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ORIGINAL ARTICLE

Allergen-Specific Immunotherapy and Biologics

Epicutaneous immunotherapy protects cashew-sensitized mice from anaphylaxis

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Abstract

Background: The prevalence of tree nut allergy has increased worldwide, and cashew has become one of the most common food allergens. More critically, cashew allergy is frequently associated with severe anaphylaxis. Despite the high medical need, no approved treatment is available and strict avoidance and preparedness for prompt treatment of allergic reactions are considered dual standard of care. In the meantime, Phase III study results suggest investigational epicutaneous immunotherapy (EPIT) may be a relevant and safe treatment for peanut allergy and may improve the quality of life for many peanut allergic children.

Objective: We aimed to evaluate the capacity of EPIT to provide protection against cashew-induced anaphylaxis in a relevant mouse model.

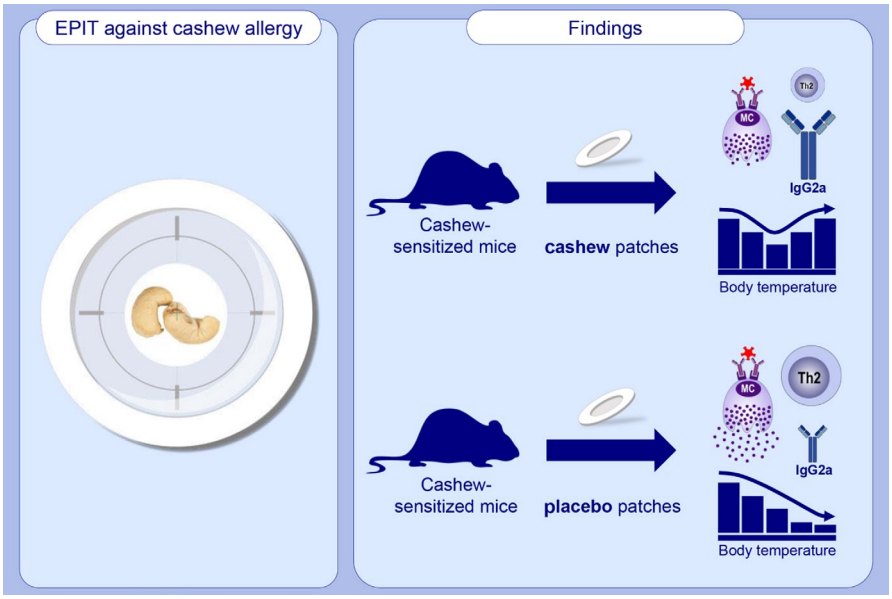
Methods: The efficacy of EPIT was evaluated by applying patches containing cashew allergens to cashew-sensitized mice. As negative control, sham mice received patches containing excipient. Following treatment, mice were challenged orally to cashew and anaphylactic symptoms, as well as plasmatic levels of mast-cell proteases (mMCP)-1/7, were quantified.

Results: Of 16 weeks of EPIT significantly protects against anaphylaxis by promoting a faster recovery of challenged mice. This protection was characterized by a significant reduction of temperature drop and clinical symptoms, 60 minutes after challenge. This was associated with a decrease in mast-cell reactivity as attested by the reduction of mMCP-1/7 in plasma, suggesting that EPIT specifically decrease IgE-mediated anaphylaxis.

Conclusion: We demonstrate that EPIT markedly reduced IgE-mediated allergic reactions in a mouse model of cashew allergy, which suggests that EPIT may be a relevant approach to treating cashew allergy.

Abbreviations: EPIT, epicutaneous immunotherapy.

KEYWORDS
anaphylaxis mouse model, cashew allergy, epicutaneous immunotherapy, Viaskin



GRAPHICAL ABSTRACT

This study demonstrates a newly developed robust mouse model of IgE-mediated cashew anaphylaxis. Cashew-sensitized mice are treated with epicutaneous patches containing cashew protein extract (cashew patches) or excipient (placebo patches) for 16 weeks. Mice treated with cashew patches present a decrease in cashew-specific Th2 response and a decrease in mast-cell reactivity and anaphylaxis symptoms following oral challenge.

Abbreviation: EPIT, epicutaneous immunotherapy.

1 | INTRODUCTION

The cashew plant (*Anacardium occidentale*) is a tropical evergreen tree belonging to the *Anacardiaceae* family, which includes mango and pistachio. It produces cashew seeds (nuts) that are regularly consumed by most of the world's population. Unfortunately, cashew is also classified as one of the most potent allergenic food, and no approved specific treatment is available to address this issue.[1] The prevalence of cashew allergy has risen over the last two decades in industrial countries with the increasing consumption of this nut.[2,3] Besides the prevalence, a high medical need for a treatment is warranted by the unique severity of anaphylactic reactions triggered by the consumption of cashew-containing food in allergic individuals.[4,5] Consequently, safe treatments minimizing contact between cashew allergens and anaphylaxis-triggering effector cells, such as mast cells, should be given paramount considerations. Investigational epicutaneous immunotherapy (EPIT) includes applying an epicutaneous system on intact skin (Viaskin).[6] This system comprised an allergen-adsorbed patch that promotes allergen delivery across the stratum corneum to epidermal Langerhans cells.[7] This route of administration is a key element of the safety profile of EPIT, making it a relevant approach for the treatment of life-threatening allergies such as cashew.[8] In that context, the aim of the present work was to assess the capacity of EPIT to protect against anaphylaxis in a mouse model of cashew allergy. To that end, a mouse model of IgE-mediated cashew anaphylaxis was first developed. Then, the ability of patches to deliver cashew allergens to skin dendritic cells was demonstrated. Finally, cashew-sensitized mice were treated with cashew patches (EPIT) for up to 16 weeks to measure the kinetic induction of specific antibodies and the level of protection afforded against anaphylaxis following oral challenge. Results showed that EPIT was able to significantly increase the level of cashew-specific IgG2a (mouse equivalent of human IgG1) all along the therapy period. More importantly, EPIT mice were significantly protected against anaphylactic symptoms following oral challenge. This protection was associated with a strong decrease in the activation of mast cells, which are the main immune effectors involved in IgE-mediated anaphylaxis.

