

Birch Pollen Immunotherapy in Mice: Inhibition of Th2 Inflammation Is Not Sufficient to Decrease Airway Hyper-Reactivity

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Key Words

Birch pollen allergy · Mucosal immunity · Murine asthma model · Immunotherapy · Airway hyper-reactivity

Abstract

Background: Suppression of Th2 cytokine production by allergen-specific Th2 cells is considered to be critical for the suppression of allergic symptoms by subcutaneous immunotherapy. The aim of this study was to develop a mouse model for birch pollen (BP) immunotherapy to elucidate the underlying mechanisms that contribute to the improvement of clinical symptoms. **Methods:** Mice with BP-induced allergic airway inflammation received weekly subcutaneous immunotherapy (SCIT) injections with BP extract (BPE) adsorbed to alum. The effect of an increasing dose of BPE adsorbed to a fixed concentration of alum on the suppression of airway inflammation and airway hyper-responsiveness (AHR) was determined. After 2, 4, 6 or 8 immunotherapy injections, the mice were rechallenged with the same allergen and all hallmarks of allergic asthma were evaluated. **Results:** Suppression of the immunological parameters by immunotherapy was dependent on the BPE dose. Two injections were sufficient to suppress IL-4, IL-5,

IL-13, IL-10 and IFN- γ production, eosinophil recruitment and peribronchial inflammatory infiltrates. BP-specific immunoglobulins were upregulated, but this was not sufficient to reduce AHR. Eight injections were needed to suppress AHR. The gradual reduction in AHR was inversely associated with the increase of BP IgG2a. **Conclusions:** BP SCIT induces an early suppression of Th2-mediated eosinophilic airway inflammation, but AHR is only effectively reduced after continued SCIT conceivably by allowing IgG2a antibody titres to build up.

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Introduction

Allergen-specific immunotherapy has been used for over 100 years as a treatment for allergic diseases and represents the only causal treatment. It consists of a series of subcutaneous injections with increasing doses of allergen, which induces desensitisation and relief of allergic symptoms. Sublingual immunotherapy is currently eval-

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uated as a safe, non-invasive alternative, but meta-analyses suggest that subcutaneous immunotherapy (SCIT) has greater efficacy when both are compared to placebo [1]. The clinical efficacy of SCIT is well documented, and much progress has been made in elucidating underlying the immunological responses, but it is still a matter of debate as to which immune changes are decisive in reaching the beneficial effect [2–4].

The reduction of allergic symptoms by SCIT has been hypothesised to be dependent on a decrease in Th2 cytokine production. Several mechanisms have been proposed to contribute to this decreased Th2 immunity: T cell anergy, a shift from a Th2 towards a Th1 response and/or the induction of Tregs [5, 6]. The induction of allergen-specific IgG antibodies, in particular IgG4, during SCIT, is another prominent observation [4]. This led to the formulation of the so-called blocking antibody hypothesis, stating that allergen-specific IgG antibodies compete with IgE for allergen binding, thereby neutralizing allergens and preventing IgE cross-linking and degranulation of mast cells. By a similar mechanism, allergen-specific IgG has been shown to inhibit IgE-facilitated antigen presentation by antigen-presenting cells to T helper cells [7]. Interestingly, in humans treated with bee-sting immunotherapy, a decreased tendency for anaphylaxis is observed very early, but the change in the IgE/IgG4 ratio, the inhibited release of mediators from eosinophils and tissue mast cells and the decrease in late-phase skin test reactivity are observed only after months [6]. This suggests that there are sequential effects of immunotherapy on the immune system preventing different clinical symptoms. It is important to establish the sequence of events for the immunological and clinical changes that occur in the path towards effective treatment [7]. To study this in detail, we used a murine model for birch pollen (BP) allergy to determine the sequential effects of BP SCIT. Most BP immunotherapy models use mice which are sensitised with recombinant Bet v 1, this being the major allergen in BP allergic humans. In this study, Bet v 1 sensitisation induced a mild inflammation and no airway hyper-responsiveness (AHR; online suppl. fig 1; for all online supplementary material, see www.karger.com/doi/10.1159/000368777) in contrast to mice sensitised with BP [8–10]. We show here that reduction of Th2 cytokine production by BP SCIT was not sufficient to immediately reduce AHR in mice, but was rather associated with the sequential effects on IL-17A production, broncho-alveolar lavage fluid (BALF) IL-5 levels and increased titres of BP IgG2a.

Materials and Methods

Mice

Female BALB/c mice, 6–8 weeks of age (from Harlan, Horst, The Netherlands) were housed under specific, pathogen-free conditions at the animal facility of the Academic Medical Center. All experiments were approved by the Center's Animal Ethics Committee.

Study Design

Sensitisation to Birch Pollen

BALB/c mice were sensitised intraperitoneally (fig. 1a) on days 0, 7 and 14 with 8.2 µg birch pollen extract (BPE; HAL Allergy BV, Leiden, The Netherlands) containing 117 µg Bet v 1/mg dry weight extract (determined by Bet v 1 ELISA) and an endotoxin level of 2.84 EU/mg (determined by the limulus amoebocyte lysate assay; Indoor Biotechnologies Inc., Cardiff, UK), using a dose equivalent to 1 µg Bet v 1, adsorbed to 1 mg of alum (Imject Alum; Pierce, Rockford, Ill., USA) or as a control with alum alone. Under isoflurane anaesthesia, the mice were challenged intranasally with 100 µg BPE (equivalent to a dose of 11.7 µg Bet v 1) in 30 µl PBS or PBS as a control on days 21–23. One day later, AHR to methacholine was assessed. On day 25, the mice were sacrificed for the analysis of eosinophil recruitment to the airways, IL-4, IL-5, IL-13, IL-10, IL-17A and IFN-γ production, airway histology and the humoral immune response (BP-specific IgE, IgG1 and IgG2a).

BP SCIT

First, a dose response experiment was performed. On days 35, 42, 49, 56, 63, 70, 77 and 84, mice with a BP-induced allergic airway inflammation underwent SCIT, adapted from a protocol for grass pollen described by Mondoulet et al. [11], administering 0.01, 0.1, 0.3 or 3 mg BPE adsorbed to 1 mg alum; control mice received PBS. To investigate the effectiveness of the immunotherapy, the mice were rechallenged with a 0.1% BPE aerosol on days 98–100 and on day 101, AHR was assessed by methacholine challenge. One day later, the mice were sacrificed to evaluate the same immunological parameters as before the start of immunotherapy (fig. 1a).

