specific IgE (sIgE), IgG1, and T-bet expression. After aerosol exposure, splenocytes from each group were cultured in the presence of peanut protein extract (PPE) and CD4+ Treg cells were sorted from the spleen cells of each group by using a magnetic bead Regulatory T Cell Isolation Kit (Miltenyi Biotec, Paris, France). Sensitization to aeroallergens (HDM or *P ileum proteus* pollen) was performed by using 2 subcutaneous injections of 100 μg of protein extract added with 1 mg of aluminum hydroxide, followed by an intranasal administration of 10 μg of protein at day 15. Each treatment group was composed of 10 mice.

**EPIT**

EPIT on intact skin was performed for 8 consecutive weeks, as already reported: 21 a Viaskin patch (DBV Technologies, Bagneux, France) was loaded with 100 μg of dedicated proteins (EPIT group) or buffer (sham) and applied for 48 hours on the intact skin of mice. Milk and HDM extracts were used for EPIT (Greer Laboratories, Lenoir, NC).

**Histologic evaluation in the digestive tract**

The esophagus was collected, fixed in formalin, and embedded for analysis, as already described. A skilled pathologist certified by the European College of Veterinary Pathologists counted eosinophils in a double-blind manner. Results were expressed as the number of eosinophils per square millimeter.

**AHR and BAL fluid**

After sensitization to peanuts or HDM, mice were submitted to a 30-minute challenge with the appropriate aerosolized allergen for 3 consecutive days. AHR was evaluated 24 hours after the last aerosol by means of whole-body plethysmography. 22 For each mouse, mean enhanced pause (Penh) values were plotted against methacholine concentrations, and the area under the curve was computed. 22

The day after plethysmography, BAL fluid was collected with sterile PBS, and eosinophil infiltration was analyzed. After completion of BAL, lungs were removed, fixed with 10% neutral formalin, and 5-μm lung sections were cut and stained with hematoxylin, eosin, and periodic-acid–Schiff. Histologic analysis was performed with a digital camera (Leica DFC420C, Nanterre, France) combined with image analysis software (Leica LAS Software).

**Specific IgE, IgG1, and IgG2a levels**

Specific IgE, IgG1, and IgG2a levels were quantified in plasma from blood samples obtained after the sensitization phase (milk or HDM), during the immunotherapy period, and after the second phase of sensitization (peanut, HDM, and pollen). Plasma were stored at −20°C until use. Specific IgE, IgG1, and IgG2a levels were determined by using ELISA. 22

**Cellular immune responses: Cytokine production and Treg cell staining**

After peanut oral exposure, splenocytes from each group were prepared, as previously described, 26 and cultured (2 × 10⁶/well/mL) in RPMI supplemented with 10% FCS, 100 U/mL penicillin/streptomycin, and 1% L-glutamine, with PPE (100 μg/mL) for 16 hours. Cytokine production was measured by ELISA on supernatants.

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with CMP, PPE, or HDM (at 100 μg/mL) for 72 hours. Cytokine levels were determined by using the Bio-Plex cytokine assay (Bio-Rad, Marnes-la-Coquette, France). The analysis of forkhead box protein 3 (Foxp3) expression was studied with an allophycocyanin-labeled anti-Foxp3 mAb using the Foxp3 staining kit (BD Bioscience, San Jose, Calif). This was performed after fixation/permeabilization allowing intracellular staining and after staining of splenocytes with phycoerythrin-labeled anti-CD4 and fluorescein isothiocyanate–labeled anti-CD25 mAbs.

**DNA methylation**

DNA was purified with the AllPrep DNA/RNA Mini Kit (Qiagen, Marseille, France). DNA methylation levels were analyzed by using the

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### FIG 1. Experimental design and sampling.

**A,** Milk EPIT prevention against PPE sensitization. Mice were sensitized to milk by means of oral gavage with CMP/CT. On day 43, sensitized mice were epicutaneously treated with a Viaskin patch loaded with milk protein extract (EPIT) or a placebo Viaskin patch (sham). On day 99, EPIT- or sham-treated mice were exposed to a protocol of sensitization by means of gavage with PPE/CT, followed on day 127 by sustained oral exposure to peanut for 10 days. Positive control animals were naive mice sensitized to PPE by using the same protocol. Negative control animals were naive mice without sensitization or immunotherapy protocols.

