

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Epicutaneous immune modulation with Bet v 1 plus R848 suppresses allergic asthma in a murine model

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Abstract

Background: Combining allergen(s) with an adjuvant is a strategy to improve the efficacy and safety of allergen-specific immunotherapy. Here, we aimed at investigating the adjuvant effects of polyadenylic–polyuridylic acid (poly(A:U)), a TLR3 agonist, and R848 (resiquimod), a TLR7 agonist, in epicutaneous immunotherapy with Bet v 1, the major birch pollen allergen, to intervene in birch pollen allergy.

Methods and Results: BALB/c mice received epicutaneous immunization (EPI) with recombinant Bet v 1 (rBet v 1) alone, or plus poly(A:U), or R848 on their depilated back using patches. Among the groups, EPI with rBet v 1 and R848 induced detectable levels of IFN- γ -producing CD4⁺ T cells in lymph nodes and Bet v 1-specific IgG2a antibodies in the sera of mice. Before or after EPI, mice were sensitized with rBet v 1 plus aluminium hydroxide adjuvant and intranasally challenged with birch pollen extract. Prophylactic EPI with rBet v 1 plus R848 inhibited the production of biologically active Bet v 1-specific IgE antibodies in sensitization. Prophylactic and therapeutic EPI with rBet v 1 plus R848 suppressed lung inflammation upon challenges. Remarkably, only rBet v 1 plus R848 reduced the development of enhanced pause (PenH), a substituted parameter for airway hyper-reactivity, in challenged mice. In contrast to R848, poly(A:U) did not present adjuvant effect on the suppression of asthmatic features.

Conclusion: Epicutaneous immunization with rBet v 1 plus R848 induced predominant Bet v 1-specific Th1 responses and efficiently suppressed asthmatic features elicited by birch pollen. R848 could be a promising adjuvant in epicutaneous immunotherapy for birch pollen-induced allergic asthma.

Allergen-specific immunotherapy (SIT) is the only specific, disease-modifying approach to treat allergic diseases. Subcutaneous immunotherapy has long been used in allergic rhinitis and asthma and is based on repeated injections of allergens bearing the risk of severe side-effects (1). Sublingual immunother-

apy is considered a safe alternative to SIT (1, 2). One of the suggested other strategies for SIT is epicutaneous immunotherapy (3).

Compared to immunization by injection, epicutaneous immunization (EPI) offers many advantages: it eliminates injection site pain, promotes better compliance with immunization schedules and offers an easier material's delivery. Several studies have shown the prophylactic and/or therapeutic effects of EPI using the major allergen or protein extract of grass pollen, house dust mites, egg white or peanuts on allergic immune responses and local inflammation using rodent models (4–7). Initial clinical trials suggested a good safety profile of epicutaneous immunotherapy (8, 9). However, recent

Abbreviations

ALUM, aluminium hydroxide adjuvant; BAL, bronchoalveolar lavage; ELISA, enzyme-linked immunosorbent assay; EPI, epicutaneous immunization; EPIT, epicutaneous immunotherapy; PenH, the enhanced pause; Poly(A:U), polyadenylic–polyuridylic acid; TLR, Toll-like receptor.

clinical studies showed the limited effects of epicutaneous immunotherapy when treating grass pollen-allergic patients using pollen extract (10), indicating the need for improved treatment regimes.

Combining allergen(s) with a novel adjuvant could be a promising strategy to improve the efficacy and safety of SIT. SIT aims at inducing desensitization or clinical tolerance, which could be achieved by inducing Th1 cells and/or regulatory immune cells producing immune-suppressive cytokines (e.g. IL-10 and TGF- β) and/or by generating blocking antibodies to antagonize the binding of IgE to the allergens (11, 12). In this context, adjuvants recognized by Toll-like receptors (TLRs) could be applied to promote the counter-regulation of Th2 responses by inducing Th1-type and/or regulatory cytokines by dendritic cells, T cells and/or B cells (12–14).

Birch pollen allergy is one of the most prevalent allergic diseases in Northern Europe, North America and Northern Japan. In this study, we aimed at investigating the adjuvant effects of polyadenylic–polyuridylic acid (poly(A:U)), a TLR3 agonist, and R848 (resiquimod), a TLR7 agonist, in epicutaneous application to intervene in birch pollen allergy. Poly(A:U), a double-stranded RNA signalling only through TLR3, is more stable and less toxic than polyinosine–polycytidylic acid (poly(I:C)), a conventionally used TLR3 agonist (15). The effect of poly(A:U) has been investigated for oncological and virus-associated diseases (16, 17), but not for allergy so far. R848 is a more potent derivative of imiquimod, a conventionally used TLR7 agonist (18, 19). The adjuvant effect of R848 on the suppression of allergic asthma had been preclinically assessed only in systemic or mucosal applications (20–24). Here, we found that EPI with a recombinant form of the major birch pollen allergen Bet v 1 (rBet v 1) plus R848, but not plus poly(A:U), predominantly induced Th1-type immune responses. Moreover, epicutaneous immunotherapy (EPIT, i.e. therapeutic EPI performed after allergen sensitization and challenge) with rBet v 1 plus R848 suppressed lung inflammation including goblet cell hyperplasia in a murine model of birch pollen allergy.