Second, a kinetic experiment was performed. The mice with a BP-induced allergic airway inflammation received 2, 4, 6 or 8 SCIT injections containing 0.3 mg alum-adsorbed BPE or PBS as a control. The concentration of 0.3 mg was found to be effective, though suboptimal compared to a 3-mg dose, but was chosen so as to limit distress to the animals caused by the severe drop in core body temperature upon injection of 3 mg. Two weeks after the last immunotherapy injection, the mice received a 30-min administration of 0.1% BPE aerosol on 3 consecutive days. AHR upon methacholine challenge was determined 24 h after the last BPE aerosol application and the mice were sacrificed 1 day later for analysis of immunological parameters (fig. 1b).

Collection and Analysis of Tissues

BALF

Broncho-alveolar inflammatory cells were obtained by intratracheal cannulation and the airway lumen was lavaged 3 times with 1 ml PBS containing 0.1 mM EDTA. Cell differentiation was done by flow cytometric analysis as described before [12]. The BALF was stored at –20°C until IL-5 and eotaxin-2 were determined by ELISA (Ready set go!; eBioscience Inc., Immunosource, Halle-Zoersel, Belgium).

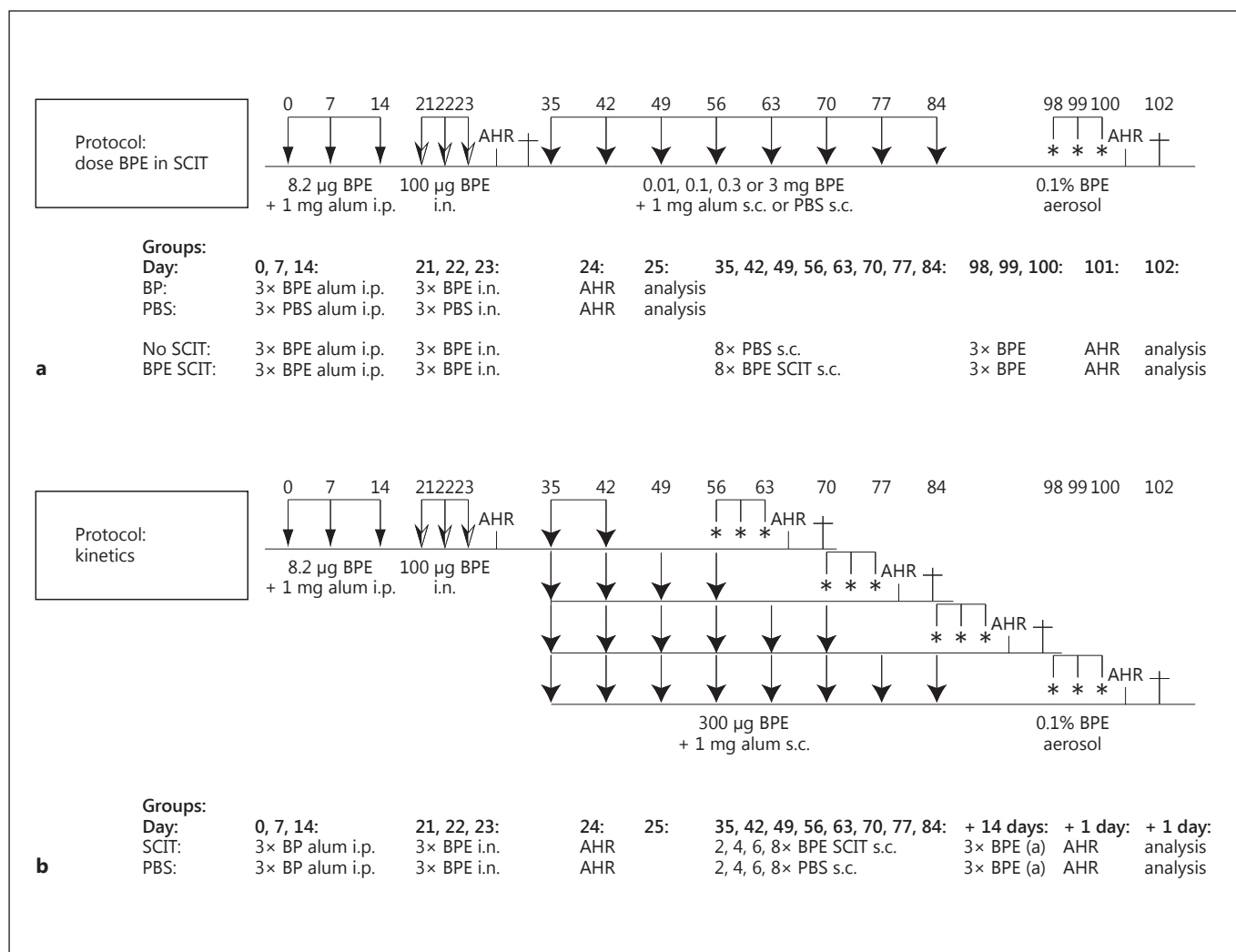


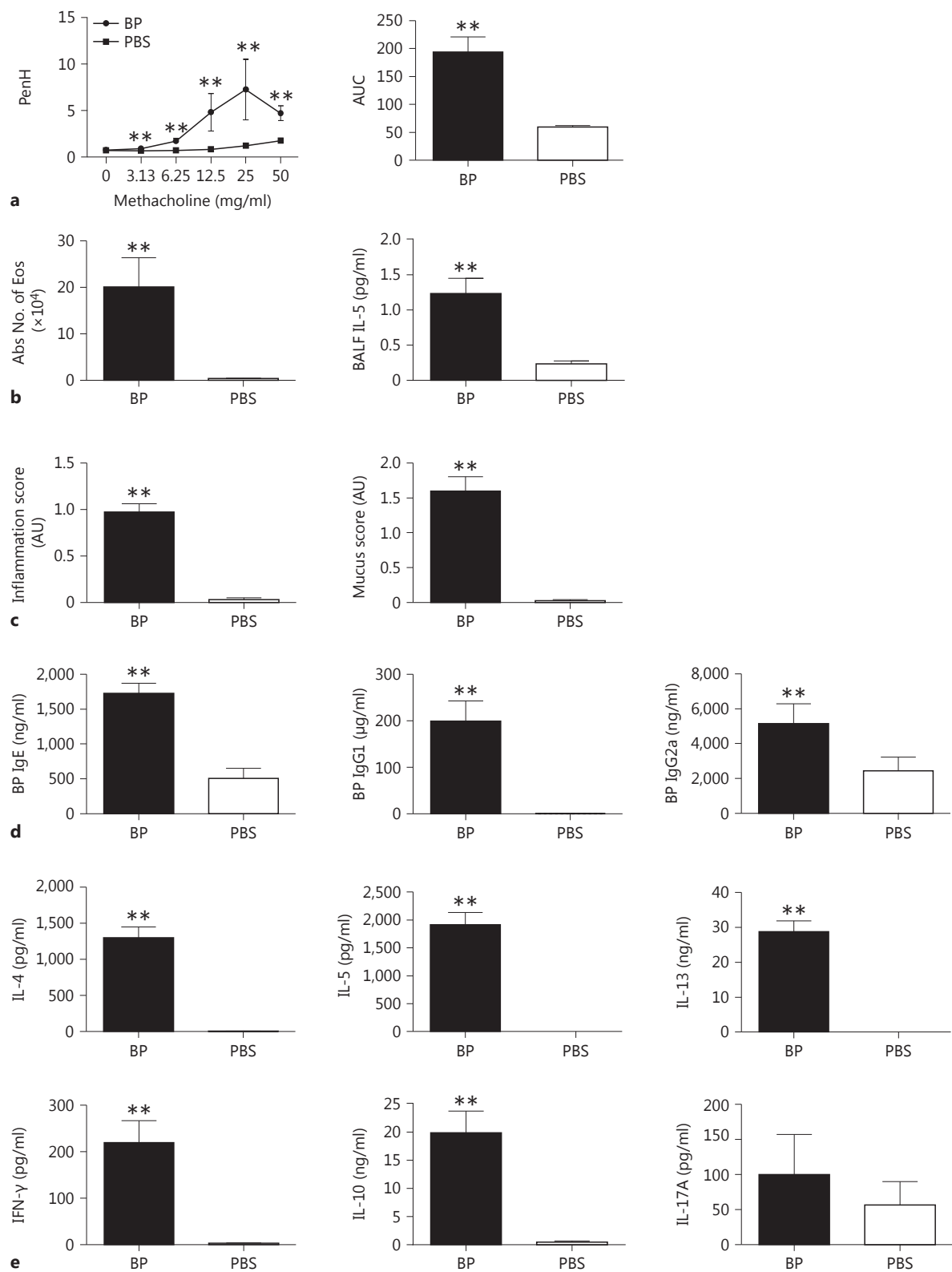
Fig. 1. Experimental designs. **a** Mice were sensitised intraperitoneally (i.p.) by BP adsorbed to alum on days 0, 7 and 14, and were challenged with BP intranasally (i.n.) on days 21–23. AHR was determined 24 h after the last BP challenge, and 1 day later, a subgroup of mice was sacrificed for analysis of immunological parameters on day 25. Ten days later, the mice received a weekly SCIT (BP adsorbed to 1 mg of alum) injection with 0.01, 0.1, 0.3 or 3 mg of BP. They were rechallenged 2 weeks after the last injection with 3 daily aerosol applications of 0.1% BPE. One day later, AHR was

determined, and 24 h later, the mice were sacrificed to examine the immunological parameters. **b** In the kinetic experiment, the mice with a BP-induced allergic airway inflammation received 2, 4, 6 and 8 BP SCIT injections (BP/alum containing 0.3 mg of BP adsorbed to 1 mg of alum). Two weeks after the last injection, they were rechallenged with a daily BPE aerosol application for 3 consecutive days. One day later, AHR was determined, and 24 h later, the mice were sacrificed to examine the immunological parameters. * 0.1% BPE aerosol.

Fig. 2. Mice developed BP-induced airway inflammation and AHR before starting BP SCIT. The mice were sensitised and challenged with BP (according to fig. 1). **a** AHR: PenH dose-response curves and average AUC of individual PenH dose-response curves were determined 24 h after the last challenge (on day 24). The mice were sacrificed 1 day later (day 25) for analysis of eosinophil recruit-

