

The contact sensitizer diphenylcyclopropenone has adjuvant properties in mice and potential application in epicutaneous immunotherapy

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Abstract

Background Epicutaneous vaccination has gained increasing interest during the past decade as it offers a safe, needle-free, and patient-friendly alternative to invasive vaccine administrations. Recently, the safety and early efficacy of epicutaneous immunotherapy were also demonstrated in patients with hay fever, as an alternative to conventional subcutaneous allergen-specific immunotherapy (SCIT). One major challenge to epicutaneous vaccination is the barrier function of the *stratum corneum*, which must be overcome either by abrasive methods or by hydration. Such barrier function of the *stratum corneum* also hampers the use of common adjuvants used to enhance the efficacy of vaccination.

Methods In a mouse model of allergy, we tested the adjuvant potential of diphenylcyclopropenone (DCP), a strong contact sensitizer, which is currently used for the treatment of a T cell-mediated hair loss disease (*alopecia areata*).

Results Diphenylcyclopropenone enhanced antigen-specific IgG2a antibody responses as well as IL-10 cytokine production after epicutaneous immunization with ovalbumin (OVA). Epicutaneous allergen-specific immunotherapy (EPIT) with OVA and DCP also protected sensitized mice from anaphylaxis and asthma. The protective effect was more robust than that of conventional SCIT, which did not significantly alleviate the symptoms of allergy in the murine models of anaphylaxis and asthma.

Conclusions This preclinical study confirmed previous clinical data that have demonstrated the potential of the skin as a target for allergen immunotherapy. The study also suggests that epicutaneous immunization or immunotherapy can be improved when an appropriate adjuvant such as DCP is used.

In the search for safe, cheap, effective, and noninvasive vaccine administration routes, the last decade has seen the development of topical and patch-based vaccines against various diseases (1, 2) including allergy (3–5). To facilitate antigen delivery and to enhance the immune responses to epicutaneously or transcutaneously administered antigens,

occlusion, tape stripping, or abrasion are used. Tape stripping and abrasion also activate keratinocytes to produce pro-inflammatory cytokines (6). The use of microneedle arrays allows targeting of the epidermal Langerhans cells (LCs) (7). Furthermore, administration of chemical adjuvants including subunits of cholera toxin, *Escherichia coli* heat-labile enterotoxin, or CpG (1, 8) can modulate the immune responses.

We recently demonstrated safety and efficacy of epicutaneous allergen-specific immunotherapy (EPIT) for the treatment of grass pollen-induced rhinoconjunctivitis using a patch containing allergen extracts (4, 5). The current study investigated the feasibility of adjuvanting EPIT in using a mouse

Abbreviations

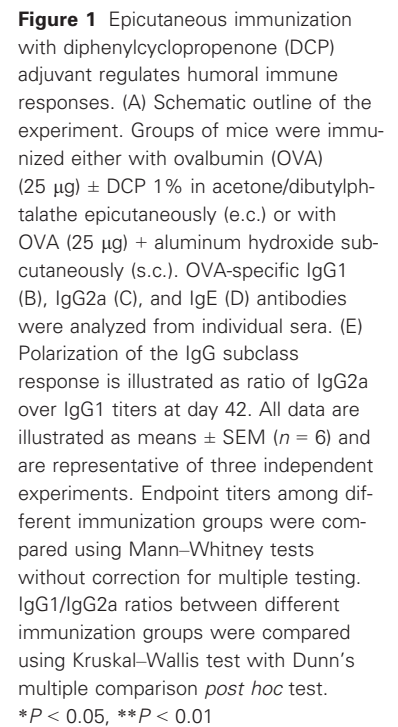
DCP, diphenylcyclopropenone; BAL, bronchoalveolar lavage; LCs, Langerhans cells; DCs, dendritic cells; EPIT, epicutaneous immunotherapy; SCIT, subcutaneous immunotherapy; i.p., intraperitoneal; s.c., subcutaneous.

The objective of the current study was to evaluate the adjuvant potential of diphenylcyclopropenone (DCP) in EPIT using a murine model. Diphenylcyclopropenone is a strong contact sensitizer with proposed immune-modulatory potential (11) and is used clinically in the treatment of *alopecia areata* and of viral warts (12).