**B,** Milk EPIT prevention against HDM sensitization. Mice were sensitized to milk by means of oral gavage with CMP/CT and then epicutaneously treated with a Viaskin patch loaded with milk protein extract (EPIT) or a placebo Viaskin patch (sham). On day 99, EPIT- or sham-treated mice were exposed to a protocol of sensitization by means of subcutaneous injections of HDM/alum, followed on day 127 by 3 aerosol challenges to HDM. Positive control animals were naive mice sensitized with HDM by using the same protocol. Negative control animals were naive mice without sensitization or immunotherapy protocols.

**C,** HDM EPIT prevention against pollen sensitization. Mice were sensitized to HDM by means of subcutaneous injections with HDM. On day 28, sensitized mice were epicutaneously treated with a Viaskin patch loaded with HDM protein extract (EPIT) or a placebo Viaskin patch (sham). On day 90, EPIT- or sham-treated mice were exposed to a protocol of sensitization by means of subcutaneous injections with pollen/alum, followed on day 117 by 3 days of aerosol to pollen. Positive control animals were naive mice sensitized with pollen extract by using the same protocol. Negative control animals were naive mice without sensitization or immunotherapy protocols.
Epitect Methyl II PCR assay (SABiosciences, San Diego, Calif) based on the detection of remaining input DNA after cleavage with a methylation-sensitive and/or methylation-dependent restriction enzyme. After digestion, the remaining DNA in individual enzyme reaction was quantified by means of real-time PCR with primers flanking a promoter region of GATA-3 (NM_008091) and T-box transcription factor (T-bet; NM_019507).

Statistical analysis
Results are expressed as means ± SDs. Antibodies, cytokine responses, and Penh values were analyzed by using the Kruskal-Wallis test, followed by the Dunn post test to compare treated mice with control animals and for overall group comparisons. For histologic analyses and anaphylaxis outcomes, comparisons were performed with the Mann-Whitney test.

RESULTS
Specific effects of EPIT on the humoral response in mice sensitized to milk or HDM
Milk sensitization induced sIgE at day 43 (see Fig E1, A, in this article’s Online Repository at www.jacionline.org). After 8 weeks of EPIT, sIgE, sIgG1, and sIgG2a levels increased compared with those in the sham group (P < .01, P < .01, and P < .05, respectively; see Fig E1, A and B). One month after the end of active EPIT (day 127), sIgE levels returned to initial values (day 43), sIgG1 levels were less than those observed after sensitization (day 43), and sIgG2a levels were maintained at values greater than those observed at day 43 (P < .05).

Milk EPIT prevention of peanut sensitization
PPE sIgE levels did not increase in milk EPIT–treated mice after the peanut sensitization procedure from days 99 to 127 after milk EPIT (Fig 3, A). On the contrary, PPE sIgE levels increased in positive control (P < .05) and sham mice. sIgG2a levels increased in milk EPIT–treated mice (P < .05) but did not vary in sham or positive control mice (Fig 3, B). PPE sIgG1 levels increased in all sensitized groups (Fig 3, C). This increase was statistically significant in the EPIT group.

Levels of IL-4, IL-5, IL-13, IL-10, and IFN-γ secreted by splenocytes (Fig 3, D-H) were low in the milk EPIT–treated group, as opposed to the high levels found in the sham groups (from P < .05 to P < .001 vs the milk EPIT–treated group). These cytokines were not detected in negative control mice. Similar effects were observed in PPE-reactivated in vitro samples. Splenocytes from milk EPIT–treated mice secreted lower levels of cytokines than splenocytes from sham mice: IL-4 (P < .05), IL-5 (P < .05), IL-13 (P < .001), IL-10 (P < .01), and IFN-γ (P < .001). In the positive control group THR cytokines were secreted at levels similar to those in the sham group after PPE but not CMP stimulation.

In CMP-incubated splenocytes (Fig 3, I) the proportion of CD4+CD25+Foxp3+ regulatory T (Treg) cells increased only for milk EPIT–treated mice. This proportion was significantly greater than in the sham (P < .05), positive control (P < .05), and negative control (P < .01) groups. In PPE-stimulated splenocytes CD4+CD25+Foxp3+ Treg cell numbers did not differ between groups (milk EPIT, sham, positive control, and negative control groups).