Materials and methods

Preparation of rBet v 1

Method for the preparation of rBet v 1 is described in Data S1.

Animals

BALB/c mice (female, 8–10 weeks) were purchased from Charles River Laboratories International (Kisslegg, Germany). Mice were housed under specified pathogen-free conditions, and animal experiments were performed in compliance with local legislations.

Immunization protocols

For EPI, the hair on the back of mice was removed using depilatory cream (Veet; Reckitt Benckiser Group plc,

Berkshire, UK). The skin of mice was dried with 5 M NaCl, before the stratum corneum was disrupted by mild abrasion with Tesa adhesive tape (ten strokes). EPI was then performed by applying 100 μ g of rBet v 1 alone, or plus 100 μ g of poly(A:U) (Invivogen, Toulouse, France), or 100 μ g of R848 (Invivogen) in 500 μ l of phosphate-buffered saline (PBS), or PBS alone onto the depilated area. The immunization area was covered with a patch for 48 h. EPI was repeated weekly a total of eight times.

For sensitization, mice were intraperitoneally immunized with 10 μ g of rBet v 1 plus 2.0 mg of aluminium hydroxide adjuvant (ALUM; Thermo Fisher Scientific, Bonn, Germany) and boosted with 0.1 μ g of rBet v 1 plus 2.0 mg of ALUM in 500 μ l of PBS twice at 2-week intervals.

For challenge to induce asthmatic features, mice were intranasally treated with 60 μ g (total protein) of birch pollen extract (Stallergenes, Antony Cedex, France) in 60 μ l of PBS on three consecutive days. Immunization schedules to assess the prophylactic EPI and therapeutic EPI (EPIT) on asthmatic features are shown in Fig. 1.

Levels of Bet v 1-specific antibodies in sera

Serum levels of Bet v 1-specific IgE, IgG1 and IgG2a antibodies were determined by enzyme-linked immunosorbent assay (ELISA) as described in Data S1.

Biological activities of Bet v 1-specific IgE antibodies in sera

Biological activities of Bet v 1-specific IgE antibodies in sera were assessed using rat basophil leukaemia (RBL)-2H3 cells (25). Method and materials are described in Data S1.

Analysis of bronchoalveolar lavage (BAL)

Two days after the final challenge, mice were sacrificed and lungs were washed with 1 or 2 ml cold RPMI1640 to prepare bronchoalveolar lavage (BAL) (26). The number of leucocytes and the concentrations of cytokines in the BAL were determined as described in Data S1.

Histological analysis

After BAL preparation, lungs were fixed and stained with haematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) for histological analysis (26).

Analysis of airway hyper-reactivity

One day after the final challenge, the enhanced pause (PenH) upon the treatment with β -methacholine was measured using noninvasive whole-body plethysmography (Buxco Electronics, Birmingham, UK). The method is described in Data S1.

Statistical analysis

Significant differences between mean values were calculated by ANOVA followed by the Tukey's HSD multiple comparison