ment and IL-5 concentration in the BALF (**b**), peribronchial infiltrates and mucus production (**c**), BP-specific IgE, IgG1 and IgG2a (**d**) and cytokine production after ex vivo restimulation of lung-draining lymph node cells with BP (**e**). * $p < 0.05$, ** $p < 0.01$ vs. PBS.

(For figure 2 see next page.)



Restimulation of Lung-Draining Lymph Node Cells ex vivo

Lung-draining lymph node cell suspensions were plated in 96-well round-bottom plates at a density of 2×10^5 cells/well and were restimulated for 4 days with 100 µg/ml of BPE. Supernatants were analysed for IL-4, IL-5, IL-13, IL-10, IL-17A and IFN-γ production by ELISA.

Serum BP-Specific Immunoglobulins

Serum was analysed for the level of BP-specific IgE, IgG1 (Opteia, BD, San Diego, Calif., USA) and IgG2a (eBioscience) by ELISA, as previously described [13]. In short, maxisorp plates were coated overnight with 100 µg of BPE. After blocking with 10% FCS, serum samples were incubated for 2 h, followed by an HRP detection step, according to the manufacturer's instructions. Serum samples of BP-allergic animals were diluted 200, 200,000 and 50 times for IgE, IgG1 and IgG2a, respectively, while the sera of PBS control animals were diluted 30, 100 and 50 times, respectively. A standard curve of murine IgE, IgG1 or IgG2a, respectively, was used as a quantitative reference.

Airway Histology

Frozen sections (6 µm) were stained with periodic acid Schiff (Sigma-Aldrich). The degree of inflammation and mucus production was semi-quantified, as described before [13].

Airway Hyper-Responsiveness

AHR to inhaled methacholine was measured by barometric plethysmography in the conscious mice (EMKA Technologies). Non-specific responsiveness was measured by exposing mice to aerosolized PBS to set a baseline value, followed by increasing concentrations of aerosolized methacholine (3.125, 6.25, 12.5, 25 and 50 mg/ml in PBS for 3 min; Sigma-Aldrich) using ultrasonic nebulizers. PenH (enhanced pause) values were measured during 5 min after each methacholine aerosol application. For each individual animal, the area under the dose-response curve of PenH to increasing concentrations of inhaled methacholine was determined.

Core Body Temperature

The core body temperature was measured with a hand-held device with a mouse rectal probe (Physitemp Instruments Inc., Clifton, N.J., USA) 30 min after administration of each SCIT injection.

Statistical Analysis

For statistical analysis, differences between groups were analysed with the Mann-Whitney U test using IBM SPSS Statistics 20 software. Association between parameters was determined by the Pearson correlation coefficient *r*. Differences were considered to be significant at a *p* value <0.05.