Animals

Immunization protocols

Alternatively, mice were sensitized by four weekly i.p. injections of 10- μ g OVA in 100- μ l PBS and 50- μ l aluminum hydroxide. Seven weeks later, the mice were treated four times in weekly intervals by epicutaneous (EPIT) or subcutaneous (SCIT) administrations as described above. Efficacy was tested after anaphylaxis (Fig. 3A) and asthmatic (Fig. 5A) challenges.



Anaphylaxis assessment

Two weeks after the therapeutic immunization, animals were challenged by i.p. injections of 200- μ g OVA in 100- μ l PBS (Fig. 3A). The temperature drop was measured with a rectal probe digital thermometer (Thermalert TH-5; Physitemp, Huron, NJ, USA) prior to and for 120 min after the challenge. Clinical signs of anaphylaxis were recorded and graded (14): 0, no symptoms; 1, mild shock including reduced activity, scratching around nose; 2, moderate shock manifesting as only slight activity after prodding and ruffled fur; 3, severe shock with additional respiratory symptoms or no activity after prodding; and 4, death.

Asthma assessment

Six weeks after immunotherapy (Fig. 5A), four mice from each group were challenged on three subsequent days by intranasal instillation of 50- μ g OVA in 20- μ l PBS. Twenty-four hours later, the mice were euthanized by i.p. injection of 10-mg pentobarbital-Na. Bronchoalveolar lavage (BAL) was performed by flushing the lungs three times with 500- μ l cold PBS via the tracheal canula (BD Insyte, BD Becton, Franklin Lakes, NJ, USA). The pooled BAL fluid was centrifuged, and the supernatants were collected and stored at -20°C until further analysis of cytokines. The RBC-free BAL cells (5×10^5 cells) were spun on cytospin slides (Cytospin 3; Thermo Shandon, Astmoor, UK) and the slides stained with May-Grünwald Giemsa stain (Merck, Darmstadt, Germany). Macrophages, eosinophils, lymphocytes, and neutrophils were counted in fields containing at least 200 cells. Lung histology was performed on tissue fixed in 4% formalin before embedded in paraffin. Tissue sections were stained with hematoxylin and eosin and periodic-acid-Schiff (PAS).

Immunogenicity assays

Cytokines in BAL, serum, or splenocyte-culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using commercial kits from eBiosciences (San Diego, CA, USA) according to the manufacturer's recommendations. Ovalbumin-specific serum antibodies were analyzed by ELISA as previously described (13). IgG1 was measured against an anti-mouse OVA IgG1 standard (Sigma-Aldrich), while IgE was measured after coating with anti-mouse IgE (Serotec, Portland, OR, USA) and against an anti-OVA IgE standard (Serotec). The IgE ELISA was detected using an in-house biotinylated OVA. Splenocyte proliferation was measured by incorporation of radioactive [^3H]-methyl-thymidine (Moravsek Biochemicals, Brea, CA, USA).

Statistical analysis

Statistical analysis was performed with Graph Pad Prism Software Inc. (La Jolla, CA, USA). For combined comparison of multiple groups, we used Kruskal–Wallis analysis with Dunn's multiple comparison *post hoc* test. Two groups were compared using Mann–Whitney *U*-test. Pre- and post-treatment data

were compared using Wilcoxon matched-pairs signed rank test. For statistical analysis of anaphylaxis experiments, individual areas under the curve were determined and compared by one-way ANOVA using Bonferroni's multiple comparison *post hoc* test. Correlations were analyzed using Pearson test. The significance level was set 95%.

Results

DCP enhanced IgG2a in epicutaneous immunization

Epicutaneous immunization with OVA was monitored in mice and compared with subcutaneous immunization (Fig. 1A). While subcutaneous OVA stimulated specific IgG1 after two injections, three to four doses of epicutaneous administrations were required to induce comparable IgG1 titers (Fig. 1B). Diphenylcyclopropanone had no effect on the IgG1 response, but significantly increased IgG2a titers ($P = 0.005$), which were higher ($P = 0.005$) than titers produced by subcutaneous OVA and aluminum hydroxide (Fig. 1C). Ovalbumin-specific IgE antibodies developed with delay and were lower after epicutaneous than after subcutaneous immunization (Fig. 1D). The ratio of IgG2a-to-IgG1 titers suggested an IgG2a-polarizing effect of DCP (Fig. 1E).