2 | METHODS

2.1 | Animals and ethic

BALB/c mice were purchased from Charles River (Lyon, France) and housed under conventional conditions (DBV Technologies, Montrouge, France, agreement number #A92-049-02). Experiments have been performed according to the European Community rules of animal care, and with permission of the French government (authorization #13305).

2.2 | Extraction of cashew allergens for sensitization and challenge

Proteins were extracted from acetone-defatted cashew flour (Stallergenes Greer) by overnight stirring in PBS 1X, at 4°C. The solution was centrifuged for 30 minutes at 3000 *g* to eliminate insoluble components. Supernatant was frozen at −80°C and lyophilized (Lyofal). Nitrogen inertization was performed. Solutions for sensitization and oral challenge were obtained by adding the required volume of PBS 1X into vials. The final protein concentration was controlled using Bradford.

2.3 | Sensitization of mice

For oral sensitization, mice received 1 mg of cashew protein extract supplemented with 10 μg of Cholera Toxin (List Biological Laboratories) per intragastric route, at a rate of one intragastric administration per week for 6 consecutive weeks. For skin sensitization, mice received 100 μg of cashew protein extract on the skin of the lower back. A liquid solution was deposited on a gauze that was maintained on depilated skin with Tegaderm® (3M) for 72 hours. Before each application, skin was pretreated using a dermatological laser (Pantec Biosolutions) at a fluence of 5.5 Joules/cm^2 to mimic atopic skin. Mice received allergen to skin twice a week, every two weeks, for 7 weeks. A schematic of the study design is presented in Figure S1.

2.4 | Injection of anti-IgE and IgG receptor-blocking antibodies

Mice received 100 μg of anti-IgE (clone EM-95, kindly provided by Fred Finkelman), 100 μg of anti-FcγRII/RIII (clone 2.4G2, Bio X Cell), or a mix of rat IgG2b and rat IgG2a as isotype controls (clones LTF-2 and 2A3, respectively, Bio X Cell) by intraperitoneal route. To avoid any nonspecific anaphylactic reaction, all mice received 200 μg of triprolidine hydrochloride (Sigma) by intraperitoneal route 30 minutes before injection of the monoclonal antibodies. Mice were challenged orally 24 hours later.

2.5 | Preparation of cashew patches and application to mice

Epicutaneous patches were loaded with 50 μg of cashew protein extract (Stallergenes Greer) or 50 μg of cashew protein extract conjugated to Fluoroprobe-647 (Interchim), prepared in phosphate buffer 0.1 M, or 50 μl of phosphate buffer 0.1 M alone (excipient) for sham mice. Patches were dried at 30°C in a ventilated oven and stored at 4°C. Before patch application, mice were anaesthetized with ketamine and xylazine (50 and 10 mg/kg, respectively) and hair on the back was removed using electric clippers and depilatory cream (Reckitt Benckiser). Patches were applied the following day and secured using an Urgoderm® bandage (Urgo Laboratories) for 48 hours, then removed. This procedure was repeated each week for EPIT during 8, 12, or 16 weeks (Figure S2).

2.6 | Collection of brachial lymph nodes (BLNs) for flow cytometry analysis

BLNs were harvested in 2 mL of RPMI containing 0.26 U/mL Liberase TL and 25 μg/mL DNase I (Sigma Aldrich). Each BLN was flushed using a syringe and incubated 20 minutes at 37°C. The enzymatic reaction was then stopped with 250 μL of EDTA 100 mM. Cells were homogenized with a 100 μm cell strainer in magnetic-activated cell sorting buffer (Miltenyi Biotec) and counted. Cells were incubated for 15 minutes at 4°C with Fc Block (BD Biosciences) and stained for 25 minutes at 4°C with fluorochrome-conjugated antibodies listed in Table S1. Additionally, dead cells were excluded with Zombie Aqua (Biolegend) staining. Cells were acquired on MACSquant 10 flow cytometer (Miltenyi Biotec), and data were analyzed using FlowJo software using the gating strategy previously described.[9]

2.7 | Collection of plasma

Blood samples were collected by submandibular puncture into microtubes containing EDTA (Greiner Bio-One) and centrifugated at 3000 x g for 10 minutes to collect plasma. Plasma samples were stored at −20°C.