Results

Airway Inflammation Preceding BP SCIT

BP-induced inflammation was induced in BALB/c mice as described in the Materials and Methods section (fig. 1a). Compared to the PBS-sensitised and challenged control mice, the BP-sensitised and challenged mice developed AHR (fig. 2a). This was accompanied by recruitment of eosinophils to the airways and increased levels of IL-5 in the BALF (fig. 2b), inflammatory infiltrates around the bronchi (fig. 2c, left panel), increased mucus production (fig. 2c, right panel), the induction of BP-specific IgE, IgG1 and IgG2a (fig. 2d) and BP-specific IL-4, IL-5, IL-13, IL-10 and IFN-γ production by lung-draining lymph node cells. IL-17A was detected at slightly higher levels in the BP-exposed mice, but this did not exceed the background level measured in the controls (fig. 2e).

Dose-Dependent Modulation of Immune Responses by BP SCIT

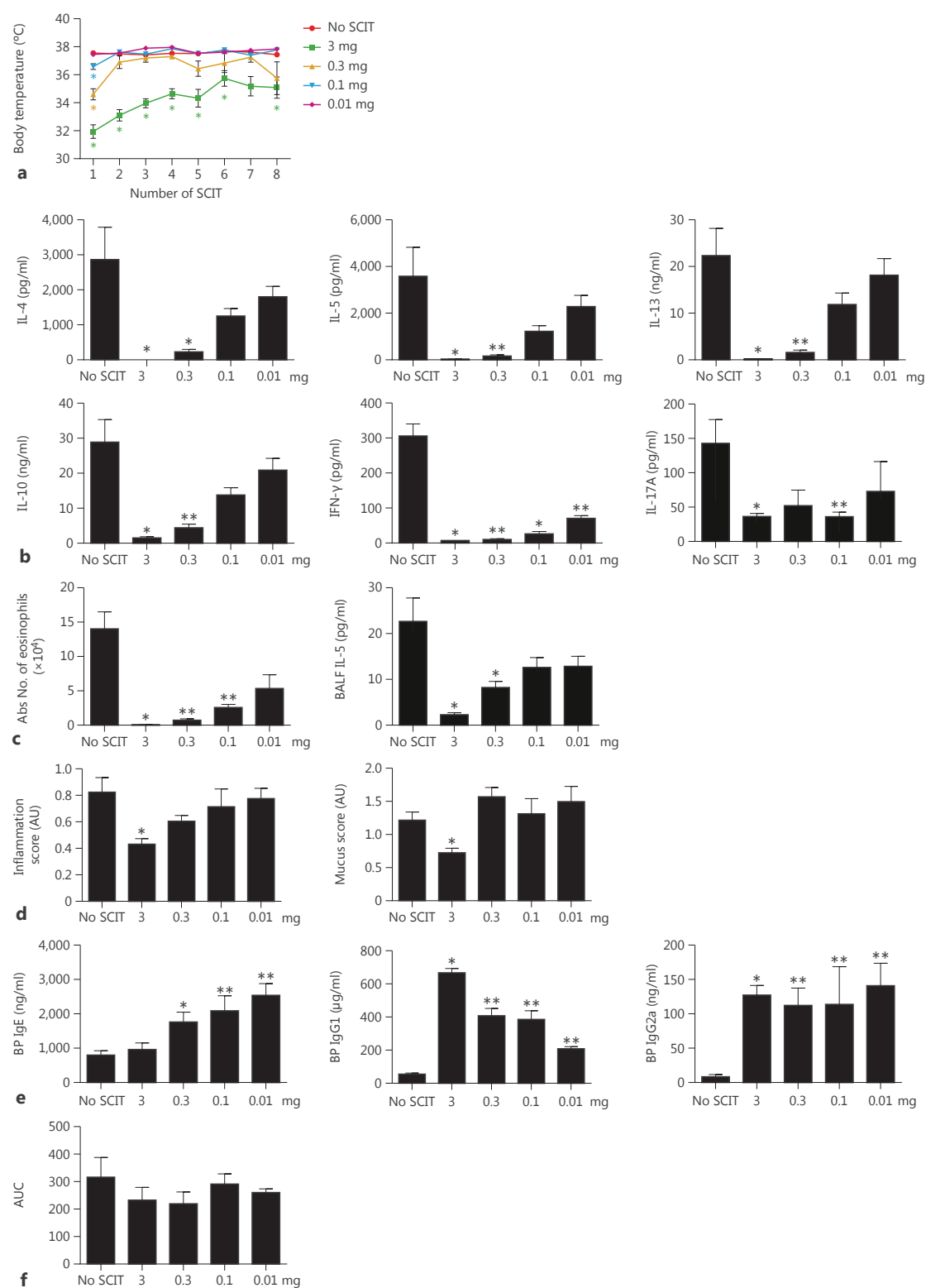
To assess the efficacy of BP SCIT to reduce the immune response to inhaled BP, the BP-sensitised mice were treated weekly with 0.01, 0.1, 0.3 or 3 mg of BPE adsorbed to 1 mg alum (s.c.) and the immune response was evaluated after inhalational challenge with BP. To determine whether injection with BP SCIT induced an anaphylactic response, the body core temperature was measured 30 min after each injection [14]. BP SCIT induced a drop in body temperature after the first injection in a dose-dependent manner, but subsequent injections led to much less pronounced effects on body temperature, except in the mice receiving the highest (3 mg) allergen dose (fig. 3a).

The efficacy of BP SCIT to suppress cytokine production by BP-specific Th2 cells was analysed. BP SCIT inhibited the Th2 cytokine production of IL-4, IL-5 and IL-13 but also of Th1-related IFN-γ, Treg-related IL-10 and Th17-related IL-17A, in a dose-dependent way (fig. 3b). The recruitment of eosinophils, the local concentration of IL-5 in the BALF (fig. 3c) and the induction of peribronchial inflammatory infiltrates were sup-

Fig. 3. BP SCIT effects are dose dependent. Mice with BP-induced allergic airway inflammation were treated with 8 SCIT injections of 0.01, 0.1, 0.3 or 3 mg of BP adsorbed to 1 mg alum. **a** The effect of the different concentrations of BP on the drop in core body temperature was assessed 30 min after each injection. Mice were re-challenged with 3 BPE aerosols. Cytokine production after ex vivo

restimulation of lung-draining lymph node cells (**b**), eosinophil recruitment and IL-5 concentration in the BALF (**c**), mucus production and peribronchial infiltrates (**d**), and BP-specific IgE, IgG1 and IgG2a levels in serum (**e**) were analysed. **f** AHR was determined 24 h after the last aerosol application. * *p* < 0.05, ** *p* < 0.01 vs. no SCIT.