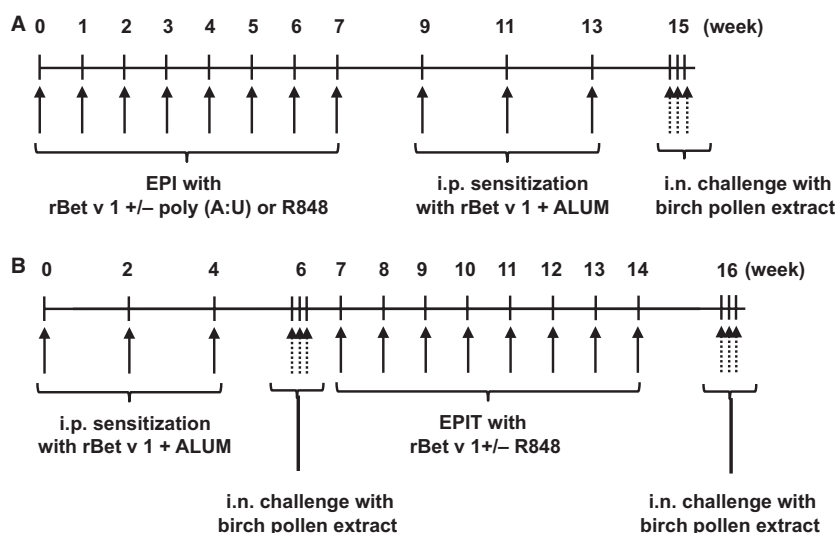


Figure 1 Immunization schedules. (A) To assess the effect of prophylactic epicutaneous immunization (EPI), mice received a total of eight weekly epicutaneous applications of rBet v 1 alone, or in combination with poly(A:U), or R848 in phosphate-buffered saline (PBS), or PBS alone, to the depilated area of their back. Two weeks after the final EPI, mice received intraperitoneal sensitization with rBet v 1 plus aluminium hydroxide adjuvant at 2-week

intervals, three times. Seventeen days after the final sensitization, mice received intranasal challenge with birch pollen extracts in PBS on three consecutive days. (B) To assess the effect of epicutaneous immunotherapy (EPIT), mice received a total of eight weekly epicutaneous applications after intraperitoneal sensitization and intranasal challenge. One week after the final EPIT, mice were intranasally rechallenged on three consecutive days.

test in GraphPad Prism 5 (SanDiego, CA, USA) or by Dunnett's test in IBM SPSS statistics (Chicago, IL, USA). A *P* value of <0.05 was considered significant.

Results

EPI with rBet v 1 plus R848 induced Th1 immune responses

To study the adjuvant effects of TLR agonists poly(A:U) and R848 in epicutaneous application, BALB/c mice were treated EPI with rBet v 1 alone, or plus a TLR agonist, weekly in total eight times (Fig. 1A). To assess T-cell responses in EPI-treated mice, proliferation and cytokine production of CD4⁺ T cells isolated from their axillary and inguinal lymph nodes after eighth EPI were measured. CD4⁺ T cells from mice treated with rBet v 1 plus R848 showed proliferation and IFN- γ production against Bet v 1, while CD4⁺ T cells from all other mice showed no detectable responses (Fig. S1). IL-4 production by CD4⁺ T cells was not detected in any EPI-treated group.

To assess antibody responses in EPI-treated mice, serum levels of Bet v 1-specific IgE, IgG1 and IgG2a antibodies were determined by ELISA. EPI with rBet v 1 alone, or plus poly(A:U), or R848 induced nearly comparable levels of Bet v 1-specific IgG1 antibodies, whereas EPI with rBet v 1 plus R848 induced Bet v 1-specific IgG2a antibodies (Fig. 2A). Bet v 1-specific IgE antibodies were detected only at marginal levels in EPI-treated groups. The results suggest that R848, but not poly(A:U), possess the adjuvant activity in EPI with rBet v 1 to induce allergen-specific Th1 responses.

Prophylactic EPI with rBet v 1 plus R848 suppressed sensitization against the allergen

Next, we examined the effect of prophylactic EPI on allergen sensitization (see immunization schedule in Fig. 1A). Intraperitoneal sensitization with rBet v 1 and ALUM induced high serum levels of Bet v 1-specific IgE antibodies in six of nine control mice epicutaneously treated with PBS (Fig. 2B). Compared with this positive control, EPI-treated mice with rBet v 1 alone, or plus poly(A:U), or R848 did not enhance Bet v 1-specific IgE antibodies. Levels of Bet v 1-specific IgG1 antibodies were comparable between the groups. However, upon sensitization, levels of Bet v 1-specific IgG2a antibodies were significantly enhanced in EPI-treated mice with rBet v 1 plus R848.

Allergen-specific IgG antibodies often interfere with the detection of allergen-specific IgE antibodies by ELISA, probably by competitive binding to allergenic epitopes. To exclude the interference by IgG antibodies and assess the biological activity of Bet v 1-specific IgE antibodies in sera of EPI-treated and sensitized mice, mediator-release assays were performed using RBL-2H3 cells (25). Cells passively sensitized with the sera of PBS-treated mice showed high levels of degranulation upon stimulation with rBet v 1 at 1.0 μ g/ml, the optimal concentration for the mediator release (Fig. 2C). In contrast, cells sensitized with the sera of EPI-treated mice with rBet v 1 plus R848 showed the lowest mediator release upon stimulation with rBet v 1 at 1.0 μ g/ml (Fig. 2C) and also at other ranges of concentrations (0.01, 0.1 and 10 μ g/ml: data not shown). This suggests that EPI with rBet

