Laser-facilitated epicutaneous immunotherapy with depigmented house dust mite extract alleviates allergic responses in a mouse model of allergic lung inflammation

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Abstract

Background: Skin-based immunotherapy of type 1 allergies has recently been re-investigated as an alternative for subcutaneous injections. In the current study, we employed a mouse model of house dust mite (HDM)-induced lung inflammation to explore the potential of laser-facilitated epicutaneous allergen-specific treatment.

Methods: Mice were sensitized against native Dermatophagoides pteronyssinus extract and repeatedly treated by application of depigmented D pteronyssinus extract via laser-generated skin micropores or by subcutaneous injection with or without alum. Following aerosol challenges, lung function was determined by whole-body plethysmography and bronchoalveolar lavage fluid was analyzed for cellular composition and cytokine levels. HDM-specific IgG subclass antibodies were determined by ELISA. Serum as well as cell-bound IgE was measured by ELISA, rat basophil leukemia cell assay, and ex vivo using a basophil activation test, respectively. Cultured lymphocytes were analyzed for cytokine secretion profiles and cellular polarization by flow cytometry.

Results: Immunization of mice by subcutaneous injection or epicutaneous laser microporation induced comparable IgG antibody levels, but the latter preferentially induced regulatory T cells and in general downregulated T cell cytokine production. This effect was found to be a result of the laser treatment itself, independent from extract application. Epicutaneous treatment of sensitized animals led to induction of blocking IgG, and improvement of lung function, superior compared to the effects of subcutaneous therapy. During the whole therapy schedule, no local or systemic side effects occurred.

Conclusion: Allergen-specific immunotherapy with depigmented HDM extract via laser-generated skin micropores offers a safe and effective treatment option for HDM-induced allergy and lung inflammation.

Keywords
depigmented extract, epicutaneous immunotherapy, house dust mite, laser, skin immunization
**INTRODUCTION**

House dust mites (HDM) are ubiquitous in the indoor environment and their allergens are the major perennial inducers of allergic rhinitis and allergic asthma worldwide.\(^1\) The high sensitization capacity of HDM allergens has been attributed to a combination of effects on the adaptive and the innate immune system. Several HDM allergens, including the two *Dermatophagoides pteronyssinus* allergens with the highest sensitization rates, Der p 1 and Der p 2, promote allergic immune responses by stimulating the innate immune system via protease-activated receptors and pattern recognition receptors.\(^2\)

Specific immunotherapy by subcutaneous injections (SCIT) or sublingual administration (SLIT) has proven efficacy in reducing symptoms and use of pharmacotherapy in HDM-induced rhinitis, whereas its ability to provide relief in asthmatic patients is limited.\(^3\) Both require long treatment schedules associated with high healthcare costs and suffer from low patient adherence. Alternative treatments with increased efficacy and patient convenience are investigated. Due to its accessibility, lack of vasculature, and richness in immunocompetent cell types, the skin has been recognized as a promising target tissue for delivery of vaccines and therapeutics. Epicutaneous immunotherapy (EPIT) of type I allergic diseases dates back to 1921 and in several studies in the middle of the last century, higher success rates and milder side effects compared to conventional subcutaneous treatment were reported.\(^4\)\(^-\)\(^6\) Recently, some clinical trials were performed, employing EPIT for treatment of grass pollen-induced rhinoconjunctivitis\(^7\)\(^-\)\(^10\) or food allergies\(^11\)\(^-\)\(^14\) using either tape-stripping or occlusive skin patches to increase permeability of the epidermis.\(^15\) Alternatively, the outermost skin barrier can overcome more efficiently by creating micropores of well-defined depth and density using fractional laser ablation to facilitate uptake of topically applied antigens.\(^16\)

It has been demonstrated that laser treatment of the skin leads to formation of an inflammation array by attracting antigen-presenting cells, providing transient immune stimulation and thereby omitting the need for external adjuvant.\(^17\)\(^,\)\(^18\)

In our current work, we investigated whether epicutaneous administration of a depigmented HDM extract via laser-generated micropores was superior compared to classical SCIT regarding induction of blocking antibodies, regulatory T cell responses, and improvement of lung function using a mouse model of allergic lung inflammation.
2.2 | Ethics statement

All studies involving human material were conducted in accordance with the guidelines of the World Medical Association's Declaration of Helsinki. The study was approved by the local ethics committee of the University of Salzburg (PLUS_Ethik_190811) and all participating subjects gave their written informed consent.