DCP facilitated antigen-specific T-cell proliferation and secretion of IL-10 and IL-4

On day 42, splenocytes were isolated and re-stimulated *in vitro* with OVA. All immunization protocols resulted in OVA-specific proliferation at comparable levels (Fig. 2A). Subcutaneous immunization triggered IFN- γ secretion (Fig. 2B), whereas epicutaneous immunization triggered significantly stronger secretion of IL-10 (Fig. 2C) and IL-4 (Fig. 2D). Both IL-10 and IL-4 were enhanced by DCP.

DCP enhanced protection against allergic anaphylaxis in mice treated with epicutaneous immunotherapy

Therapeutic efficacy of epicutaneous immunization was tested in a model of allergic anaphylaxis (Fig. 3A). Sensitized mice were treated by epicutaneous (EPIT) or subcutaneous (SCIT) immunotherapy as described above and challenged with high-dose OVA i.p. Sensitized but otherwise untreated mice experienced anaphylaxis with an average body temperature drop of 7.1°C (Fig. 3B), accompanied by other clinical symptoms (Fig. 3C). Epicutaneous allergen-specific immunotherapy suppressed anaphylaxis with an average temperature drop of 4.5°C , and DCP enhanced the protection (3.8°C) (Fig. 3B,C). Epicutaneous allergen-specific immunotherapy induced a more robust protection against anaphylaxis than did SCIT, which was barely protective. The average body temperature drop after SCIT was 6.0°C , and SCIT produced only little symptom alleviation (Fig. 3B,C). When the change in temperature was analyzed as the areas under the curve over 120 min, only EPIT with DCP revealed a significant suppression of anaphylaxis as compared to untreated mice ($P < 0.05$; Fig. 3D).