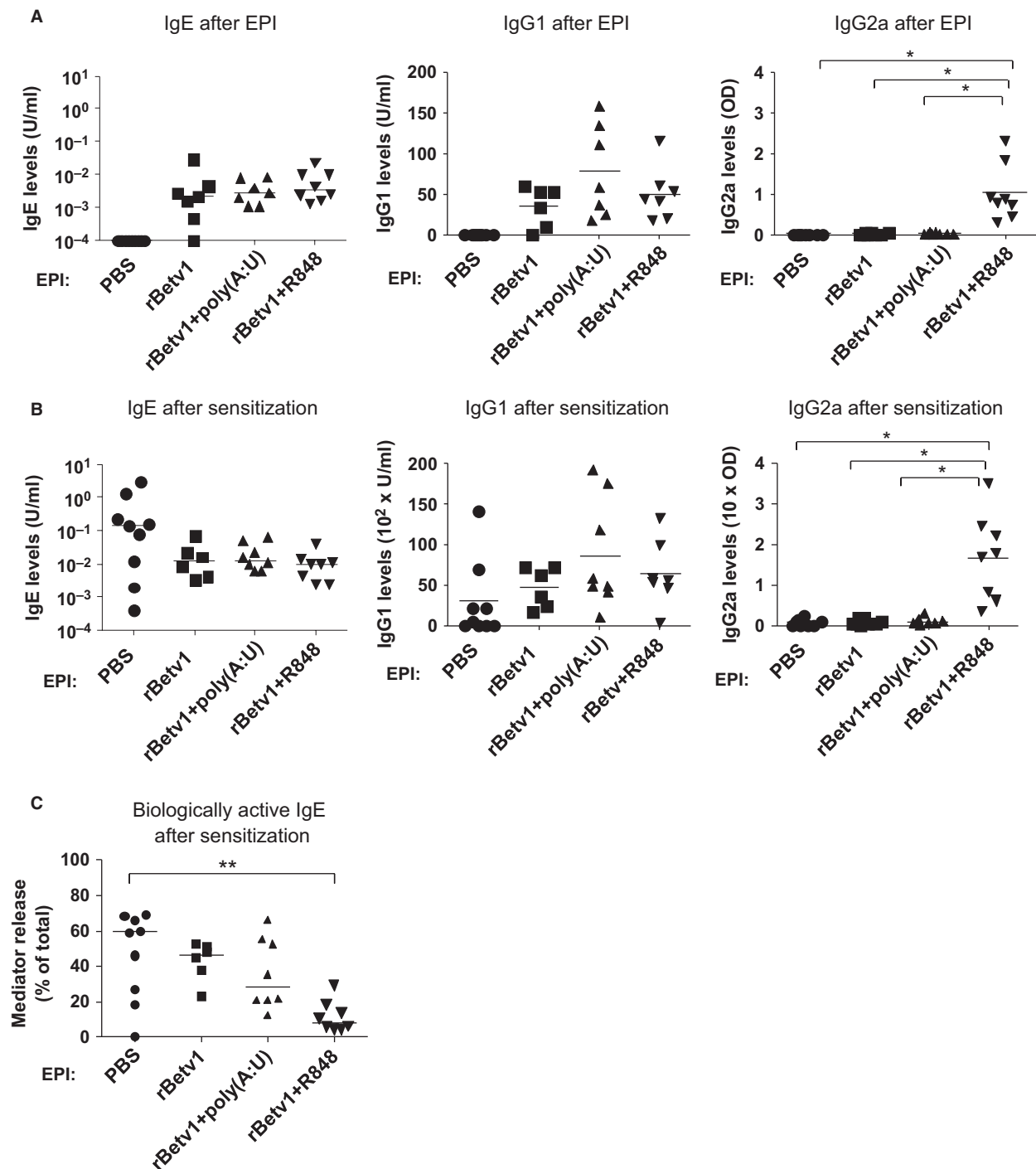


Figure 2 Effect of epicutaneous immunization (EPI) with rBet v 1 plus R848 on antibody responses. Two weeks after the final EPI with rBet v 1 alone, or in combination with poly(A:U), or R848, mice were i.p. sensitized with rBet v 1 plus aluminium hydroxide adjuvant (Fig. 1). (A) One week after the final EPI, and (B) 1 week after the final sensitization, the serum levels of Bet v 1-specific IgE, IgG1 and IgG2a antibodies in the mice were determined by

enzyme-linked immunosorbent assay. $*P < 0.001$ (C) Biological activities of Bet v 1-specific IgE antibodies in sera of EPI-treated and sensitized mice were measured by mediator-release assays using rat basophil leukaemia-2H3 cells. Error bar; SD $**P < 0.01$ Data are representative for three independent experiments. Each symbol represents an individual mouse.

v 1 plus R848 suppresses the production of biologically active Bet v 1-specific IgE antibodies.