All animal experiments were conducted in compliance with EU Directive 2010/63/EU and have been approved by the Austrian Ministry of Education, Science and Research, permit number BMBWF-66.012/0006-V/3b/2018.

2.3 | Animal experiments

Female BALB/c mice aged 6-8 weeks were obtained from Janvier (Le Genest-Saint-Isle, France) and maintained at the animal facility of the University of Salzburg in a specific pathogen-free environment according to local guidelines.

Blood samples were taken on day 29 (immunogenicity) or on days 58 and 69 (therapy) by puncture of the vena saphena, and sera were stored at −20°C until analysis.

For studying immunogenicity, mice (n = 5) were immunized on days 0 (on the back) and 15 (on the abdomen) with 10 or 100 µg depigmented HDM extract in PBS either by subcutaneous (s.c.) injection or epicutaneous immunization (EPI) using a PLEASE Professional laser microporation device (Pantec Biosolutions) with the following settings: 0.7W, 3 pulses per pore, 9% pore density, total fluence of 8.3 J/cm² (two application sites per immunization).

For studying therapeutic efficacy, mice (n = 8) were first sensitized on days −18 and −11 by s.c. injection of 10 µg native HDM extract in PBS containing 50% (v/v) Alu-Gel-S (Serva). For induction of lung inflammation, on day −4 mice were intranasally instilled with 40 µL of PBS containing 10 µg of native HDM extract under isoflurane anesthesia. Therapeutic immunizations with 100 µg depigmented HDM extract were performed on days 0, 7, 14, 21, 28, 35, 42, and 49 either by EPI or by s.c. injection. For sham treatment PBS was applied to skin micropores. Laser settings were the same as described for immunogenicity study. S.c. injections were given in PBS with or without 50% (v/v) Alu-Gel-S.

After the last treatment, mice were challenged by exposure to aerosolized native HDM extract (10 mL of 1% native HDM extract in PBS) on days 65, 66, and 67 using a Pariboyst SX jet nebulizer.

2.4 | Lung parameters

Twenty-four hours after the last aerosol challenge, whole-body plethysmography (WBP) was performed using a 6-chamber unrestrained Buxco WBP system (Data Sciences International). At this point, two naïve animals were included for baseline assessment. Their values were excluded from statistical analysis. The next day, mice were killed and bronchoalveolar lavage fluid (BALF) was collected and centrifuged. The supernatants were mixed with 1/10 vol. of DPBS, 10% BSA, 1% NaN₃, and stored at −20°C for cytokine measurement. Cells were stained for Ly6G, Siglec F, CD45, CD4, CD25, and FoxP3 followed by analysis on a FACSCanto II flow cytometer (BD Biosciences).

2.5 | Immunoglobulin measurement

HDM-specific IgG subclasses and IgE were determined by direct ELISA. Total IgE was measured by sandwich ELISA. Biologically active, HDM-specific IgE in sera was measured by a rat basophil leukemia (RBL-2H3) cell assay. Cell-bound IgE was measured ex vivo using a basophil activation test performed on the day of killing using either washed or nonwashed whole blood. Cells were stimulated with 30 ng/mL HDM extract for 2 hours at 37°C. Subsequently, cells were stained for IgE, CD4, CD19, and CD200R. Basophils were gated as IgE⁺CD19neg CD4neg cells and activation status was assessed by the median fluorescence intensity of CD200R.