In the model of peanut-induced esophageal inflammation (Fig 3, J), the mucosa was investigated after a 10-day exposure to peanut. Eosinophil infiltration was low in the milk EPIT–treated group, which was similar to that seen in the negative control group. This contrasts with the high levels found in the positive control and sham-treated groups (P < .05 vs the milk...
FIG 3. Serologic and cellular responses to peanut sensitization after milk EPIT. A-C, Sera were harvested before (day 99) and after (day 127) PPE sensitization of the positive control, sham, and EPIT groups, respectively, for the measurement of IgE, IgG2a, and IgG1 reactive to PPE. D-H, Measurement of secreted cytokines by reactivated splenocytes. I, The percentage of CD4+CD25+Foxp3+ cells was evaluated among reactivated splenocytes. J, Eosinophilic infiltration into the esophageal mucosa was determined in all groups after a 10-day period of exposure to peanuts and expressed as mean ± SD numbers of eosinophils per square millimeter. *P < .05, **P < .01, and ***P < .001.
EPIT group). In the 2 additional experiments conducted 1 and 2 months after the end of EPIT to evaluate the sustainability of protection (see Fig E2 in this article’s Online Repository at www.jacionline.org), eosinophilic infiltration was lower in the milk EPIT group than in the positive control (P < .01 and P < .05, respectively) or sham-treated (P < .05) groups.

Milk EPIT prevention of HDM sensitization

After sensitization to HDM, although HDM-specific IgE levels increased in all groups (Fig 4, A), sIgG2a levels increased only in milk EPIT-treated mice (0.40 μg/mL, P < .05; Fig 4, B). The variations of cytokines secreted by splenocytes (Fig 4, D-H) were similar to those observed after PPE sensitization in milk EPIT–treated mice. AHR measurements performed after HDM aerosol challenges and exposure to increasing doses of methacholine (Fig 4, J) showed that milk EPIT–treated mice had decreased Penh values (P < .05). These values were significantly less than those in positive control (P < .01) or sham (P < .05) mice but similar to those in negative control mice. Infiltration of eosinophils in BAL fluid was less in milk EPIT–treated mice than in sham or positive control mice (P < .01; Fig 4, K). Lung hematoxylin and eosin staining showed decreased cellular infiltration around the airways in the EPIT group compared with that seen in the positive control and sham groups but similar to that seen in the negative control group (Fig 4, L-O).

HDM EPIT prevention of pollen sensitization

Sensitization to pollen in HDM EPIT–treated mice was associated with a humoral response pattern that was identical to the humoral response pattern obtained after sensitization to HDM in milk EPIT–treated mice (see Fig E3 in this article’s Online Repository at www.jacionline.org). The profiles of the cytokines secreted by reactivated splenocytes were also similar (data not shown). No analysis of Treg cells was performed. During AHR, milk EPIT–treated mice had low Penh values (see Fig E3) that were slightly higher than those in negative control mice and significantly less than those in sham-treated or positive control mice (P < .05).

Milk EPIT–induced Treg cells prevented sensitization to peanut

In contrast with mice that received cells from sham mice or nontransferred mice (positive control mice), naive mice receiving cells from an EPIT group did not show increased sIgE levels after sensitization to peanut (P < .05, Table 1). Only the EPIT Treg groups showed increased sIgG2a levels compared with sham or positive control mice (P < .05). PPE sIgG1 levels were similar in all groups. After transfer of Treg cells from milk EPIT–treated mice, PPE reactivation of the splenocytes of recipient mice induced low IL-4, IL-5, and IL-13 levels. These levels were substantially lower than those observed after splenocyte reactivation after transfer of Treg cells from sham mice (sham Treg cells) or in the absence of transfer (positive control; P < .05, P < .01, and P < .001, respectively, vs milk EPIT–treated mice; see Fig E4 in this article’s Online Repository at www.jacionline.org). Whatever the treatment group, no cytokines were detected when splenocytes were reactivated with medium alone or with milk-enriched medium.

EPIT Treg cell transfer protected against infiltration of eosinophils in the esophageal mucosa (Fig 5, A) compared with that seen in the sham Treg or positive control groups (19 vs 58 or 57 eosinophils/mm², respectively; P < .05). No infiltration of eosinophils was observed in negative control mice or in the absence of sensitization to peanut.

After 3 days of aerosol challenge with PPE, AHR was significantly decreased in the EPIT Treg group compared with that seen in the sham Treg or positive control groups (306 vs 398 [P < .01] and 391 [P < .05], respectively). Higher values were also observed in the positive control (no transfer) and sham Treg groups than in naive mice (P < .01; Fig 5, B). This was in accordance with the significant reduction of eosinophilic infiltration in BAL fluid of the milk EPIT Treg group compared with the positive control or sham Treg groups (Fig 5, C).