Prophylactic EPI with rBet v 1 plus R848 suppressed asthmatic features

To examine the prophylactic potential of EPI on the development of asthmatic features, mice received intranasal challenges with birch pollen extract after the final sensitization (Fig. 1A). Upon challenges, enhanced numbers of eosinophils were observed in BAL of EPI-treated mice only with PBS, namely allergic control mice (Fig. 3A). EPI-treated mice with rBet v 1 alone, or plus R848, tended to present fewer eosinophils in BAL, compared to PBS-treated mice. Neutrophil numbers were basal in all groups. EPI-treated mice with rBet v 1 plus R848 also tended to present the lowest levels of Th2 cytokines IL-4 and IL-13, in BAL among the EPI-treated groups, and significantly lower levels of IL-5, compared with PBS-treated mice (Fig. 3B). Levels of IL-10 in BAL were comparable between the groups (Fig. 3B), whereas levels of Th1 cytokine IFN- γ were close to the detection limit (<1.0 pg/ml) in all groups (data not shown).

Histological analysis was then performed to assess lung inflammation of the challenged mice. Massive cell infiltration and increased numbers of PAS-positive cells, that is, mucus producing goblet cells, were induced in the lung of PBS-treated mice (Fig. 4A). The cell infiltration and goblet cell hyper-

plasia in tissues are indications of inflammation. In contrast, EPI-treated mice with rBet v 1 alone, or plus adjuvant, tended to reduce the number of PAS-positive goblet cells and suppress the lung inflammation (Fig. 4A,B).

Next, PenH, a substituted parameter for airway hyper-reactivity, in the challenged mice and naive mice was assessed. PBS-treated and Bet v 1-sensitized mice increased PenH upon methacholine exposure, in comparison with naive mice (Fig. 5A). Among EPI-treated groups, only mice treated with rBet v 1 plus R848 showed a significant reduction in PenH (Fig. 5B). The results suggest that EPI with rBet v 1 plus R848 could prophylactically suppress asthmatic features.

Therapeutic EPI with rBet v 1 plus R848 suppressed asthmatic features

Finally, we examined the effect of therapeutic EPI, namely EPIT, on established asthmatic features. After sensitization and challenge, mice received therapeutic EPI with rBet v 1 alone, or plus R848, and rechallenged (Fig. 1B). EPI-treated mice with rBet v 1 plus R848 showed a significant reduction in the number of eosinophil (Fig. 6A) and in concentrations of IL-4, IL-5, and IL-13 in BAL (Fig. 6B), compared to mice epicutaneously treated with PBS. Lung inflammation in EPI-treated mice with rBet v 1 plus R848 tended to be milder, compared to PBS-treated mice and EPI-treated mice with rBet v 1 alone (Fig. 7A). The number of goblet cells in lungs