2.6 | Blocking IgG measurement

IgE blocking capacity of murine sera was assessed by RBL cross-linking inhibition assay as previously described or direct inhibition ELISA. Detection of HDM-specific IgE in sera from allergic patients (RAST class 6, 1300 ± 322 ng/mL of Der p 2 specific IgE) was performed as described for murine IgE (see supplementary materials and methods), except that anti-human IgE biotin (clone MHF-18, Biologend) was used as secondary antibody. To test IgE binding inhibition, plates were preincubated for 1 hour with murine sera of treated mice, before patient sera were added to the wells.

2.7 | Lymphocyte cultures, flow cytometry, and cytokine measurements

Splenocytes and skin draining lymph node (SDLN) cells from mice were prepared and cultured at a concentration of 4 × 10⁶ cells/mL in T cell medium (RPMI 1640, 10% FCS, 25 mmol/L HEPES, 2 mmol/L L-Glu, 100 µg/mL streptomycin, 100 U/mL penicillin) with or without 100 µg/mL depigmented (immunogenicity experiment) or native (therapy experiment) HDM extract. Cells were incubated for 3 days at 37°C, 5% CO₂, 95% humidity. After that, supernatants were harvested, diluted 1:2 with PBS and analyzed for cytokines using the LEGENDplex Mouse Th Cytokine Panel 13-plex (Biologend) according to the manufacturer's instructions. Cell pellets from restimulated spleen cells were washed with DPBS, blocked with an anti-CD16/32 containing hybridoma supernatant and stained for CD62L, CD44, CD4, CD25, FoxP3, GATA3, and Tbet. Samples were analyzed on a CytoFLEX S flow cytometer (Beckman Coulter).
2.8 | Statistical analysis

Statistical differences between groups were calculated by one-way ANOVA, followed by Tukey’s post hoc test (α = 0.05) using GraphPad Prism 7. Effect of restimulation (medium vs extract) was assessed by paired t test. Comparison of AHR and IgE levels before and after challenge was done using repeated measurement two-way ANOVA followed by Sidak’s multiple comparison assay.

3 | RESULTS

3.1 | EPI induces similar IgG antibody responses compared to s.c. injection

Fourteen days after two s.c. or epicutaneous immunizations with 10 or 100 μg depigmented HDM extract, significant levels of allergen-specific IgG could be detected by direct ELISA. Subclass analysis showed that IgG consisted mainly of IgG1 and IgG2b, while only low amounts of IgG2a were detected. Application via laser-generated micropores induced similar amounts of IgG compared to s.c. injection (Figure 1). Because application of antigen to barrier-disrupted skin potentially leads to sensitization,16,20 we also measured total serum IgE and HDM-specific IgE by ELISA as well as biologically active HDM-specific IgE using an RBL release assay. As shown in Figure S1, EPI induced a modest dose-dependent increase in total IgE, which did not significantly differ from SC immunized groups. Despite depletion of inhibitory IgG, we were not able to detect HDM-specific IgE by direct ELISA, even at a 1:10 serum dilution. RBL assay was more sensitive and we found that only the EPI 10 group induced detectable IgE levels, whereas no specific IgE was found in all other groups.

3.2 | Laser microporation favors generation of memory T cells and regulatory T cells

SDLN cells and splenocytes from immunized mice were restimulated in vitro for 3 days with depigmented HDM extract. As shown in Figure 2, splenocytes, but not SDLN cells from mice that were treated by laser microporation showed significantly elevated levels of memory T cells compared to naïve mice or mice immunized subcutaneously. This effect was independent of the topical administration of HDM extract and thus is a result of the laserporation process itself. The laser-induced increase in memory T cells corresponded to a significantly elevated number of FoxP3+ regulatory T cells in the spleen (see Figure S2 for gating strategy).