(For figure 3 see next page.)



pressed in a dose-dependent way (fig. 3d, left panel). Mucus production in response to exposure to BPE aerosols was only inhibited after the 3-mg BP SCIT treatment (fig. 3d, right panel). The induction of BP-specific IgE was inversely correlated with BP SCIT dose, whereas BP-specific IgG1 showed a positive dose-response relation (fig. 3e). BP-specific IgG2a was increased but did not show a clear dose response. BP SCIT did not suppress AHR with a similar dose-dependency as was seen for the other parameters. Although a modest decrease in AHR was observed, significance was not reached in this particular experiment as in the other experiments (fig. 5d), likely because of a lack of statistical power due to the insufficient number of mice left in the 'no SCIT' group for AHR measurement ($n = 5$; fig. 3f).

Kinetics of the Inhibition of Th2 Inflammation and Amelioration of AHR

We determined how many BP SCIT injections were needed to suppress the immunological parameters and AHR. Mice were rechallenged with BPE aerosols after 2, 4, 6 or 8 BP SCIT injections. As in the previous experiment, the first SCIT injection induced a sharp decrease in body temperature which was not observed after following injections, although a small but significant drop was still observed at 4, 6 and 8 injections (fig. 4a). Two SCIT injections suppressed IL-4, IL-5, IL-13, IFN- γ and IL-10 production (fig. 4b), eosinophil recruitment (fig. 4c) and peribronchial inflammation (fig. 4d, left panel) to a level similar to that seen after 8 BP SCIT injections. IL-17A suppression was observed after 4 and 8 BP SCIT injections (fig. 4b). Interestingly, BALF IL-5 was not inhibited until the 8th BP SCIT injection (fig. 4c, right panel). In contrast to the cellular response, additional SCIT injections did have an effect on the humoral immune response. An increasing number of SCIT injections induced a rise in BP-specific IgE, IgG1 and IgG2a in the serum. The increase of BP-specific IgE levelled out after 6 injections, and that of BP-specific IgG1 levelled out after the 4th injection (fig. 4e). In contrast, BP-specific IgG2a steadily

increased up to the 8th injection. Interestingly, AHR was only inhibited significantly after 8 injections compared with mice receiving PBS (fig. 5). Both groups of BP-sensitised mice (receiving either 8 PBS or 8 SCIT injections) had a similar AHR before starting therapy (day 24) and mice receiving PBS had a similar increased AHR at day 102 after BP rechallenge compared with day 24 (fig. 1b, 5e). The level of BP-specific IgG2a correlated strongly with the suppression of AHR (fig. 5g: $r^2 = 0.78$ and $p = 0.0034$), while BP-specific IgE and BP-specific IgG1 were less strongly correlated with the suppression of AHR (both $r^2 = 0.64$, $p < 0.05$). Eight times BP SCIT decreased the BP IgE/BP IgG2a ratio compared to 2 $\times$ and 4 $\times$ SCIT and, although not significantly, to 6 $\times$ BP SCIT (fig. 5h).

Discussion

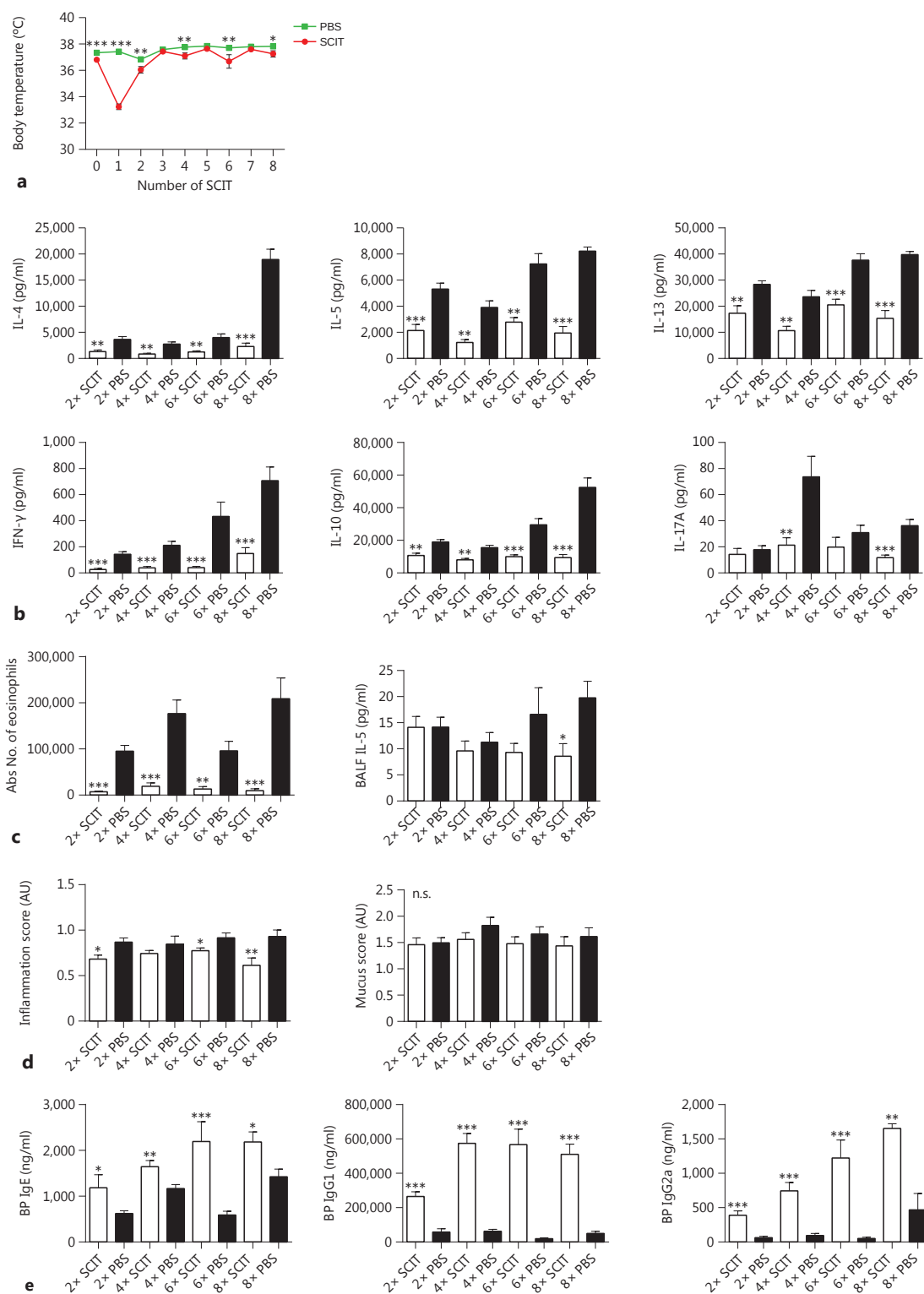
In this study, it was shown that the suppression of Th2 cytokine production and eosinophil recruitment was not sufficient to reduce AHR, but that a prolonged period of BP SCIT was needed.