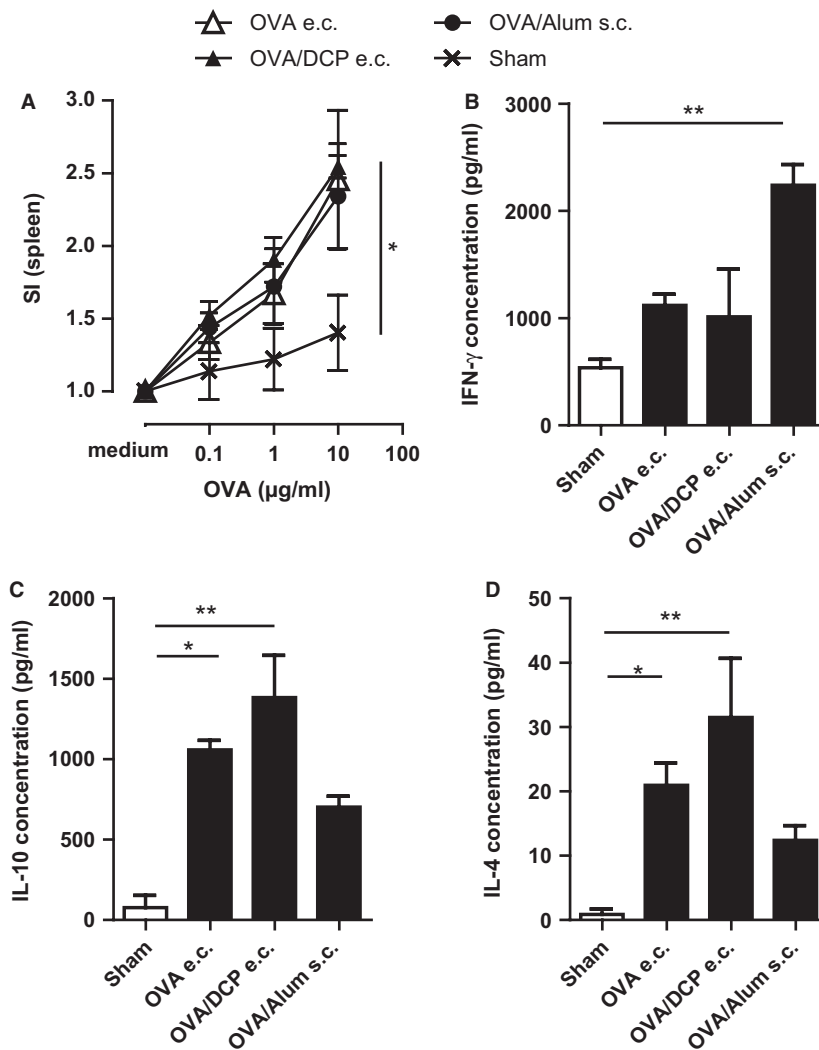


Figure 2 Epicutaneous immunization with diphenylcyclopropanone adjuvant facilitates T-cell responses. Mice were immunized four times as in Fig. 1A. (A) Ovalbumin (OVA)-specific T-cell proliferation was analyzed on splenocytes 2 weeks after the last immunization. Concentrations of IFN-γ (B), IL-10 (C) and IL-4 (D) cytokines after *in vitro* re-stimulation with 10 μg/ml OVA. All data are expressed as means ± SEM ($n = 4$), and the data are representative of three experiments performed. Comparison between different immunization groups and sham treated mice was made by Kruskal–Wallis test with Dunn's multiple comparison *post hoc* test. * $P < 0.05$, ** $P < 0.01$

Anaphylaxis protection correlated with IgG2a antibody titers

None of the treatment protocols enhanced the OVA-specific IgG1 responses beyond that triggered by the sensitization (Fig. 4A). Epicutaneous allergen-specific immunotherapy with DCP caused an 11-fold ($P = 0.034$) increase in OVA-specific IgG2a titers (Fig. 4B). Neither EPIT without DCP nor SCIT enhanced the IgG2a response. Epicutaneous allergen-specific immunotherapy caused no further boosting of IgE, whereas SCIT increased IgE ($P = 0.031$) (Fig. 4C). We found a significant negative correlation between the therapy-induced change in IgG2a and the degree of anaphylaxis ($P = 0.04$; $R = -0.42$; 95% CI, -0.70 to -0.14) (Fig. 4D).

EPIT with OVA and DCP suppressed asthma

Four weeks after the anaphylaxis test, the mice were challenged by intranasal administrations of OVA (Fig. 5A). One day later, the mice were euthanized and BAL fluid and lungs were collected. We observed a reduced number of

inflammatory cells in the BAL of mice treated with EPIT and DCP as compared to SCIT-treated mice (Fig. 5B). Epicutaneous allergen-specific immunotherapy with DCP caused significant reduction ($P = 0.032$) in eosinophils (Fig. 5C), and the reduction in eosinophils was associated with a threefold reduction in eotaxin (CCL11), a chemokine secreted by epithelial cells during allergic asthma reactions (Fig. 5D). Epicutaneous allergen-specific immunotherapy without DCP had no effect on eosinophils (Fig. 5C) or eotaxin (Fig. 5D) levels in the BAL. Epicutaneous allergen-specific immunotherapy with OVA and DCP was associated with increased IgA (Fig. 5E). IL-13, IL-4, IL-5, IL-17, and IL-10 were not affected (Fig. 5F and data not shown). Histological analyses of lung sections revealed a reduction in perivascular and peribronchial eosinophilic inflammation after EPIT with OVA and DCP (Fig. 5G) when compared to untreated mice (Fig. 5H). Subcutaneous allergen-specific immunotherapy reduced neither eosinophilic airway inflammation (Fig. 5B,C) nor eotaxin as compared to untreated mice (Fig. 5D).