3.3 | Depigmented HDM extract induces innate cytokine responses in splenocytes that are partly suppressed by EPI using laser microporation

When restimulating splenocytes from naïve mice, we observed that depigmented HDM extract unselectively induced IFN-γ, TNF-α, IL-6, IL-10, and IL-22, suggesting innate immune cells as a source of these cytokines (Figure 3A). These HDM extract-induced unspecific responses were partly reduced in mice that had been treated by laser microporation, independent whether extract was applied or not (Figure 3B). This effect was only statistically significant for IFN-γ and IL-6 in the EPI PBS vs. SC 10 groups. The only vaccination-induced cytokine responses were detected in the subcutaneously immunized groups, and these were mostly TH2 (IL-4, IL-5, and IL-13), but also IL-2 and IL-17A were found. EPI again did not prime these HDM-specific TH2 responses (Figure 3C). Suppression of IFN-γ responses in EPI groups and induction of TH2 responses in the SC groups resulted in a significant shift in IFN-γ/TH2 and IL-10/TH2 ratios (Figure S3). While the former were reduced in EPI groups (due to lower IFN-γ), the latter were reduced in SC groups (due to higher TH2 cytokines). SDLN cells produced much lower cytokine levels (mostly close to the detection limit), and we only found slightly elevated IL-4 and IL-5 levels in the vaccination groups (data not shown).

Taken together, cytokine responses that can be attributed to vaccination-induced T cells were mainly TH2 driven and only induced by subcutaneous immunization. In contrast, laser microporation in general tended to suppress innate cytokine responses induced in HDM extract stimulated splenocytes, correlating with the enhanced number of Tregs in these groups. In a next step, we therefore evaluated the efficacy of laser-mediated epicutaneous immunotherapy (EPIT) in a mouse model of allergic lung inflammation. We chose the 100 μg dose for the therapeutic approach, as this dose did not exhibit IgE inducing potential when applied epicutaneously.

3.4 | EPIT induces lower levels of therapy-associated IgE than SCIT, induces IgG with blocking capacity, downregulates cytokine responses, and improves lung function.

After sensitization and intranasal provocation, mice were stratified into five groups with similar levels of airway hyperresponsiveness (AHR) and HDM-specific IgE (see Figure S4). HDM sensitization induced very potent AHR, which prompted us to use low methacholine challenge doses (5, 7.5, and 10 mg/mL) in compliance with animal welfare and with respect to data quality. Nevertheless, even at these low doses, many mice reached Penh values of 10 or higher, indicating strong sensitization (Figure S4). Subsequently, mice were treated 8 times with depigmented HDM extract applied epicutaneously via laser microporated skin (EPI), subcutaneously with (SC/alum) or without (SC) alum, were sham treated with PBS on laser microporated skin (EPI PBS), or left untreated. After treatment, mice were challenged by exposing them to aerosolized native HDM extract in a chamber for 20 minutes on three consecutive days (see Figure 4A for schedule).