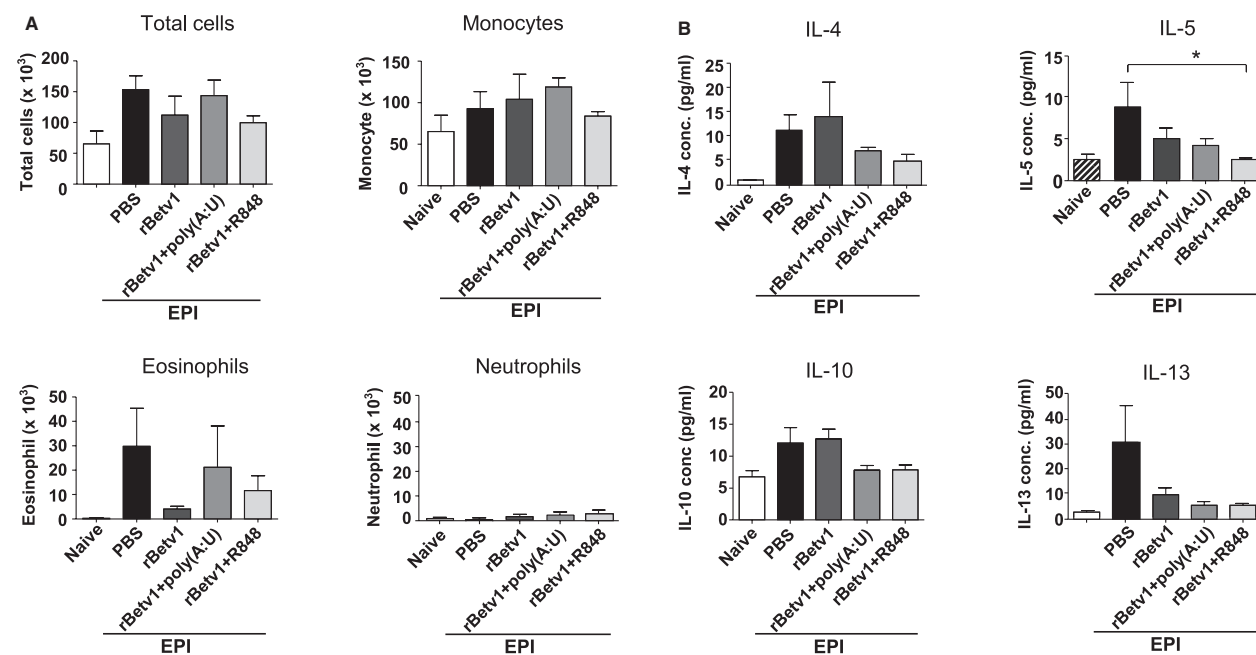


Figure 3 Effect of prophylactic epicutaneous immunization (EPI) with rBet v 1 plus R848 on eosinophilia in lungs upon allergen challenge. After EPI and sensitization, mice were intranasally challenged with birch pollen protein extract, as shown in Fig. 1A. (A) The numbers of monocytes, eosinophils and neutrophils in bronchoalveolar lavage (BAL) of challenged mice and naive mice

(a negative control) were determined by immunochemistry. (B) Concentrations of cytokines IL-4, IL-5, IL-10 and IL-13 in BAL were determined by enzyme-linked immunosorbent assay. Data are representative for three independent experiments with three to four mice per group. Error bar; SD * $P < 0.05$.

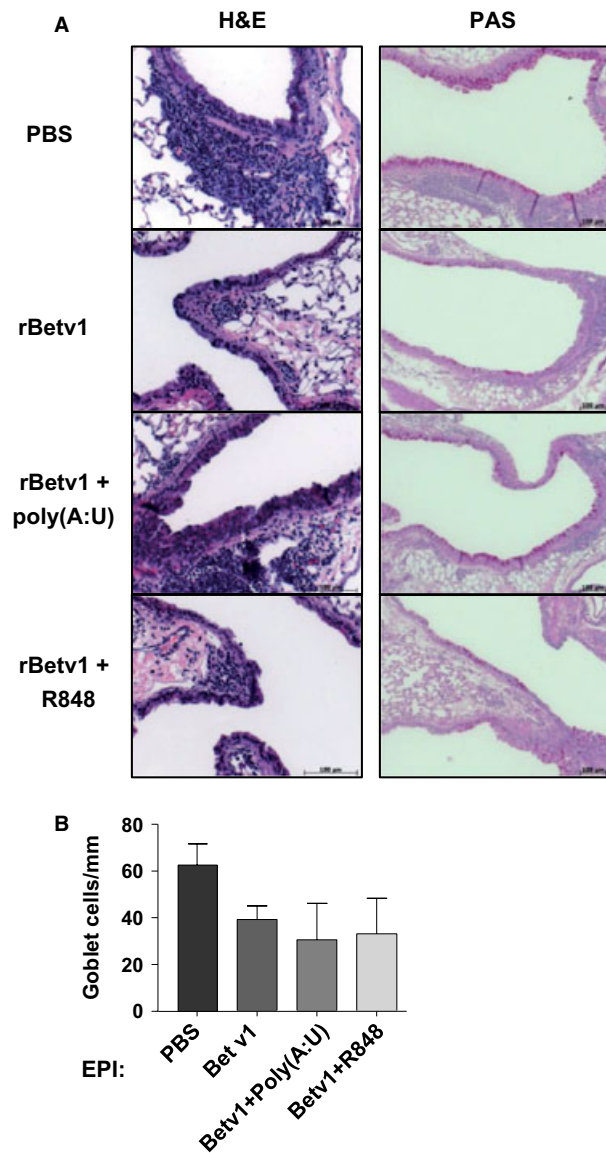


Figure 4 Effect of prophylactic epicutaneous immunization (EPI) with rBet v 1 plus R848 on lung inflammation upon allergen challenge. After EPI and sensitization, mice were intranasally challenged with birch pollen protein extract, as shown in Fig. 1A. Two days after the final challenge, pulmonary tissues were harvested. (A) Tissue sections were stained with haematoxylin and eosin and periodic acid-Schiff (PAS). (B) The numbers of PAS-positive cells in 400–600 μ m in the sections were counted. The data are representative for three independent experiments.

of EPI-treated mice with rBet v 1 plus R848 was considerably lower than those in lungs of EPI-treated mice with rBet v 1 alone (Fig. 7B). The results suggested that R848 possesses the adjuvant activity in EPIT with rBet v 1 to reduce goblet cell hyperplasia and thereby suppresses established lung inflammation. Taken together, EPIT with rBet v 1 plus R848 could have the potential to suppress asthmatic features caused by the birch pollen allergen.