Interestingly, although BP SCIT induced an early suppression of Th2 cytokine production and eosinophil recruitment not sufficient to inhibit AHR, the anaphylactic response, as demonstrated by a drop in body temperature after the first SCIT injection, was almost completely absent after the second BP SCIT injection, except for with the highest BP concentration. In human immunotherapy, this early protective effect against anaphylaxis has been explained by desensitisation of mast cells and basophils [6, 15]. Although AHR showed a trend to decrease with the number of injections (fig 5f, g), for significant suppression of AHR compared to 'no SCIT' treatment, 8 injections were needed. Interestingly, the gradual decrease in AHR was inversely correlated with an increase in BP-specific IgG2a. A similar gradual increase in allergen-specific IgG2a during SCIT treatment was reported in an ovalbumin (OVA) SCIT model [16]. Whether BP-specific IgG2a antibodies have a functional role or whether they

Fig. 4. Kinetics of suppression of allergic airway inflammation during BP SCIT. Mice with BP-induced allergic airway inflammation were treated with 2, 4, 6 or 8 SCIT injections consisting of 0.3 mg BP absorbed to 1 mg alum, and were then rechallenged 2 weeks later with 3 BPE aerosols. **a** The core body temperature was assessed 30 min after each BP SCIT injection. The effects of BP SCIT

was determined on cytokine production of lung-draining lymph node cells (**b**), eosinophil recruitment and IL-5 concentration in the BALF (**c**), peribronchial inflammation and mucus production (**d**), and BP-specific IgE, IgG1 and IgG2a levels in serum (**e**) were analysed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. mice receiving corresponding number of PBS injections instead of BP SCIT.

(For figure 4 see next page.)



are more an epi-phenomenon of another pathway still has to be explored. A possible mechanism could be via competition for IgE-mediated basophil release, as recently described in BP-sensitised mice, in which treatment with rabbit anti-Bet v1 IgG inhibited IgE-mediated basophil degranulation [17]. In support of a functional role for BP-specific IgG2a, intratracheal instillation of OVA-specific IgG2a or IgG1 in mice with OVA-induced allergic asthma was sufficient to suppress AHR after OVA aerosol challenge [18]. Likewise, in a Fel d 1 immunotherapy model, it was demonstrated that allergen-specific IgG1 and IgG2a, but not CD4⁺ T cells, were critical for the alleviation of local and systemic symptoms [19]. In humans, the induction of allergen-specific IgG antibodies, especially of the IgG4 subclass, is a hallmark of SCIT, and its bioactivity to block IgE-allergen interactions has been associated with clinical improvement [20–22]. In contrast, Shirinbak et al. [23] showed that, in mice, the suppression of a Th2-driven airway inflammation after OVA SCIT was not dependent on the induction of IgG or IgA responses, Fcγ-RIIB signalling or B-cell function, but the effect on AHR was not examined. Our study suggests that the induction of immunoglobulins might play a role in the suppression of AHR following the suppression of a Th2 cytokine response.

The serum IgE concentration was inversely correlated with the concentration of allergen used in the BP SCIT formulation, in contrast to the dose-dependent suppression of IL-4. We cannot exclude that this result might have been caused by binding of the coated BPE by BP-specific IgG1 in the ELISA. This needs further investigation.

Both eosinophil recruitment and IL-5 production in lung-draining lymph nodes was suppressed early in BP SCIT, whereas concentrations of IL-5 in the BALF decreased only after 8 SCIT injections. This did not hamper the suppression of eosinophil recruitment because eotaxin-2 was suppressed; this is considered to be the main chemoattractant for eosinophils while IL-5 is involved mainly in the activation and survival of eosinophils [24]

(online suppl. fig. 2). Eosinophils have been thought to be instrumental in AHR, but in this study, the inhibition of eosinophilic airway inflammation after 2 SCIT injections was not accompanied by reduced AHR. Eosinophils can be dispensable for AHR as recently shown in eosinophil-deficient mice that developed AHR in response to house dust mite extract [25]. Hamelmann et al. [26] described 15 years ago that IgE is important for AHR development especially when there is a limited number of eosinophils. It is therefore tempting to speculate about dependency on an eosinophil/BP-specific IgE/IgG2a axis for the amelioration of AHR. Although BP SCIT suppressed eosinophil recruitment very early, BP-specific IgE did not decrease (or even increased), as seen in humans after starting SCIT [27], and the BP-IgG2a might had to increase sufficiently to compete with the BP-specific IgE in order to result in the amelioration of AHR.

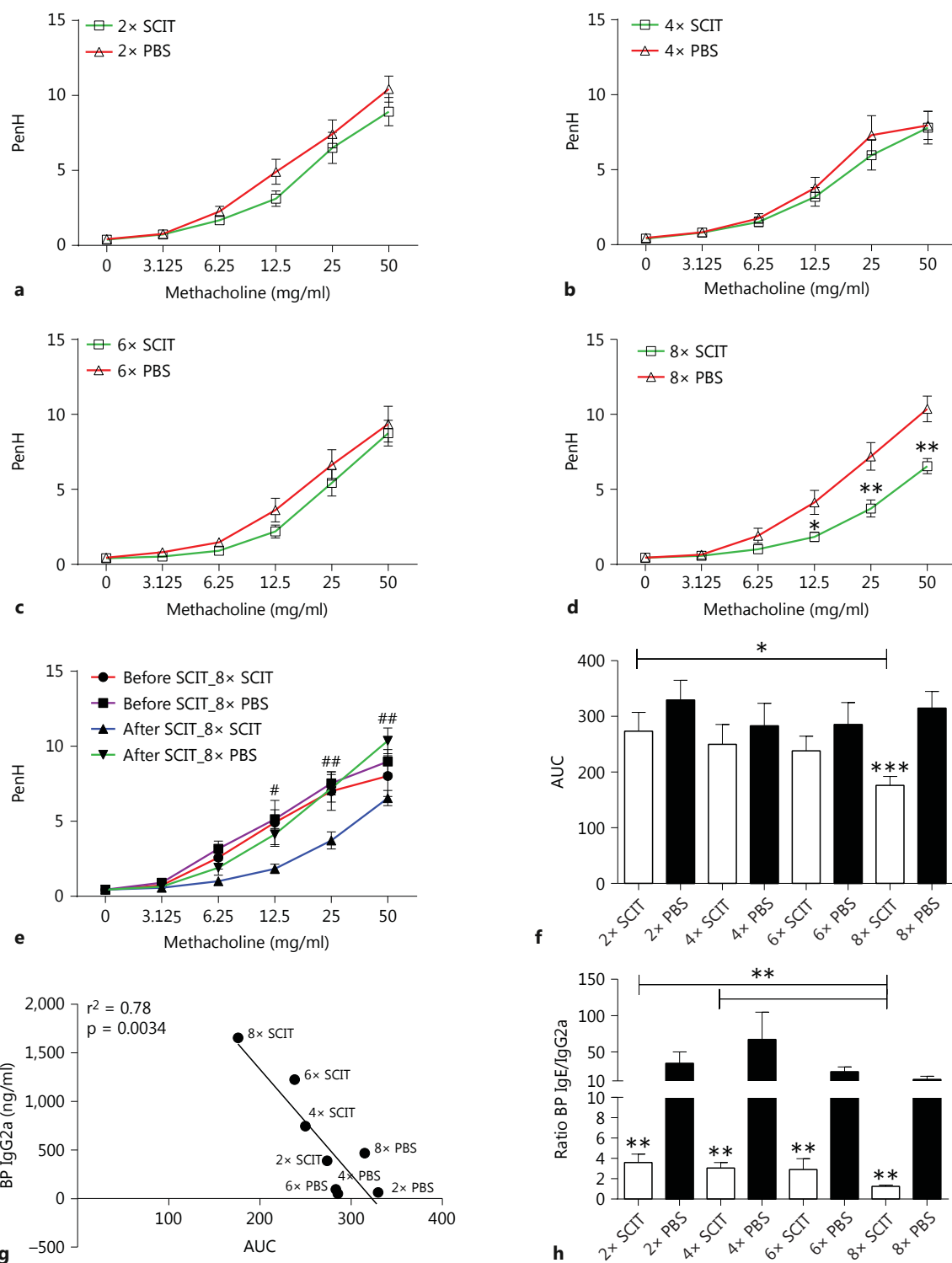
As increased IL-17A levels have been associated with an increase in AHR in both mice and humans, the levels of IL-17A were analysed before and after BP SCIT [28, 29]. Although IL-17A production was only slightly, but not significantly, enhanced in BP-sensitised mice before starting BP SCIT, 8 injections of BP SCIT did suppress IL-17A production significantly. This suggests that the suppression of IL-17A contributed to the observed amelioration of AHR.