EPIT and SCIT resulted in a significant upregulation of biologically active HDM-specific serum IgE, as measured by RBL assay (Figure 4B). After aerosol challenge, IgE levels in the untreated
groups significantly increased, whereas IgE levels in the EPI 100 group (and to a lesser degree in the SCIT groups) dropped (Figure S5A and B). However, SC-treated groups were still significantly higher compared to the untreated control groups postchallenge (Figure 4C). Similarly, treated groups showed a significant increase in total serum IgE compared to untreated or sham-treated groups (Figure S5C), but interestingly, HDM-specific IgE measured by direct ELISA was only increased in the SCIT groups, whereas the EPI 100 group displayed the lowest HDM-specific IgE levels of all treatment groups (Figure S5D). Basophils analyzed ex vivo at the time point of killing showed a similar activation status as indicated by the median fluorescence intensity of CD200R (data not shown). When basophils were washed, in vitro restimulation for 2 hours with native HDM extract induced a strong upregulation of CD200R indicating the presence of cell-bound HDM-specific IgE (Figure S6). However, restimulating full blood samples in vitro (in the presence of autologous serum) resulted in a significantly lower activation of basophils in the groups treated with depigmented HDM extract in the absence of alum, indicating the presence of blocking IgG (Figure 4D). By calculating the ratio of basophil activation in the presence and absence of autologous serum, a blocking capacity index (1 = no blocking capacity) could be calculated (Figure 4E). The presence of biologically active blocking IgG correlated with the amount of total IgG measured by ELISA (Figure 4F) that was significantly higher in all treatment groups, which showed a more than 10-fold higher antibody titer compared to untreated or sham-treated controls (P < .001, Figure S7). Again, total IgG consisted mainly of IgG1 and IgG2b subclasses. EPIT induced significantly more IgG compared to SCIT with and without alum (Figure 4F), and also resulted in the lowest levels of serum IgE and the highest blocking capacity. To confirm that the mouse sera also inhibit naturally induced IgE, we took sera from allergic patients and tested the inhibition of IgE binding in
Figure 2. Naive, central memory (TCM), effector memory (TEM), and FoxP3 + CD25 + regulatory T cells (Treg) in SDLN (top) and splenocyte (bottom) cultures restimulated for 3 d with depigmented HDM extract. Data are shown as percentage of live CD4 + T cells from mice immunized epicutaneously (EPI) or subcutaneously (SC) with 10 or 100 µg of depigmented HDM extract. Sham treated (EPI PBS) and naïve mice served as controls. Data are shown as means ± SD and individual data points (n = 5). ****P < .0001, ***P < .001, **P < .01, *P < .05.

Figure 3. A, Innate cytokine responses in splenocytes of naïve mice restimulated with depigmented HDM extract (E) or with medium alone (M), **P < .01, *P < .05 (stimulated vs. unstimulated). Innate (B) or vaccination-induced (C) cytokine responses in mice immunized epicutaneously (EPI) or subcutaneously (SC) with 10 or 100 µg of depigmented HDM extract. Data are shown as individual data points or box (25th to 75th percentile) and whiskers (min to max) plots (n = 5). **P < .01, *P < .05.

Figure 4. A, Mouse model of allergic lung inflammation. B, HDM-specific serum IgE posttreatment and postchallenge (C) measured by RBL assay. D, BAT of in vitro stimulated basophils at the time point of killing. E, Blocking capacity of therapy-induced IgG measured by BAT. F, HDM-specific serum IgG levels postchallenge measured by ELISA. G, Cytokine levels in supernatants of splenocytes restimulated with native HDM extract. Data are shown as mean ± SD and individual data points or box (25th to 75th percentile) and whiskers (min to max) plots (n = 8). Serum dilution in B, C, and E was 1:100. ****P < .0001, ***P < .001, **P < .01, *P < .05.
RBL-2H3 cells transfected with the human high affinity FccR and direct ELISA. As shown in Figure 5, sera from treated mice could efficiently inhibit up to 80% of binding as well as cross-linking with no significant difference between treatment arms. Treatment also resulted in a significant suppression of cytokine secretion by splenocytes restimulated in vitro for 3 days with native HDM extract, which correlated with the expression of Fox P3, and in the case of IL-22 also with the percentage of memory T cells (Figure S8). Though there was no significant difference between treatment groups, the suppressive capacity in general was EPI > SC > SC/alum (Figure 4G).

No significant changes in IFN-γ/TH2 or IL-10/TH2 cytokines were found (data not shown). In vitro stimulation significantly (P < .001) induced the expression of key transcription factors FoxP3, GATA3, and Tbet. The latter was significantly suppressed in the EPI100 group compared to untreated controls (Figure S9).