Whatever the sensitization status before adoptive transfer (ie, naive or sensitized to milk), EPIT Treg cell transfer protected against the decrease of body temperature after intravenous challenge of PPE (Fig 5, D and F) compared with the sham Treg or positive control groups, in both of which anaphylaxis occurred under the same experimental conditions (P < .05 and P < .001, respectively, for naive recipients and P < .001 for milk-sensitized recipients). No modification in body temperature was observed in negative control mice. Consistently, plasma mMCP1 levels in the EPIT Treg group were significantly less than in the sham Treg or positive control groups (P < .001; Fig 5, E and G). No difference was found between the negative control and EPIT groups.

Milk EPIT induced methylation of the GATA-3 promoter

In PPE-sensitized milk EPIT–treated mice, spleen cells revealed marked alterations in the DNA methylation levels of the promoter regions of GATA-3. Splenocytes from milk EPIT–treated mice had significantly increased methylation in the GATA-3 promoter compared with that seen in sham mice (Fig 6, A). No modification of methylation was observed for the T-bet promoter (Fig 6, B).
These results were maintained after the second phase of sensitization to peanut, with increased methylation of the GATA-3 promoter for the milk EPIT–treated group (Fig 6, C) and no effect on the T-bet promoter (Fig 6, D).

**DISCUSSION**

EPIT is a technique designed for the treatment of an established food allergy. The results of the present studies performed in an animal model of sensitization indicate that EPIT might also play a role in the prevention of further sensitization to other allergens when applied early to a young allergic subject.

As recently underlined, several decades of intense clinical and basic research in allergy prevention led to little progress in identifying effective strategies to reduce the burden of allergic conditions. Despite some optimistic reports, most of the concepts and proposals have failed to be effective, underlining the need for completely novel ideas and approaches. In a recent study tolerance principles were applied to boost resistance to primary allergic sensitization to inhalant allergens, but the protocol of sublingual administration of a mixture of soluble aeroallergens still did not provide any evidence of efficiency for allergy prophylaxis. Identifying secondary prevention strategies to stop the atopic march remains a realistic means of investigation. The fact that food allergy and atopic dermatitis in infancy are progressively replaced by asthma and rhinoconjunctivitis indicates a potential effect of age on the progression of allergic diseases, the so-called allergic march. EPIT is the most recent immunotherapeutic approach to date. It is safe in both adults and children with nonsevere or severe peanut allergy. Such a safety profile opens the door to greater use of EPIT in the youngest children.

The design and allergy models used in the current studies were chosen in relation to the clinical events observed in young children, in whom allergy seems to exhibit successive phases of sensitization and the concepts and proposals have failed to be effective, underlining the need for completely novel ideas and approaches. In a recent study tolerance principles were applied to boost resistance to primary allergic sensitization to inhalant allergens, but the protocol of sublingual administration of a mixture of soluble aeroallergens still did not provide any evidence of efficiency for allergy prophylaxis. Identifying secondary prevention strategies to stop the atopic march remains a realistic means of investigation. The fact that food allergy and atopic dermatitis in infancy are progressively replaced by asthma and rhinoconjunctivitis indicates a potential effect of age on the progression of allergic diseases, the so-called allergic march. EPIT is the most recent immunotherapeutic approach to date. It is safe in both adults and children with nonsevere or severe peanut allergy. Such a safety profile opens the door to greater use of EPIT in the youngest children.

The design and allergy models used in the current studies were chosen in relation to the clinical events observed in young children, in whom allergy seems to exhibit successive phases of sensitization. In addition, because allergy to milk represents the first signal for allergic march enrollment and considering the lifespan of mice, young 3-week old animals were used. Our experiments are based on established models of mice sensitized to food allergens or aeroallergens, investigating disorders as close as possible to human diseases: aeroallergen-induced AHR, peanut-induced esophageal eosinophilia, and anaphylaxis. Even if AHR is better explored by using invasive methods, we decided to restrict the evaluation to whole-body plethysmography (Penh), a noninvasive method. This decision was made because we had already shown the complete equivalence between this approach and the invasive one in the same model of mice sensitized to peanut. In addition, the assessment of eosinophilia in BAL fluid was needed 48 hours after the last allergen aerosol challenge, and therefore no invasive technique could be previously performed.