2.8 | Measurement of antibody titers

IgG1 and IgG2a titers were measured by direct ELISA: 96-well plates were coated overnight at 4°C with cashew protein extract (5 μg/mL, protein equivalent) or with anti-IgG1 or anti-IgG2a (5 or 1 μg/mL, respectively) for binding of standards. Plates were washed in PBS 1X-Tween® 0.05% and blocked 1 hour at room temperature (RT) with phosphate 0.1 M pH 7.4-NaCl 0.15 M-BSA 0.1%-sodium azide 0.01% (EIA buffer). Plates were then incubated overnight at 4°C with mouse plasma (in duplicates) or serially diluted standards (Rabbit F(ab')2 anti-mouse IgG1 or human anti-mouse IgG2a, Bio-Rad), and then 1 hour at 37°C with relevant secondary antibodies conjugated to alkaline phosphatase (AP) (Bio-Rad). Finally, plates were incubated for 30 minutes at RT with AP substrate (p-Nitrophenyl Phosphate, pNPP), and optical densities (OD) at 405 nm were recorded. Antibody concentrations were calculated with Boltzmann sigmoidal equation using OD obtained from standards (GraphPad Prism®), and after subtracting OD obtained from nonspecific points. IgE titers were measured by indirect ELISA to avoid competition with IgG1 binding: 96-well plates were coated with 2μg/mL of rat anti-mouse IgE (Bio-Rad) and incubated overnight at 4°C. Plates were washed in PBS 1X-Tween® 0.05% and blocked 1 hour at RT with EIA buffer. Following washing, plates were incubated overnight at 4°C

with mouse plasma (in duplicates) or serially diluted standard (mouse IgE, Bio-Rad), and then 1 hour at RT with biotinylated cashew proteins for plasma samples or biotin anti-mouse IgE (1:2000, Biolegend) for standard. Plates were finally incubated 1 hour at RT with AP-conjugated streptavidine (1:5000, Jackson Immuno Research Lab), and then 15 minutes at RT with pNPP. OD were recorded, and antibody concentrations were measured as described above.

2.9 | Oral challenge and monitoring of anaphylaxis

Mice were challenged orally with 45 mg of cashew protein extract (protein equivalent). Anaphylactic reactions were characterized by a drop in body temperature and the occurrence of clinical symptoms. Body temperature was measured before challenge and every 5 minutes for 60 minutes following challenge using subcutaneous transponders (Plexx). Clinical symptoms were recorded following the same frequency following Tables S2 and S3.

2.10 | Measurement of mMCP-1 and mMCP-7

Of 96-well plates were coated overnight at 4°C with anti-mouse MCPT-1 (eBioscience) or MCPT-7 (R&D systems). Plates were washed in PBS 1X-Tween® 0.05% and blocked 1 hour at RT with blocking buffer 1X (eBioscience). Plates were incubated overnight at 4°C with plasma samples or recombinant MCPT-1 (eBioscience) or MCPT-7 (R&D systems) as standards. Following washing, plates were incubated 1 hour at RT with biotinylated anti-mouse MCPT-1 (eBioscience) or MCPT-7 (R&D systems), then 30 minutes at RT with HRP-conjugated avidin (eBioscience). HRP was developed using TMB substrate (eBioscience), for 15 minutes at RT. The reaction was stopped with 1 N chloride acid, and OD at 450 nm was recorded. Concentrations were calculated with Boltzmann sigmoidal equation using OD obtained from standards (GraphPad Prism®), and after subtracting OD obtained from nonspecific points.

2.11 | Statistical analysis

Data are presented as median with interquartile ranges or mean with SEM. The nonparametric Mann-Whitney test was used to compare unpaired values (GraphPad Prism®). Values of $P < .05$ were considered significant. The level of significance is indicated with asterisks: *, $P < .05$; **, $P < .01$; ***, $P < .001$; ****, $P < .0001$ and n.s., nonsignificant.

3 | RESULTS

3.1 | Development of a mouse model of cashew anaphylaxis

First, our cashew protein extraction method was characterized by analyzing subsequent protein extracts on SDS-PAGE (Figure S3). The obtained electrophoretic profile was identical to that of the initial cashew flour as well to that of commercial cashew protein extracts. Moreover, major allergens (Ana o 1/2/3) were retrieved at molecular weights and proportions identical to what has been described previously.[10,11] Using this extract, two routes of sensitization were investigated: oral and skin (Figure S1). The evaluation of skin as a sensitization route was prompted by previous data suggesting that skin is a proposed portal of entry for peanut allergens, especially in patients suffering from atopic dermatitis.[12] Here, mice were treated by laser microporation to mimic eczematous skin before topical application of allergens. Both routes of sensitization induced cashew-specific IgE, IgG1 and IgG2a (Figure 1A). However, IgE titers were significantly higher in mice sensitized orally. Conversely, IgG2a titers were significantly higher in mice sensitized cutaneously. Following oral challenge, anaphylactic reactions were similar between the two routes (Figure 1B-D). Importantly, both routes were able to promote mast-cell degranulation following oral challenge, as evidenced by a significant increase in mMCP-1 and mMCP-7 levels. However, the greatest increases were observed in orally sensitized mice (Figure 1E,F). There is strong evidence for IgE-dependent anaphylaxis and little evidence for IgG-dependent anaphylaxis in humans. Conversely, a consistent role for IgG in anaphylaxis has been demonstrated in mice.[13] Thus, we considered the oral route as the most clinically relevant since it gave the surest indicators of IgE-mediated anaphylaxis (highest IgE titers and strongest mast-cell activation). To validate this, orally or skin-sensitized mice received anti-IgE or anti-IgG blocking antibodies 24 hours prior to oral challenge. As expected, anti-IgE was able to limit anaphylactic symptoms and significantly reduced mast-cell degranulation in orally sensitized mice (Figure 2). Although anti-IgG was also able to reduce temperature drop (Figure 2A), it had no impact on diarrhea nor mast-cell activation (Figure 2B-D). On the other hand, anti-IgE had less impact at reducing temperature drop and failed to reduce mMCP-1 secretion in skin-sensitized mice (Figure S4A,C). Overall, these data confirmed that IgE is the main mediator of anaphylaxis in orally sensitized mice, but that IgG is also involved, albeit to a more modest level.