A critical issue for interpretation of the kinetic experiment is whether the AHR in the PBS-treated mice was stable during the full 8 weeks of treatment. In fact, AHR was identical after 2, 4, 6 and 8 weeks of PBS treatment. It can therefore be ruled out that the inhibition of AHR observed in the mice receiving 8 weeks of active immunotherapy could be explained by a 'natural' loss of AHR. It was due to the BP SCIT treatment. Interestingly, the PBS-treated control mice demonstrated increased IL-4, IL-5, IL-13, IL-10, IL-17A and IFN-γ production, eosinophil influx and IgE production after BP rechallenge after 8 weeks compared with after 2 weeks ($p < 0.05$), suggesting that the increased interval without exposure to the allergen increased the Th2 inflammation after the inhalation

Fig. 5. Inhibition of AHR correlated with the induction of BP IgG2a. Mice treated with 2 (a), 4 (b), 6 (c) or 8 (d) BP SCIT injections were rechallenged with 3 BPE aerosols, and 24 h later, AHR was determined. AHR was also determined before starting BP SCIT or PBS treatment and compared with AHR after 8 BP SCIT or 8 PBS treatments (e). f The average AUC of the PenH values depicted in a–d were determined and plotted against the average level of BP IgG2a in serum found in mice receiving 2, 4, 6 or 8 BP

SCIT injections or PBS as a control. g Correlation was assessed by determining the correlation coefficient. h The BP IgE/BP IgG2a ratio was compared between the mice in the BP SCIT group (2, 4, 6 or 8 injections) and those in the PBS control group and between the different numbers of SCIT injections. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. mice receiving corresponding number of PBS injections instead of BP SCIT. # $p < 0.05$, ## $p < 0.01$, After SCIT_8x SCIT vs. After SCIT_8x PBS $p < 0.01$.

(For figure 5 see next page.)



of BPE aerosols. The mechanism underlying this aggravation of Th2 inflammation is unclear. Our study shows that BP SCIT was capable of suppressing this expansion of Th2 inflammation to levels similar to those observed in mice receiving 2 BP SCIT injections.

One goal of clinical research into allergen-specific SCIT is to identify a marker for successful SCIT. Our study suggests that a decrease in Th2 cytokine production in the T cells after SCIT may confirm that the immune system is being redirected in a desirable manner. This, however, does not automatically result in an improved clinical outcome. In human SCIT, the functional activity of IgG4 seems to be one of the most useful markers at the moment [7, 22]. Mice do not produce IgG4 and murine IgG1 is considered to be its murine homologue [30, 31]. In our mouse model, we found that BP-specific IgG1 was the most abundant subclass after BP SCIT; however, BP IgG2a was more strongly correlated to the amelioration of AHR and may play a similar role to human IgG4.

In conclusion, we showed, for the first time, that BP SCIT induced sequential effects in mice. The suppression of IL-4, IL-5, IL-10, IL-13 and IFN- γ production, peri-

bronchial inflammatory infiltrates and induction of allergen-specific immunoglobulins was established before AHR was suppressed. The suppression of AHR was accompanied by the suppression of IL-5 levels in the BALF, the suppression of IL-17A and the induction of BP IgG2a. In contrast, mice became resistant to anaphylactic responses to SCIT injections, measured by a drop in body temperature, after only 1 injection. This model may be helpful as a preclinical model to improve immunotherapy for BP allergy and to identify therapeutic targets to ameliorate clinical symptoms.