In accordance with our previous results with various allergens (pollen, OVA, HDM, and peanut), the therapeutic effect of EPIT was highly specific and associated with a decrease in specific IgE levels, increase in specific IgG2a levels, lower production of both Th2 and Th1 cytokines, suppression of AHR, and decreased recruitment of local eosinophils. The discrepancy between low Th1 cytokine levels and high IgG2a secretion might be explained by the modification of class-switch recombination and thus by the type of secreting plasma cells. This is supported by the decrease we observed in part of the Th2 cytokines and of other regulatory signals modifying the gene molecular rearrangement in B cells (eg, secretion of IL-21).

EPIT to a first allergen (milk or HDM) resulted in a protective effect against the second sensitization. These results appeared very consistent and reproducible irrespective of the role and order attributed to the different allergens according to experiments. These data are in accordance with published studies on prevention of de novo sensitization to unrelated antigens by using other routes of immunotherapy. For example, immunization with hen’s egg lysozyme after induction of tolerance with OVA, an unrelated antigen, did not elicit a primary Th2 response to lysozyme.

In a model of mice simultaneously immunized to OVA through the intraperitoneal route and HDM through the intranasal route, a recent study reported the effectiveness of HDM-peptide intranasal immunotherapy in patients with OVA-induced allergic airways disease.

Three salient points emerge from the present results. They concern the timing of sensitizations, the role of Treg cells, and the sustainability of the preventive effect after the end of treatment.

First, the protective effect of EPIT was observed only when animals were first sensitized to 1 allergen (milk or HDM), treated with EPIT, and submitted to a second unrelated allergen. No effect was observed on the second sensitization in case of simultaneous immunization (unpublished data).

Second, the mechanistic process leading to prevention of further sensitization probably relies on Treg cells. These cells are critical for the therapeutic effect of EPIT, especially in its ability to reduce Th2-mediated inflammation and maintain immune tolerance.
suppression of eosinophilic airway inflammation in a murine model of intranasal tolerance induction. A higher number of milk-specific Treg cells in the PBMCs cultured with milk allergen ensures a milder clinical disease and a more favorable outcome. In our previous experiments, EPIT induced a significant increase in numbers of spleen CD4+CD25+Foxp3+ Treg cells. A previous experiment based on adoptive transfer of CD4+CD25+ T cells from the spleen, which comprise Treg cells,
further showed their capacity to block specific IgE responses and eosinophilia.\textsuperscript{25} The present study also suggests a central role for Treg cells in protection against further sensitizations: adoptive transfer of CD4\textsuperscript{+}CD25\textsuperscript{+} T cells from animals sensitized and treated with EPIT was as effective as EPIT itself in preventing further sensitizations. Treg cells act either directly or indirectly at the site of antigen presentation to create a regulatory milieu that promotes bystander suppression and infectious tolerance.\textsuperscript{44,45} In a recent work published by our group,\textsuperscript{25} it was clearly shown that transfer of CD4\textsuperscript{+}CD25\textsuperscript{+} T cells from EPIT-treated mice into Foxp3-IRES-mRFP reporter knock-in mice induced a significant increase in the proportion of CD4\textsuperscript{+}CD25\textsuperscript{+}mRFP\textsuperscript{+} cells in the spleen. Because only host Treg cells express mRFP, the increase in CD4\textsuperscript{+}CD25\textsuperscript{+}mRFP\textsuperscript{+} cells suggests that EPIT-induced Treg cells could promote de novo induction of Treg cells. Additional experiments will be needed to assess whether enhancement of the number of Treg cells, enhancement of their activity, or both is involved, such as suggested with specific immunotherapy.\textsuperscript{46}