3.2 | Development and validation of cashew patches

To identify the optimal excipient for patch manufacturing, lyophilized cashew protein extract was solubilized in either PBS 1X, NaCl 0.45% or phosphate buffer 0.1M and deposited on epicutaneous patches. Deposits were dried and then resolubilized using distilled water and analyzed by ELISA or SDS PAGE (Figure S5). Cashew-specific IgG1 or IgE generated in orally sensitized mice reacted with all extract deposits, demonstrating that their immunogenicity was preserved (Figure S5A). Moreover, the electrophoretic profiles of all deposits were comparable to that of the initial protein extract (Figure S5B). However, the best protein integrity was obtained with phosphate buffer, as demonstrated by the absence of aggregated proteins in the pellet, that was selected as the preferred excipient. Patches loaded with fluorescent cashew proteins were applied to cashew-sensitized mice for 48 hours to

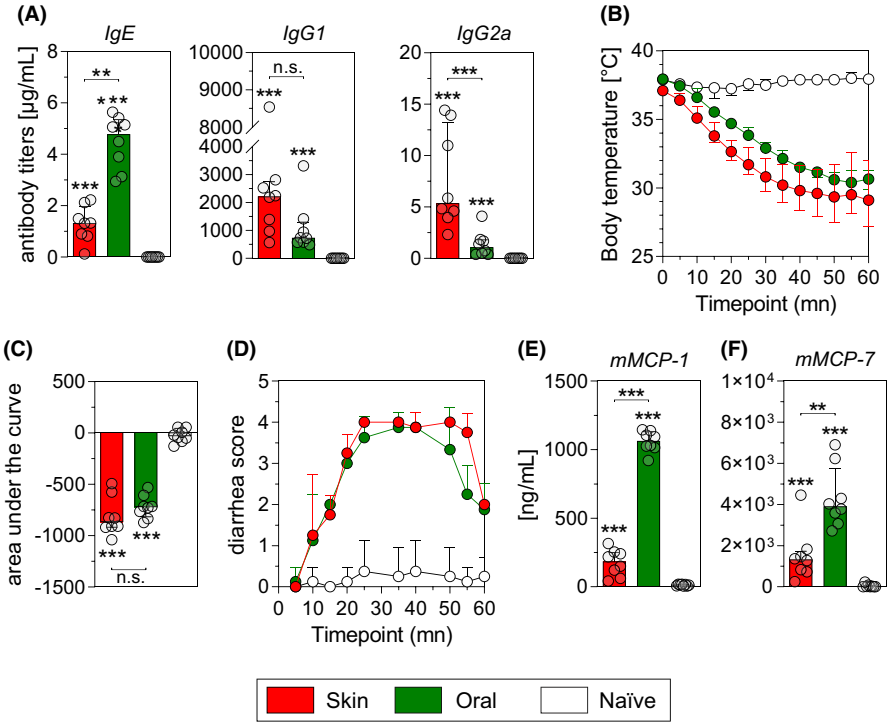


FIGURE 1 Development and characterization of mouse model of cashew sensitization and anaphylaxis. Mice were sensitized to cashew through skin (in red) or oral (in green) routes. As a negative control, a group of naïve mice (in white) was included. (A) Cashew-specific IgE, IgG1, and IgG2a antibody responses were evaluated by ELISA, from blood samples collected one week after the end of the sensitization. Mice were orally challenged to cashew one week after the end of the sensitization. (B) Body temperature was measured every 5 minutes following challenge for 60 minutes. (C) Area under the curve was calculated for each individual percentage of temperature variation curve using 100% as a baseline. (D) Diarrhea occurrence and severity was scored based on Table S2. mMCP-1 (E) and mMCP-7 (F) concentrations were measured by ELISA from plasma collected immediately after the challenge, (n = 8 per experimental group). Data are median with interquartile range of individual values. P values were determined using the Mann-Whitney unpaired *t* test (***P* < 0,01; ****P* < 0,001; n.s., nonsignificant). For A, C, E, and F panels, the level of significant measured between each sensitized group and the negative control group is indicated above each graph

evaluate the capacity of cashew patches to deliver allergens to skin dendritic cells (DCs). To control for the degree of sensitization, cashew-specific IgG1, IgG2a, and IgE were quantified from plasma collected before patches were applied. All mice presented similar antibody titers that were consistent with previous experiments (data not shown). The number of fluorescent DCs was then measured from BLNs (Figure 3). A significant increase of cashew-positive DCs was observed in mice that received cashew patches compared to those receiving excipient patches. Of note, this increase was more pronounced in cDC2 dermal DCs and Langerhans cells than in cDC1 dermal DCs. Overall, these data demonstrate that cashew patches can be produced while maintaining allergen integrity and that they are able to deliver allergens to skin DCs, especially Langerhans cells and cDC2.