EPIT also resulted in a significant improvement of lung function as indicated by a reduction of Penh (enhanced pause), a surrogate parameter measured by WBP that correlates with airway hyperresponsiveness in BALB/c mice.21 Similar to the other read-outs, EPIT and s.c. injection with depigmented extract showed a stronger reduction in Penh compared to s.c. treatment together with alum (Figure 6A). In all three treatment groups, but not in sham-treated or untreated mice, a significant reduction of Penh compared to pretreatment values was observed (Figure 6B). This improvement of lung function correlated with a reduction of cellular infiltrate into BALF (Figure 6C and D). Treatment also resulted in an increase in the percentage of FoxP3 + T cells in the BALF, which was significantly higher compared to untreated mice only in the EPIT group (Figure 6E).

**DISCUSSION**

Commonly, successful therapeutic intervention against type 1 allergic diseases has been associated with production of high-titered blocking IgG antibodies, induction of regulatory T cells, or immune deviation from TH2 toward TH1, or combinations thereof.22 In our current study, mice were sensitized and challenged with native extract to mimic the natural allergen exposure, and experimentally desensitized with depigmented *D. pteronyssinus* extract via laser-generated micropores. This depigmented extract consists of a highly purified protein allergen extract, lacking low molecular weight non-allergenic substances. This extract exhibits a higher potency due to increased purity and higher content of proteins and major allergens compared to native extract. Both, native and depigmented extracts were manufactured and released under GMP conditions and fulfilled the same requirements than those established for the manufacturing of immunotherapy products for human use. We found strong evidence for the induction of blocking IgG and some indications for the generation of regulatory T cell responses, but did not observe immune deviation. With regard to EPIT, it has been demonstrated that repeated application of OVA on intact skin of previously sensitized mice mainly resulted in induction of regulatory T cells, which downregulated specific local and systemic responses and that this process required allergen uptake and transport to skin draining lymph nodes by epidermal Langerhans cells.23,24 Notably, in these studies patches containing dry allergen were placed on untreated skin, forming an occlusive chamber. The authors underlined that they found no evidence for passive passage or systemic delivery of the allergen23 and emphasized that (at least in case of
peanut-induced food allergy) integrity of the skin was crucial for the tolerogenic effect of EPIT, as tape-stripping before patch application boosted TH2 type responses (IgE, cytokines). Here, we show that removal of superficial skin layers with infrared laser pulses followed by EPIT of sensitized mice with depigmented HDM extract induces only a temporary IgE increase in treated animals, that declines after aerosol challenge. In contrast, SC injections (especially together with alum) result in higher levels of therapy-induced IgE and a weaker decline postchallenge. As expected, untreated groups show a dramatic boost of IgE responses after challenge (Figure 4B and C and Figure S5). This closely resembles the effects seen after immunotherapy in humans. While treatment had a strong boosting effect on total serum IgE (Figure S5C), there was only little difference in HDM-specific IgE between treatment and untreated groups (Figure S5D), indicating the generation of IgE against nonrelated antigens due to a bystander effect. In all cases, EPI 100 induced the lowest levels of therapy-associated total as well as HDM-specific IgE. Despite this temporary boost in IgE, EPI 100 led to a general suppression of cytokines (including TH2 cytokines) that correlated with increased expression of FoxP3. Suppression of IL-22 also correlated negatively with the percentage of memory T cells (Figure S8). Together, this strongly indicates regulatory responses rather than immune deviation. It has been demonstrated that wound healing in the skin is associated with the expansion of regulatory T cells, which subsequently migrate to secondary lymphoid organs. In line with this, we found that laser microporation, independent of application of HDM extract, led to increased numbers of FoxP3 + regulatory T cells in the spleen (Figure 2) and partial suppression of innate cytokines (Figure 3B, statistically significant only for IFN-γ and IL-6, EPI PBS vs. SC 10). Also, in the therapeutic setting, only the group treated via laser microporated skin (EPI 100) showed a significantly higher percentage of Tregs in the lung after aerosol challenge (Figure 6E) and the strongest suppression of all tested cytokines in the spleen (Figure 4G). Thus, our data clearly indicate that HDM EPIT via