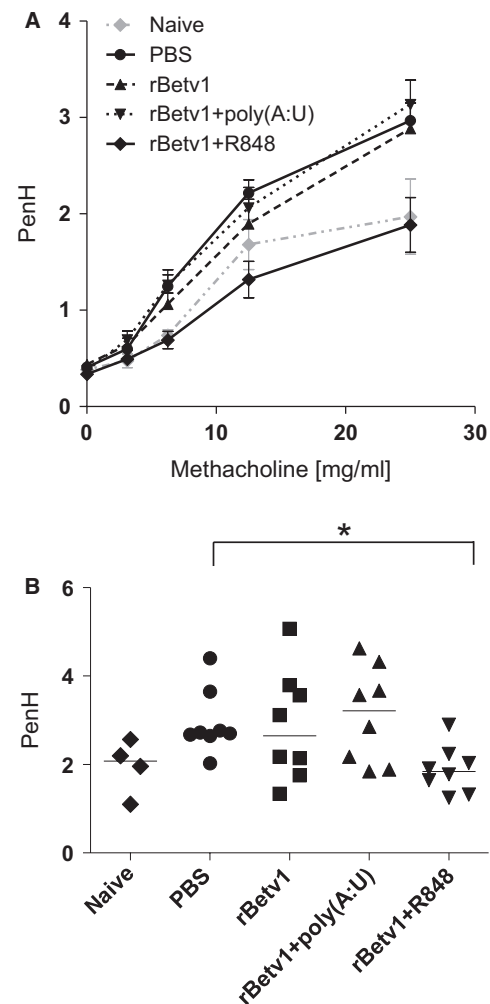


Figure 5 Effect of prophylactic epicutaneous immunization (EPI) with rBet v 1 plus R848 on the enhanced pause (PenH) upon allergen challenge. After EPI and sensitization, mice were intranasally challenged with birch pollen protein extract, as shown in Fig. 1A. (A) The challenged mice and naive mice (a negative control) were treated with saline aerosol followed by increasing concentrations (3.125, 6.25, 12.5 and 25 mg/ml) of β -methacholine aerosol, and PenH was calculated. Error bar; SD (B) PenH of each mice at 25 mg/ml of β -methacholine aerosol was extracted. The data are representative for three independent experiments. * $P < 0.05$.

Discussion

Toll-like receptor agonists are capable of modulating the immune system and have been assessed as potential adjuvants in immunotherapy for allergic diseases. Actions of TLR agonists are different depending on their receptors and application routes, because compositions of immune and structural cells expressing TLRs are different among tissues. Here, we found that R848, but not poly(A:U), presented the adjuvant effect in (i) EPI with rBet v 1 to induce Bet v 1-specific Th1 immune responses and (ii) EPIT with rBet v 1 to suppress goblet cell hyperplasia in established lung inflammation, an