3.3 | EPIT with cashew patches increased cashew-specific IgG2a in sensitized mice

Mice were sensitized orally to cashew as described above and treated as described in Figure S2. Plasma was collected every two weeks during treatment to measure the evolution of cashew-specific IgE, IgG1, and IgG2a (Figure 4). A transient increase of cashew-specific IgE was observed in EPIT mice compared to the sham group, with a peak level at week 12 post-treatment followed by a significant decrease (Figure 4A). Similarly, an increase of cashew-specific IgG1 was observed in EPIT mice, with a peak level at week 10 post-treatment followed by a significant decrease (Figure 4B). Finally, a progressive and continuous increase in cashew-specific IgG2a was observed in treated mice compared to the sham group (Figure 4C). Overall, these data indicate that EPIT to cashew can strongly modulate cashew-specific antibody responses.

3.4 | EPIT with cashew patches protects sensitized mice against IgE-mediated anaphylaxis

Mice were sensitized and treated as described above. At the end of the treatment period (*ie*, 8, 12, or 16 weeks), mice were challenged orally to cashew (Figure 5). A clear protective effect was observed in EPIT mice compared to the sham group, and this protective effect was further improved with increasing length of treatment. This protection was characterized by a decrease

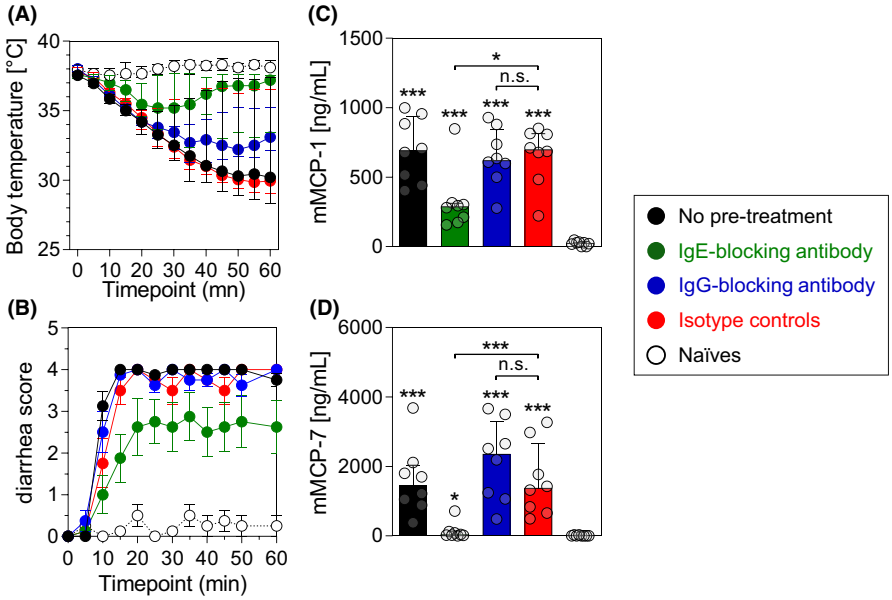


FIGURE 2 Validation of the oral route of sensitization as a trigger of IgE-mediated anaphylaxis in mice. Mice were orally sensitized to cashew. One week after the end of the sensitization, mice received IgE blocking antibody (clone EM-95, in green), IgG blocking antibody (anti-FcγRII/RIII clone 2.4G2, in blue) or relevant isotype controls (in red). The day after, mice were challenged orally to cashew. (A) Body temperature was measured every 5 minutes following challenge for 60 minutes and (B) diarrhea occurrence and severity was scored based on Table S2. mMCP-1 (C) and mMCP-7 (D) concentrations were measured by ELISA from plasma collected immediately after the challenge, (n = 8 per experimental group). Data are median with interquartile range of individual values. P values were determined using the Mann-Whitney unpaired t test (*P<.05; ***P<.001; n.s., nonsignificant). For C and D panels, the level of significant measured between each sensitized group and the negative control group is indicated above each dot plot

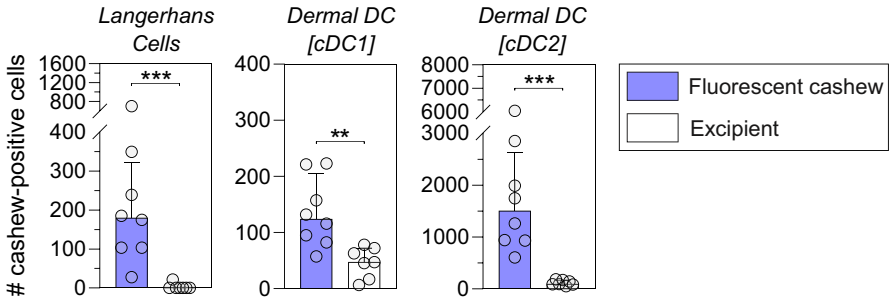


FIGURE 3 Cashew allergens are efficaciously delivered by epicutaneous patches, leading to allergen capture by skin dendritic cells. Mice were orally sensitized to cashew. One week after the end of the sensitization, mice received a patch loaded with cashew protein extract conjugated to Fluoroprobe-647 (F-647) for 48 hours. As negative controls, mice received a patch containing excipient. Brachial draining lymph nodes were collected, and cells were isolated and labeled for FACS analysis. The absolute number of cashew-positive (F-647-positive) cells was measured among each DC subsets, (n = 7-8 per experimental group). Data are median and interquartile ranges of individual values. P values were determined according to the Mann-Whitney test (**P<.01; ***P<.001)

in both temperature drop (Figure 5A,C,E) and clinical symptoms (Figure 5B,D,F). To evaluate the capacity of EPIT to protect against mast-cell activation, plasma was collected immediately after oral challenge to measure the level of mMCP-1 and mMCP-7 (Figure 6). A sharp and significant decrease of both mMCP-1 and mMCP-7 was observed in EPIT mice compared to the sham group. Of note, the prolongation of the treatment period does not induce further decrease in the plasmatic concentration of these two proteases. Overall, these data indicate that EPIT to cashew can efficiently protect mice against IgE-mediated anaphylaxis induced by oral challenge.