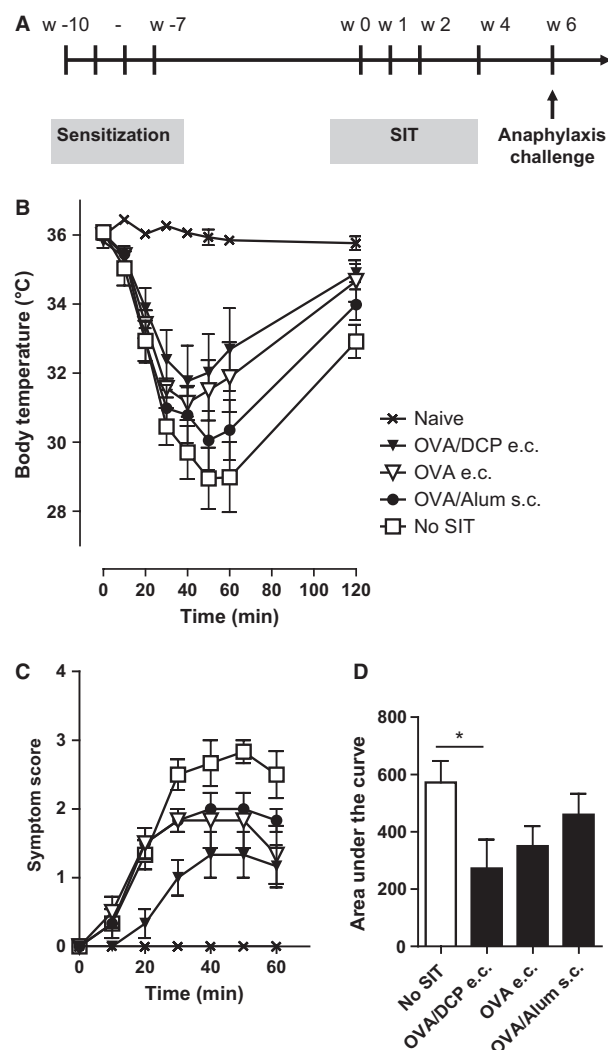


Figure 3 Epicutaneous immunotherapy with diphenylcyclopropenone (DCP) adjuvant protects from anaphylaxis. (A) Schematic outline of the experiment. Groups of mice were sensitized by four i.p. injections of ovalbumin (OVA) (10 μ g) + aluminum hydroxide. Thereafter, mice were treated either with OVA (25 μ g) $\pm$ DCP 1% in acetone/dibutylphthalate epicutaneously (e.c.) or with OVA (25 μ g) + aluminum hydroxide subcutaneously (s.c.). One group of mice was sensitized but received no immunotherapy. Two weeks after the last immunotherapy, mice were challenged i.p. with 200- μ g OVA. (B) Changes in rectal body temperature drop in degrees Celsius and (C) clinical symptom scores were recorded in regular intervals. The data represent the means $\pm$ SEM ($n = 6$) from one of two independent experiments. (D) For group comparison, individual areas under the temperature drop curves were determined and compared by one-way ANOVA using Bonferroni's *post hoc* test. * $P < 0.05$, ** $P < 0.01$

Discussion

The skin is our largest organ and functions as physical barrier against infections and injuries. However, the skin has also developed a second line of defense comprising epidermal

LCs, dermal dendritic cells (DCs), T lymphocytes, and keratinocytes, located in the different layers of the skin (15). This renders the skin an interesting site for the administration of vaccines, provided that the strong barrier function of the *stratum corneum* can be overcome. Not surprisingly then, the skin has been shown to be an attractive application route for vaccination (1) and more recently also for allergen immunotherapy (3–5). To further improve epicutaneous vaccination, we tested the adjuvant potential of the small organic molecule DCP, an obligate contact sensitizer, which is used to treat *alopecia areata*, autoimmune hair loss. It has been suggested that DCP may have immune-regulatory properties with regard to cytokine secretion (11). Diphenylcyclopropenone is known to induce strong proliferative T-cell responses in the murine local lymph node assay and the mouse ear-swelling test (16, 17).

In our hands, DCP influenced the quality of induced immune responses and enhanced the therapeutic efficacy EPIT in mice. Epicutaneous allergen-specific immunotherapy with DCP produced an IgG2a-dominated antibody response, IgG2a being associated with a Th1 response (18). Alleviation of clinical symptoms of IgE-mediated anaphylaxis and asthma correlated with increased levels of IgG2a in the serum and IgA in the BAL fluid. These effects were strongly dependent on DCP, because EPIT without DCP did not produce a comparable degree of protection or immunological correlates.