The third point relates to the fact that the protective response induced by EPIT is sustained. IL-4 secretion by an allergen-specific T\textsubscript{H}2 population facilitates further T\textsubscript{H}2 sensitization to new allergen.\textsuperscript{47} By decreasing IL-4 production in sensitized animals, EPIT probably inhibits the long-term progression of the disease through the interruption of “collateral priming,”\textsuperscript{47-49} a process likely involved in the progression toward polysensitization. An epigenetic mechanism also seems involved. Indeed, EPIT increased the methylation of the GATA-3 promoter from whole spleen cells, which was in correlation with a decreased mRNA expression of GATA-3 in the same samples (unpublished data). This methylation status seems to be long lasting because it is sustained over at least 2 months (unpublished data), probably in relation to the large preventive action of EPIT against further sensitization. The binding sites of GATA-3 are found in the promoter region of IL-5 and IL-13, indicating a crucial role in the transcription of these interleukins.\textsuperscript{50} Inhibition of GATA-3 in T\textsubscript{H}2 responses implies an inhibition in the expression of IL-5 and IL-13 and results in the decrease in excessive T\textsubscript{H}2 lineage specification.\textsuperscript{51} We might hypothesize that EPIT-induced Treg cells, acting through cell contact mediation by cytotoxic T lymphocyte–associated antigen 4 expression,\textsuperscript{25} directly play a role on the methylation level of the GATA-3 gene. It was shown that cytotoxic T lymphocyte–associated antigen 4 modulates GATA-3 protein levels per cell and contributes to keeping this factor under the threshold required to initiate differentiation into a T\textsubscript{H}2 effector cell.\textsuperscript{52} Thus it affects IgE and IgG\textsubscript{2a} production and contributes to the outcome of allergen-specific immune responses.\textsuperscript{52} More specific analyses on epigenetic modifications are needed to precisely determine the different steps of immunomodulation.

The meaning of these data for a preventive action of EPIT in human subjects needs to be investigated. The development of early disorders, such as food allergies and atopic dermatitis, predicts the later development of the airway allergic diseases and the occurrence of new sensitizations.\textsuperscript{3,7} The long-term solution to the asthma epidemic is thought to be prevention and not treatment of the established disease.\textsuperscript{48} However, the exact timing of allergen sensitization remains controversial, with conflicting evidence for priming: it might involve the in utero period and more certainly the first years of life. In an attempt to elucidate the kinetics of sensitization in high-risk children during their first 2 years of life, Rowe et al\textsuperscript{53} showed that HDM induced T\textsubscript{H}2 responses in PBMCs from 6 months onward, particularly for IL-4 and IL-5, with a positive correlation with sensitization outcomes at 2 years. Interventions have to take place early. In that respect the time frame of our model is in good accordance with the allergic march, in which milk or HDM allergy precedes and often heralds the occurrence of other allergies to food allergens or aeroallergens.\textsuperscript{29,54}
In conclusion, we demonstrated in mice the efficiency of EPIT in inducing protection against further sensitization to food or inhaled allergens. The induced protection was clearly Treg cell dependent, thereby demonstrating a critical role of these cells in tolerance to new allergen sensitizations. Epigenetic modifications are also suggested. A potential use of EPIT to prevent development of new sensitizations in allergic children in the appropriate window of opportunity can be considered.

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Key messages
- Specific EPIT with Viaskin induced protection against sensitization to new food or respiratory allergens.
- Specific EPIT in children might influence the allergic march (ie, the natural course of allergy).

REFERENCES
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FIG E1. Humoral response to milk protein during EPIT in milk-sensitized mice. Sera were harvested after sensitization (day 43), after 4 (day 78) or 8 weeks (day 99) of EPIT, and 1 month after EPIT (day 127). A-C, Specific IgE response (Fig E1, A), specific IgG1 response (Fig E1, B), and specific IgG2a response (Fig E1, C) for the negative control, sham, and EPIT groups. Results are expressed as means ± SDs. *P < .05 and **P < .01.
FIG E2. Eosinophilic infiltration into the esophageal mucosa was determined in the negative control, positive control, sham, and EPIT groups after a 10-day exposure to peanuts. A, Evaluation of protection 1 month after the end of EPIT. B, Evaluation of protection 2 months after the end of EPIT. Results are expressed as mean ± SD numbers of eosinophils per square millimeter. *P < .05 and **P < .01.
FIG E3. Serologic and cellular responses to pollen sensitization after HDM EPIT. A-C, Sera were harvested before (day 99) and after (day 127) pollen sensitization of the positive control, sham, and EPIT groups, respectively, for the measurement of IgE, IgG2a, and IgG1 reactive to PPE. D, AHR was investigated in the negative control, positive control, sham, and EPIT groups after 3 days of aerosol challenges to pollen and exposure to increasing doses of methacholine. Values are expressed as the mean ± SD area under the curve (AUC) with individual mice. *P < .05, **P < .01, and ***P < .001.
FIG E4. Cytokine (IL-4, IL-5, and IL-13) secretions in the negative control, positive control, sham Treg, and EPIT Treg groups after 3 days of in vitro stimulation with milk or PPE. Values are expressed as means ± SDs. *P < .05.