4 | DISCUSSION

In this study, we aimed to evaluate the potential of EPIT to treat cashew allergy. To that end, we first developed a clinically relevant mouse model of anaphylaxis, for which IgE is the main mediator and in which challenge is performed by oral administration of allergens, similar to what occurs in humans. Using this model, we demonstrated that epicutaneous patches are able to deliver cashew allergens to skin DCs, especially Langerhans cells and cDC2 that have been demonstrated as the main promotors of tolerance to topical allergens.[14,15] More importantly, EPIT with

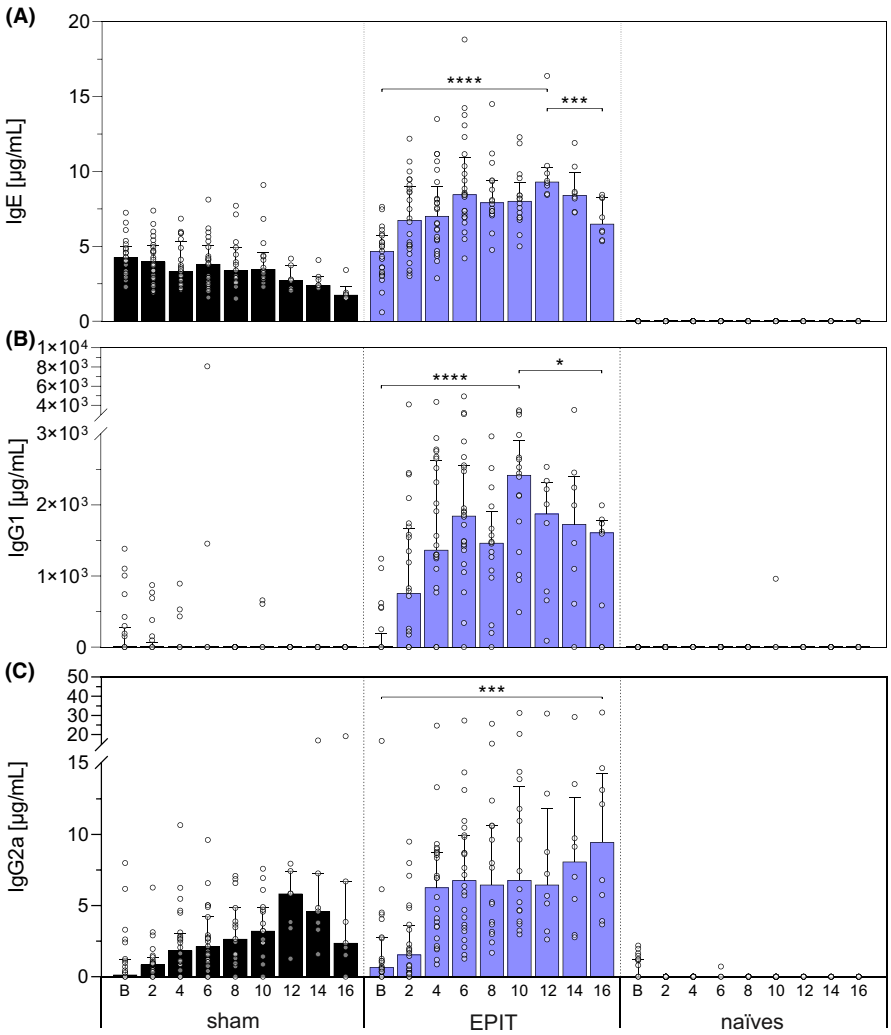


FIGURE 4 Kinetic modulation of cashew-specific antibody response following EPIT to cashew nut in mice. Mice were orally sensitized to cashew. One week after the end of the sensitization, mice were submitted to EPIT. To that end, mice received cashew patches containing 50 µg of cashew protein extract, once a week for up to 16 weeks (in blue). Patches were applied for 48 hours. As negative controls, mice received patches containing excipient (sham, in black) or were kept untreated (naïves, in white). Blood samples were collected before EPIT (B) or every two weeks during treatment to isolate plasma (weeks 2, 4, 6, 8, 10, 12, 14, and 16), as indicated on C panel. Cashew-specific antibody titers were measured from plasma by indirect ELISA (IgE, panel A) or direct ELISA (IgG1 and IgG2a, panel B and panel C, respectively), (n = 8-24 per experimental group – 8 mice of each group were challenged at weeks 8 and 12 and killed). Data are median and interquartile ranges of individual values