Immune response polarization after skin immunization has been shown to be governed by activated keratinocytes (19) and functionally diverse subsets of DCs (15). It has been proposed that the degree of epithelial cell stress or damage determines the cytokine milieu, which again plays a key role in shaping the adaptive immune responses induced. While minor chemical or mechanical stress to the epithelium promotes Th2- or Treg-type immune responses, more severe stress or tissue damage favors Th1-type responses (19). Furthermore, LCs in the outermost layer of the skin can trigger generation of Th2- and Treg-type responses as well as cytotoxic CD8 T-cell responses (20). In contrast, activation of dermal DCs has been demonstrated to elicit IgG2a and IgA isotype class switch in B cells (20, 21). These immunological features match our results observed after EPIT. While EPIT without adjuvant DCP might induce only superficial epithelial stress by exclusively activating LCs, EPIT with DCP may represent a more stressful procedure and enabled triggering of IgG2a and IgA production in addition to IL-4 and IL-10 secretion. The action of DCP might therefore be related to its potential to activate dermal DCs, a property possibly related to its role as a contact sensitizer (22). Indeed, effective adjuvants for topical application have been shown to activate and mobilize LCs and DCs similar to that of contact obligate sensitizers (23).

The therapeutic relevance of the DCP-mediated shift from IgG1 to IgG2a dominated antibody responses is underlined by the observed correlation between IgG2a antibody titers and protection from anaphylaxis both here and elsewhere (13, 24). The exact protective role of different antibodies subclasses is incompletely understood, but antibodies of the

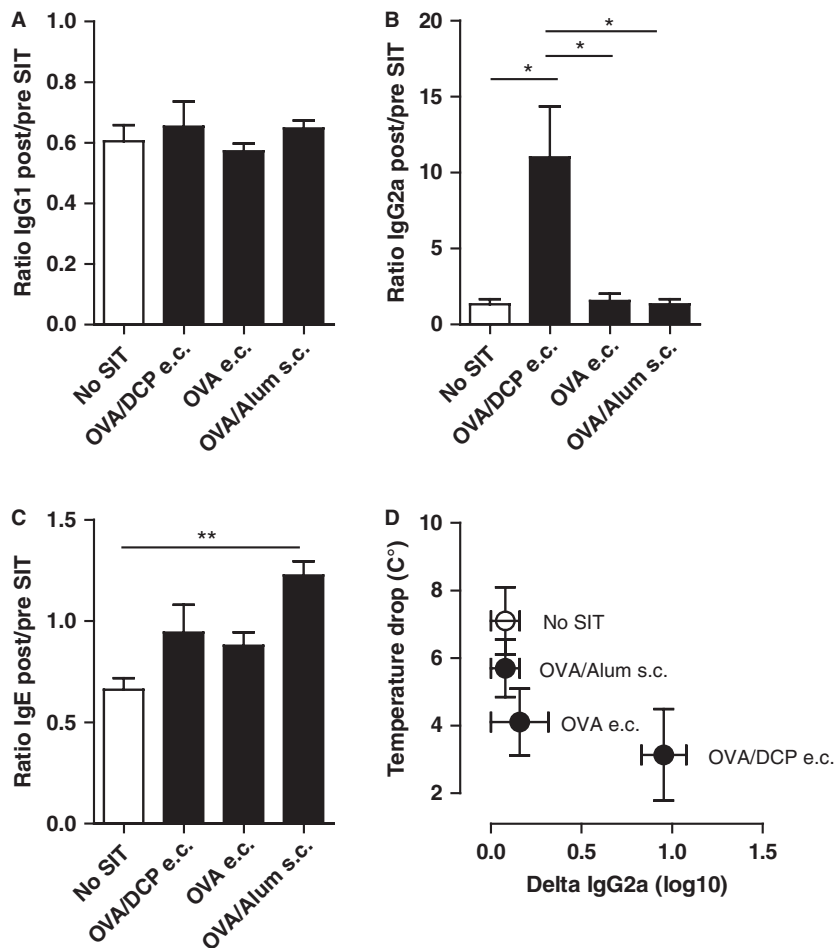


Figure 4 Induction of IgG2a titer correlates with protection against anaphylaxis in mice. Mice were sensitized, treated, and challenged as in Fig. 3A. Before and 2 weeks after the last immunotherapy, sera were analyzed for ovalbumin (OVA)-specific antibodies. (A) Changes in OVA-specific IgG1 concentration, (B) OVA-specific IgG2a titers, and (C) OVA-specific IgE concentrations were compared between different groups with Kruskal–Wallis test with Dunn’s multiple comparison *post hoc* test. * $P < 0.05$, ** $P < 0.01$. All data are represented as mean $\pm$ SEM ($n = 6$). (D) Correlation between the mean temperature drop at 60 min as a function of the mean change in IgG2a titers and as depicted for the different treatment groups ($P = 0.04$ by Pearson test).