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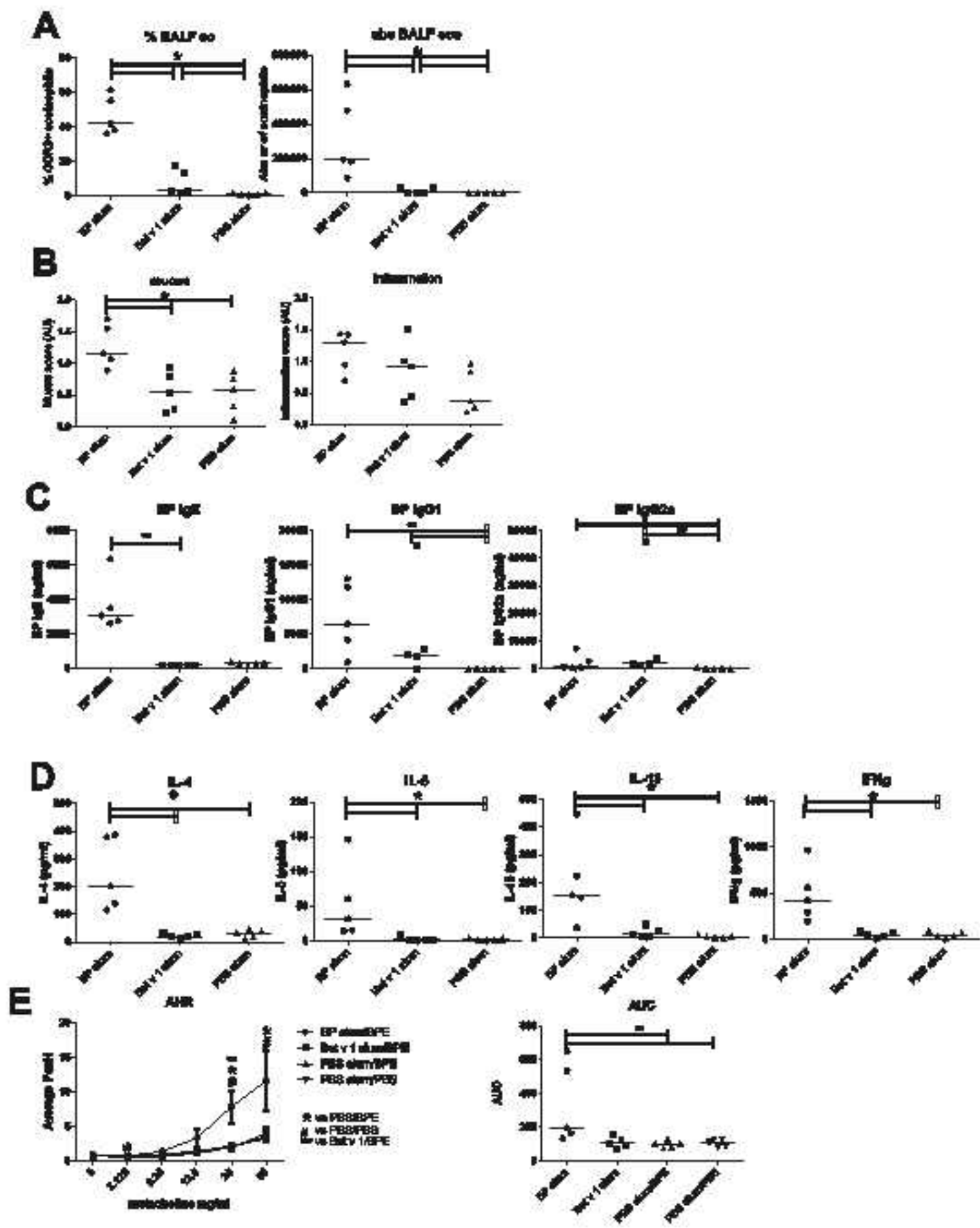
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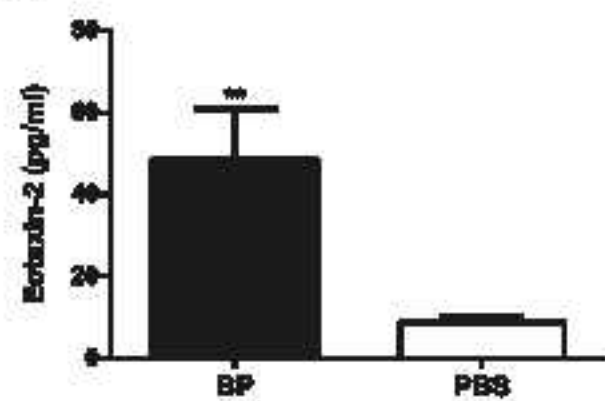
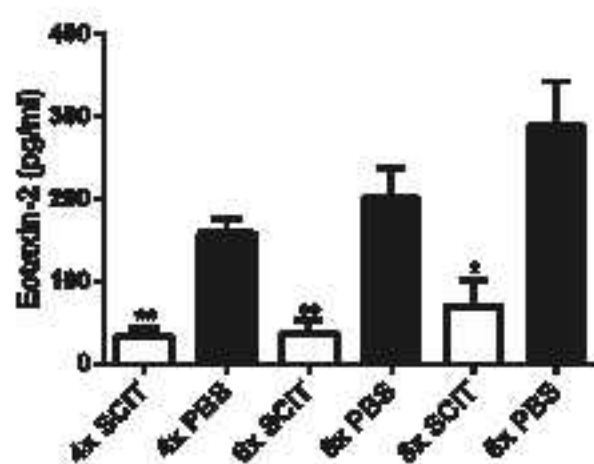
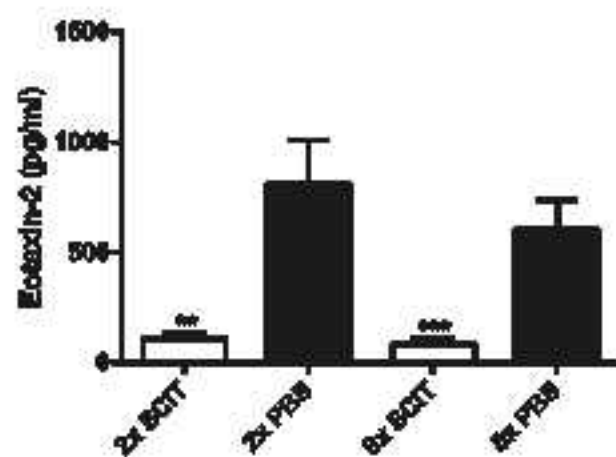
D.-J.O. and H.P.M.v.d.K. are employees and R.v.R. is a consultant of HAL Allergy Leiden, The Netherlands.

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A**B**

Supplementary figure 1. Mice received at day 0, 7, 14 an i.p. injection with Bet v 1 (10 µg)/alum (1 mg) or BPE (8.2 µg BP containing 1 µg Bet v 1)/alum(1 mg). Mice were challenged at day 21, 22, 23 with 100 µg BP intranasally. AHR was determined at day 24 and mice were sacrificed at day 25 to analyze the immunological parameters.

Supplementary figure 2. Eotaxin-2 levels were determined in individual BALF samples of mice with a BP induced eosinophilic airway inflammation (A), or in BALF of mice which received 2x BP SCIT or 8x BP SCIT (left panel) or in pooled BALF samples of 2-3 animals after treatment with 4, 6 or 8x BP SCIT (right panel). The pooled samples were obtained from two independent experiments and were analysed together to achieve a sufficient number of data points for statistical analysis (B). BP SCIT treated mice were rechallenged with 3 BPE aerosols and 48 hours later the airways were lavaged to determine Eotaxin-2 levels. *P<0.05, **P<0.01, ***P<0.001 vs mice receiving corresponding number of PBS injections instead of BP SCIT.