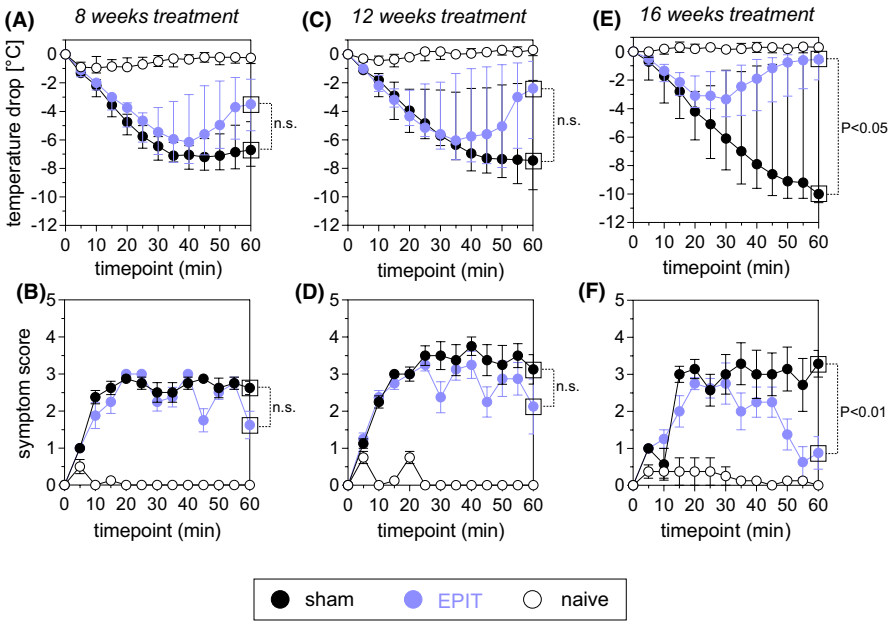


FIGURE 5 Evaluation of the level of protection afforded by EPIT against anaphylaxis following 8, 12, or 16 weeks of treatment. Mice were orally sensitized to cashew and treated as described in Figure 4. Following 8 (A, B), 12 (C, D), or 16 (E, F) weeks of EPIT, 8 mice of each group were challenged orally to cashew. (A, C, E) Body temperature was measured every 5 minutes following challenge for 60 minutes. Data are median and interquartile ranges of individual values. (B, D, F) Clinical symptoms were monitored every 5 minutes following challenge for 60 minutes, based on Table S3. (n = 8 per experimental group). Data are mean with SEM of individual values. P values were determined according to the Mann-Whitney test

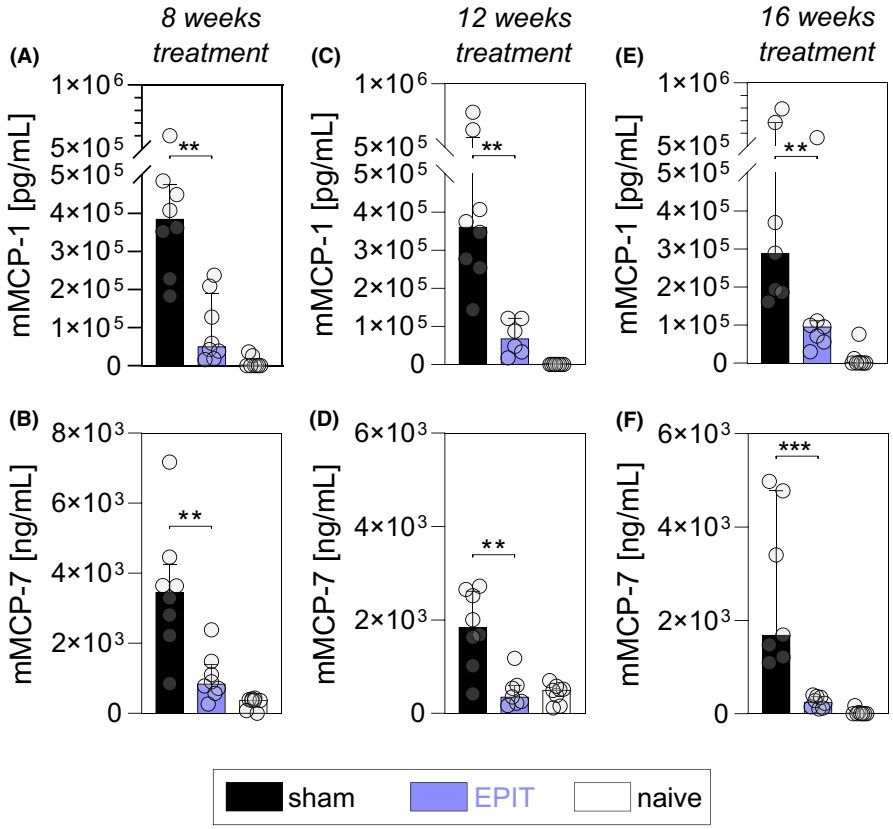


FIGURE 6 Evaluation of mast-cell activation induced by oral challenge following 8, 12, or 16 weeks of EPIT. Mice were orally sensitized to cashew, treated as described in Figure 4, and finally challenged as described in Figure 5. Blood samples were collected 60 minutes after the challenge to isolate plasma. mMCP-1 (A, C, E) and mMCP-7 (B, D, F) concentrations were measured from plasma by ELISA, (n = 8 per experimental group). Data are median with interquartile range of individual values. P values were determined using the Mann-Whitney unpaired t test (**P<.01; ***P<.001)