IgG2a subclass can capture allergens before these cross-link IgE on mast cells and basophils. As blocking antibody, IgG2a prevent the degranulation of such cells. IgG2a antibodies are also efficient opsonins by activation of macrophages through binding to Fc γ RI and Fc γ RIII (25). IgG2a antibodies might also prevent mast cell and basophil degranulation by signaling through the inhibitory receptor Fc γ RIIB (26); Fc γ RIIB-deficient mice indeed suffer from increased severity of anaphylactic reactions (27). In addition to the critical role of IgG2a in preventing anaphylaxis, the increased secretion of IL-10 can reduce the responsiveness of mast cell and basophil to signals triggered via allergen, IgE and Fc γ receptors (28).

Epicutaneous allergen-specific immunotherapy with DCP also protected mice against an allergic late-phase reactions. We observed reduction in eosinophilic airway inflammation and increased levels of IgA in the BAL fluid of mice treated with DCP-adjuvanted EPIT. IgA can reduce eosinophilic airway inflammation by capturing and neutralizing allergen before it encounters mucosal mast cells (29). This is further supported by clinical data reporting increased frequency of asthmatic airway disease in individuals suffering from selective IgA deficiency (30).

In the current investigation, we demonstrated that DCP is a potent adjuvant for topical use in EPIT with strong potential to induce an antibody response characterized by IgG2a. As the currently clinically tested topical adjuvants such as cholera toxin and heat-labile enterotoxin preferentially induce strong IgG1 polarized antibody responses, DCP fulfills an unmet need. Furthermore, DCP is nonmutagenic, lacks cross-sensitization with substances in the natural or technical environment of humans (31, 32), and has a good safety record after more than 30 years in clinical use as treatment for *alopecia areata* (12). Patients are initially sensitized with a concentration of 2% DCP in acetone. The subsequent applications are at lower concentrations ranging from 0.01% to 0.1%. These elicit only mild and transient eczematous reactions, typically mild erythema and itching. Treatment discontinuation because of side-effects is rare (31, 32).

Immunization with haptens together with proteins has been shown to induce immune responses to (i) the hapten determinant, (ii) the unaltered epitopes on the carrier protein, and (iii) new epitopes formed by regions of both hapten and carrier molecule. In allergen-specific immunotherapy, the goal is the production of antibodies induced to the co-administered