**FIGURE 6** Lung function and cellular composition of BALF. Enhanced pause (Penh) was measured by whole-body plethysmography before (B) and after (A and B) therapy. Data are shown as the area under the curve (AUC) of Penh during a challenge with increasing doses of methacholine. Statistical significance shown in panel B compares treatment effects within the individual group (before vs. after treatment). Cellular composition of BALF after treatment was analyzed by flow cytometry and the number of eosinophils (C) and CD4 + T cells (D) recovered per BAL is shown. E) Percentage of FoxP3 + cells of total CD4 + cells in BALF. Data are shown as means ± SD and individual data points (n = 8). ***P < .001, **P < .01, *P < .05, NS = not significant.
laserporated skin does not boost TH2 responses, but rather favors regulatory T cell responses. This confirms data from laser-mediated EPIT with the grass pollen allergen Phl p 5, which in contrast to SCIT did not boost TH2 cytokines, but also resulted in a general suppression of cytokine responses.\(^{31}\) In contrast to findings that Langerhans cells are the key players in antigen transport from skin to draining lymph nodes following EPIT on intact skin,\(^{26}\) we have previously identified CD207−CD11b+ dendritic cells to preferentially transport antigen due to their large size, which limits transport.\(^{32}\) The immunogenicity of vaccine formulations applied to skin micro-pores, while simultaneously providing the precision and reproducibility of its laser scanning technology. In contrast to other technologies, the laser settings can be easily adapted to different properties of the skin such as skin type, body location, and hydration level. These features may compensate for the at first glance complex methodology. Moreover, by creating a local and temporary inflammatory chemokine/cytokine milieu at the treated area, a substantial adjuvant effect is generated due to the release of danger-associated molecular patterns (DAMPs), such as dsDNA, which can in turn promote the production of STING (stimulator of interferon genes) agonist, 2’3’- cyclic GMP-AMP (cGAMP). cGAMP has been recently identified as a main contributor to the laser-mediated adjuvant effect.\(^{17,18}\) Thus, the laser-induced injury can serve as a natural cutaneous adjuvant that can substitute traditional adjuvants such as colloidal aluminum hydroxide (alum). Moreover, alum, which is also a typical constituent in allergen extract preparations for standard immunotherapy, has been shown to negatively affect the immunogenicity of vaccine formulations applied to skin micro-pores.\(^{33}\) It has been speculated earlier that alum together with MPL and MF59 are generally not useful adjuvants for skin-based immunization due to their large size, which limits transport.\(^{32}\)

Moreover, in this study we could demonstrate that EPIT in the context of skin microporation was associated with production of high titers of IgG antibodies, which proved their specific blocking activity ex vivo (Figure 4) and in vitro, by inhibition of patient IgE binding (Figure 5). In accordance with others applying HDM patches to intact skin for EPIT,\(^{15}\) lung functions as indicated by decreased Pefh values were significantly improved by laser-facilitated treatments as shown in Figure 6B. Interestingly, in sensitized untreated animals, IgE titers further increased during the time course, pointing toward the high sensitization potency of the HDM extract, whereas in other mouse models using recombinant allergen(s) for sensitization, titers dropped with time.\(^{31,34}\)

As the superficial skin layers are not vascularized, one of the advantages of EPIT over standard SCIT is superior safety due to avoidance of direct contact of applied allergens with the bloodstream. However, in clinical studies of EPIT performed on tape-stripped skin, some dose-dependent local adverse events were recorded.\(^{9}\) Furthermore, in a mouse model using ovalbumin for laser-assisted EPIT, we also observed local side effects, which could be alleviated by conjugation of the allergen to glucans, rendering the allergen hypoallergenic (Korotchenko et al, manuscript in preparation). Interestingly, the depigmented HDM extract employed in the current study did not induce any adverse reactions, irrespective of the application method or the dosage.