cashew patches affords a substantial level of protection against IgE-mediated anaphylaxis to cashew. We focused this study on a 50-μg dose per patch. This dose was selected based upon preliminary experiments, showing that 100, 250, or 500 μg doses did not afford additional benefits for protection (see results obtained with a 500-μg dose on Figure S6). To date, few studies focused on immunotherapy against cashew allergy. In one of those works, Kulis et al demonstrated in mice that allergy to cashew can be managed by immunotherapy.[16] In that paper, authors showed that cashew immunotherapy succeed in decreasing specific Th2 response and reducing anaphylactic symptoms following challenge. More interestingly, cashew immunotherapy was also able to afford protection against other cross-reactive tree nuts such as pistachio and walnut. More recently, Pereira *et al* evaluated the immunogenicity of orally administered poly(anhydride) nanoparticles loaded with cashew allergens in BALB/c mice.[17] These authors showed that nanoparticles were able to promote a strong specific Th1 response to cashew in association with an induction of FoxP3 + and LAP + T regulatory cells (T-Reg). Unfortunately, the clinical efficacy of this treatment has not been reported. In our preclinical model, we showed that EPIT induced a transient peak of cashew-specific IgE at the beginning of immunotherapy. Interestingly, the same phenomenon has been observed in our previous preclinical data evaluating EPIT against milk allergy[18] and in patients during clinical trials investigating EPIT against peanut allergy.[19] Moreover, and in a similar way to what has been observed in humans, this increase is followed by a gradual progressive decrease of IgE titers with continued treatment. In the present study, of note, IgE titers did not return to baseline and were not reduced in EPIT mice compared to sham group following 16 weeks of treatment. This suggests that a longer treatment period might be required to pursue that downward trend. Of note, an extended duration of treatment might be limited by the advanced age of animals at the end of the study. Additionally, our results revealed that EPIT induces an increase of cashew-specific IgG (IgG1, mouse equivalent of human IgG4 and IgG2a, mouse equivalent of human IgG1). Again, these data should be viewed in the context of clinical data investigating EPIT for peanut allergy, in which a progressive increase of peanut-specific IgG4 levels has been observed during the first 12 months of treatment.[19] This increase in IgG4 response in humans is usually interpreted as a beneficial impact of the treatment since IgG4 antagonizes effector functions mediated by IgE through competition and neutralization of allergens.[20] The permanent increase of IgG2a observed in our study should also be interpreted as a positive outcome since it is the main marker of Th1 immune orientation in mouse, which is known to be associated with nonallergic responses.[21,22] Mast cells express a high amount of high affinity IgE receptors (FcεRI) and are unequivocally considered as key players in IgE-dependent anaphylaxis.[23] Additionally, it has been previously shown that IgE-dependent activation of mast cells plays an important role in disease induction in mouse models of allergy.[24,25] Here, we demonstrated that EPIT strongly reduced mast-cell stimulation and degranulation following oral challenge. Interestingly, this inhibition occurs despite a relatively high level of IgE, suggesting that EPIT modulates mast-cell reactivity to IgE signaling. Beyond the competition between IgE and

IgG for allergen binding we described above, this modulation could be linked to an inhibition of the signal triggered by FcεRI as described in previous studies.[26,27] However, it is unlikely that this desensitization results from constant stimulation of gut mast-cell with small, sub-activating amounts of allergen, since EPIT does not lead to systemic diffusion of allergen. Despite almost complete suppression of mMCP-1 and mMCP-7 levels in blood following 8 and 12 weeks of treatment, EPIT mice still presented substantial anaphylactic symptoms following oral challenge. Therefore, we cannot exclude a role for specific IgG in anaphylactic reactions, as suggested by Figure 2. Consequently, our mouse model may underestimate the potential efficacy of EPIT in humans, for which the role of IgG in anaphylaxis is still a matter of considerable debate. Also, we observed that EPIT promotes a faster recovery of mice from anaphylactic reaction but does not abolish the initial occurrence of symptoms (from 0 to 30 minutes after challenge). This suggests that EPIT induce effectors able to downmodulate anaphylaxis mediators, allowing a quicker return to homeostasis. Therefore, we are currently performing in vivo experiments to elucidate the mechanisms underlying this modulation and the induction of tolerance to cashew, with a special focus on regulatory T cells which have been demonstrated as key players in murine models of peanut allergy.[28,29] Our preliminary data showed that EPIT to cashew induced a modest increase of CD62L-positive T-regs in mesenteric lymph nodes (data not shown) and was able to decrease cashew-specific Th2 response as soon as 8 weeks of treatment (Figure S7). Further investigations are warranted to confirm these trends, especially after a longer period of treatment.

EPIT may offer a safe approach to treat food allergy,[30] primarily due to the absence of systemic dissemination of allergens, thus avoiding direct contact with anaphylaxis-triggering effector cells, for example, mast cells. Therefore, we suggest that EPIT may be a relevant treatment strategy for cashew allergy. Indeed, the unique severity of reactions to cashew reported in allergic patients could be a source of concerns regarding other immunotherapeutic strategies that require subcutaneous injections or oral administration of allergens.

Overall, our data suggest that the potential of EPIT to safely treat cashew allergy deserves confirmation in clinical trials. They also bring up new insights and new questions regarding EPIT mechanisms that would require more extensive studies to be fully elucidated.

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CONFLICT OF INTEREST

Benjamin Pelletier, Camille Plaquet, Jean-Louis Labernardière, Mélanie Ligouis, Dr. Dioszeghy, Dr. Wavrin, Dr. Matthews, Dr. Sampson, and Dr. Hervé report personal fees from DBV Technologies, during the conduct of the study; personal fees from DBV Technologies, outside the submitted work. Audrey Perrin, Noémie Assoun, Nathalie Oreal, Laetitia Gaulme, Adeline Bouzereau, and Dr. Porcheray report personal fees from DBV Technologies, during the conduct of the study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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