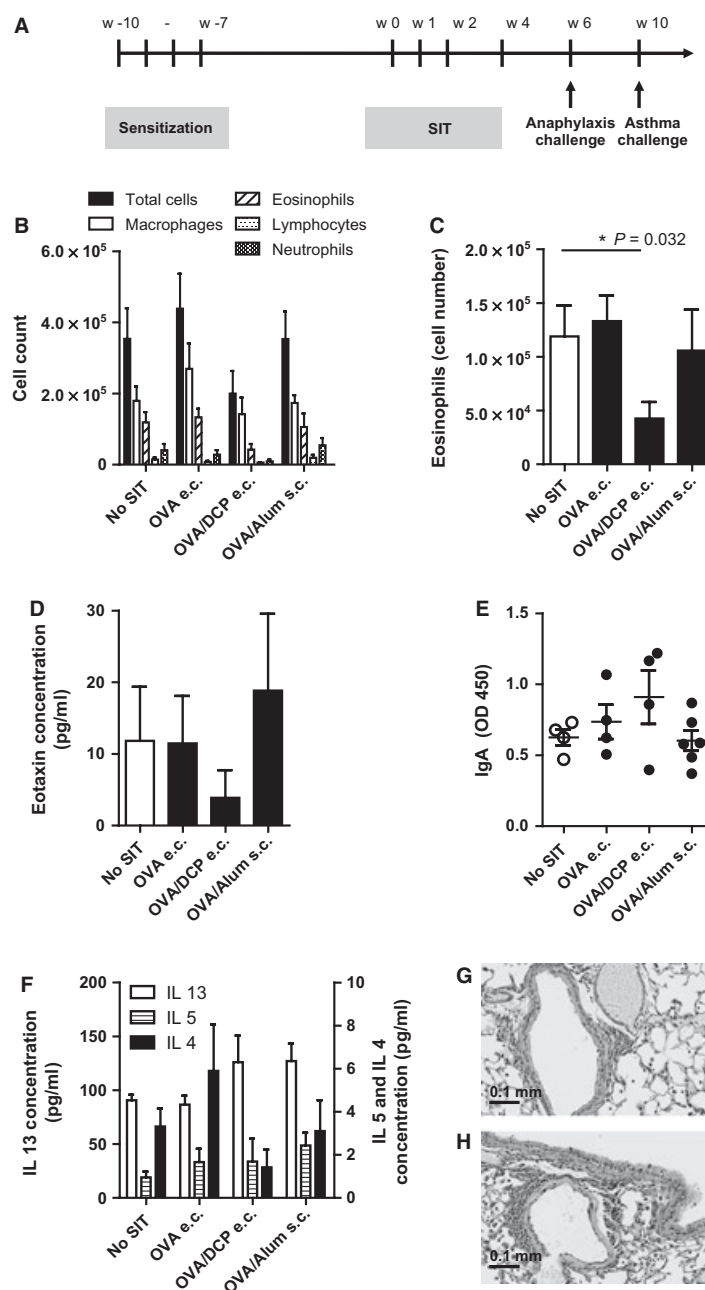


Figure 5 Epicutaneous immunotherapy with diphenylcyclopropanone (DCP) suppresses allergic airway inflammation. (A) Mice were sensitized and treated either with ovalbumin (OVA) (25 µg) ± DCP epicutaneously (e.c.) or with OVA (25 µg) + aluminum hydroxide subcutaneously (s.c.) or left untreated. Six weeks after the last vaccination, mice were re-challenged on three subsequent days with 50-µg OVA intranasally. Twenty-four hours later, BAL was performed and lungs were fixed for histology. (B) Total cell counts and absolute numbers of macrophages, eosinophils, neutrophils, and lymphocytes as counted in the BAL fluid. (C) Numbers of eosinophils were compared between untreated mice and different treatment groups by Mann–Whitney test without correction for multiple testing. * $P < 0.05$. (D) Eotaxin, (E) secretory IgA, and (F) IL-4, IL-5 and IL-13 in the BAL fluid as determined by ELISA. All data are illustrated as means ± SEM ($n = 4$). (G, H) Periodic-acid-Schiff stained lung sections show perivascular and peribronchial cell infiltrates with special focus on eosinophils with donut-shaped nuclei (Scale bar 100 µm). (G) Lung section of a mouse treated with OVA (25 µg) + DCP epicutaneously. (H) Lung section of an untreated mouse.

allergen (here OVA), which we have been shown to be enhanced in the presence of the contact sensitizer DCP. Although antibody responses against DCP might also be induced, such antibodies are unlikely to be of clinical relevance because exposure to DCP in daily life will not occur as this substance is absent in the natural or technical environment of humans, and no cross-sensitization to other substances are known (31, 32).

Research for topical adjuvants is currently focusing on small toll-like receptor ligands agonists such as CpG oligodeoxynucleotides and imidazoquinolines. Topical administration of CpG has been demonstrated to be efficient in priming

IFN- γ producing CD8 T cells, whereas stimulation of antibody responses was poor (8, 10). Similarly, imidazoquinolines have been reported to enhance the induction of cytotoxic T cells (33). Epicutaneous immunization using contact sensitizers as adjuvants may also induce cytotoxic T-cell responses. This property has been exploited in the treatment of metastatic melanoma (34) and viral infections (35). Diphenylcyclopropanone might therefore be a promising adjuvant in skin vaccination and enable further development of patch-based vaccines especially for diseases that necessitate strong neutralizing IgG2a- or IgG4-polarized antibody responses such as IgE-mediated allergy, but also for diseases requiring efficient

Th1-like or cytotoxic T-cell responses such as cancer immunotherapy and vaccination against infections. In the future, efficacy of epicutaneous vaccination is likely to increase by the rational choice of the adjuvant co-administered with the antigen, thereby enabling a better tailoring of the triggered immune responses.

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Conflict of interest

G. Senti and T. M. Kündig are named as inventors on a patent on epicutaneous immunotherapy; the patent is owned by the University of Zurich.

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