Like other airborne allergens, HDM fecal pellets are easily inhaled and it has been shown that by entry of aeroallergens via the respiratory epithelium, systemic sensitization can be induced.\(^{35}\) However, as Dermatophagoides spp. feed on human skin, it is obvious that recognition of HDM by skin cells could also promote sensitization. Deckers et al\(^{36}\) recently demonstrated that epicutaneous application of HDM to untreated ear skin of mice led to TH2 sensitization and eosinophilic airway inflammation following intranasal challenge, even if the enzymatic activity was blocked. Notably, when we immunized naïve mice twice with depigmented HDM extract either by s.c. injection or via laser-generated micropores, TH2 associated cytokines were only detected in the s.c. groups and whereas IgE levels were comparable, we found induction of HDM-specific IgE only in mice epicutaneously receiving 10 μg (Figure 3). HDM extracts contain a plethora of molecules providing pathogen-associated molecular patterns (PAMPs) and can also induce release of DAMPs from skin cells. While low concentrations of PAMPs and DAMPs are associated with TH2 responses, high concentrations can modulate immune reactions toward inflammatory response types such as TH1 and TH17.\(^{37}\) This might explain why in the context of laser microporation only the low HDM dose induced IgE responses. Accordingly, we have previously shown that stimulation with Toll-like receptor (TLR) 3 or TLR9 agonists can downregulate IgE responses induced by EPI.\(^{16,38}\)

Several allergens in HDM extracts are naturally highly glycosylated and thus have the potential to address skin dendritic cells via their specific receptors. We have recently shown that targeting dendritic cells in the skin with Bet v 1-mannan neoglycoconjugates applied to micropores induced potent immune responses that were superior compared to those after intradermal injection of the same conjugates or to laser-assisted immunization with unconjugated
allergen.\textsuperscript{19} Thus, laser microporation synergistically enhances immunogenicity when combined with an antigen formulation targeting C-type lectin receptors. The observed superior immunogenicity of HDM extract applied via micropores compared to s.c. injection might also be attributable to this additive effect.

Following application of antigen to laser-generated micropores, only around 10\% of the solution can be estimated to reach the dermis, whereas the remainder gets deposited in between the micropores on intact parts of the skin.\textsuperscript{19} Considering that such a substantial portion will most probably get lost, the equal or even superior efficacy of this immunization technique compared to injection methods seems even more impressive.

Taken together, we could convincingly demonstrate that EPIT via laser-generated skin micropores represents a promising alternative to classical SCIT or SLIT for treatment of HDM-allergy and lung inflammation. Due to the unique immunostimulatory potential of the laserporation process itself, the use of adjuvants might be dispensable. Moreover, by delivery of allergens to superficial skin layers, the risk of severe side effects can be avoided. In human subjects, EPIT has already been used for treatment of allergic patients and its safety and efficacy have been proven.\textsuperscript{13} However, high doses and frequent applications over long periods (daily for 12 months) seem to be required to achieve significant efficacy compared to placebo controls.\textsuperscript{14} While intact skin has been presumed to be a prerequisite for successful immunotherapy,\textsuperscript{29} here we demonstrate that regulatory responses can also be elicited by EPIT following microporation with the PLESE laser. Recently, a clinical study revealed that skin patches containing pertussis toxin were only able to induce antibody responses over placebo after controlled epidermal skin preparation with the PLESE device.\textsuperscript{39} Collectively, these data underline the potential of laser microporation to facilitate EPIT with improved efficacy in humans, while maintaining the high safety profile of this approach.

CONFLICT OF INTEREST
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AUTHOR CONTRIBUTIONS
EK, SS, IU, MH, RW, DC, and RM performed experiments. RW, JHH and SS analyzed data. RW, VI and JC designed the study. RW, SS, RM, and JC wrote the manuscript. DC, JHH, and RW critically revised the manuscript.

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

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