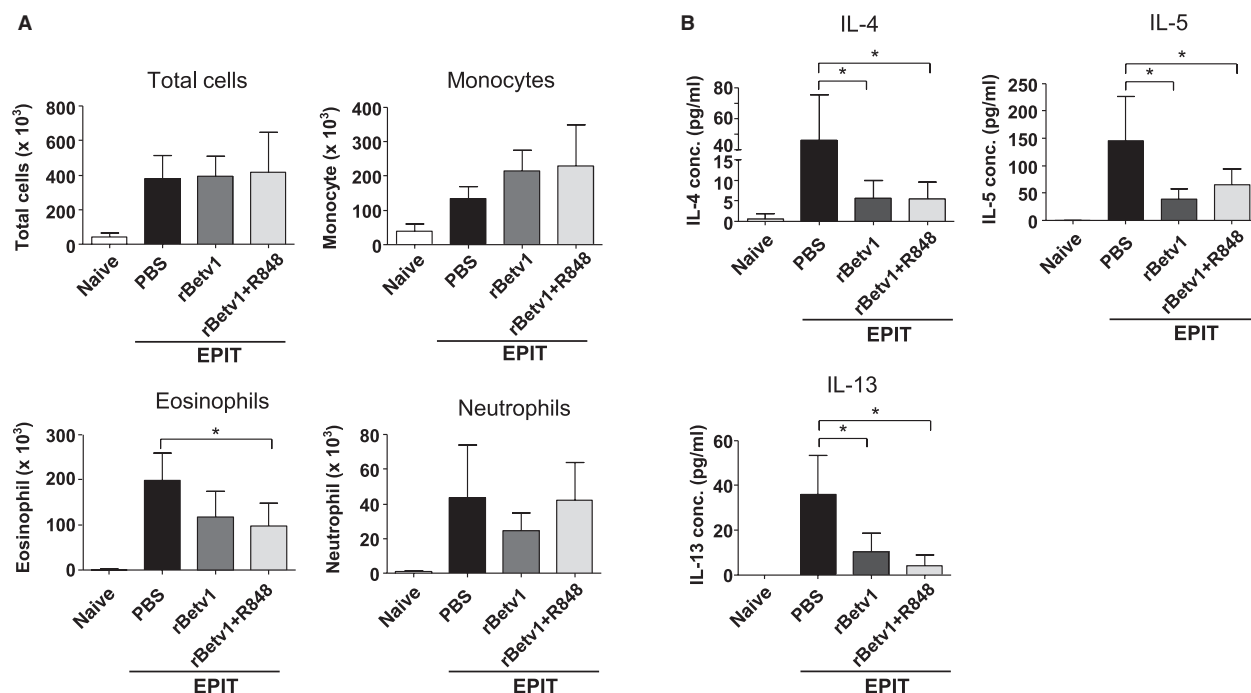


Figure 6 Effect of epicutaneous immunotherapy (EPIT) with rBet v 1 plus R848 on eosinophilia in lungs. After sensitization and challenge, mice received epicutaneous immunization with rBet v 1 alone, or in combination with R848, and rechallenged as shown in Fig. 1B. (A) The numbers of monocytes, eosinophils and neutrophils in bronchoalveolar lavage (BAL) of rechallenged mice and naive

mice (a negative control) were determined by immunochemistry. (B) Concentrations of cytokines IL-4, IL-5 and IL-13 in BAL were determined by enzyme-linked immunosorbent assay. Data are representative for two independent experiments with three to four mice per group. Error bar; SD * $P < 0.05$.

asthmatic feature in birch pollen allergy. Additionally, R848 tended to present the adjuvant effect in (i) the EPI to suppress the sensitization and development of airway hyper-reactivity against allergen and (ii) the EPIT to reduce IL-4, IL-5 and IL-13 concentrations and eosinophil number in the BAL of asthmatic lung tissues. The reduced levels of IL-5 and IL-13 in the pulmonary fluid could be particularly important, because these cytokines are major components to develop and maintain inflammation and airway hyper-reactivity (27, 28).

Epicutaneous immunization with rBet v 1 plus R848 predominantly induced Bet v 1-specific IFN- γ -producing CD4⁺ T cells and Th1-type IgG2a antibodies, suggesting that allergen-specific Th1 cells could play a crucial role in the EPI to suppress the sensitization and development of asthmatic features. It is also noteworthy that the efficacy of SIT could be associated with a rise in IFN- γ -producing T cells (1). IFN- γ inhibits antigen-presenting capacity and cytokine production of dendritic cells (29), proliferation of Th2 cells (30), antibody class switch to IgE in B cells (31) and goblet cell hyperplasia and eosinophilia in allergic inflammation (32). Several studies have shown the suppressive effect of IFN- γ on established lung inflammation (29, 33). Thus, it is likely that the adjuvant effect of R848 in the EPIT is associated with its IFN- γ -inducing activity. A risk in the induction of IFN- γ in SIT has also been suggested as this Th1 cytokine might trigger neutrophil-mediated inflammation (34). However, EPIT

with rBet v 1 plus R848 did not increase neutrophil number in the BAL and did suppress lung inflammation in our experiment. Although a potential risk of IFN- γ induction still needs to be assessed carefully, our results suggest a possible clinical application of R848 for EPIT of birch pollen-induced allergic asthma.

Several studies have shown that systemic or mucosal application of R848 alone, or together with allergen during sensitization or challenges, results in reduced allergic airway inflammation (20–24). IL-10 neutralization and IL-12 deficiency partially abrogated the suppressive effect of R848 on airway inflammation (21). Induction of invariant NKT cells or regulatory T cells was observed upon intraperitoneal application with R848 (23, 24). In our prophylactic approach, the concentration of IL-10 in the BAL and the frequency of NK-positive cells and CD4⁺CD25⁺Foxp3⁺ cells in the pulmonary lymph nodes of challenged mice were nearly comparable between EPIT-treated groups (Fig. S2). However, EPI with rBet v 1 and R848 increased the frequency of CD11c⁺B220⁺ cells, a dendritic cell population in the lungs after allergen challenge (Fig. S3). A recent study has shown that R848 stimulated CD11c⁺ cells to produce IL-1 α/β production, cytokines inducing Th1 differentiation (35). Further study is necessary to determine whether and how CD11c⁺B220⁺ cells are involved in the adjuvant effect of R848 on preventing asthmatic features. Furthermore, the potential contribution of

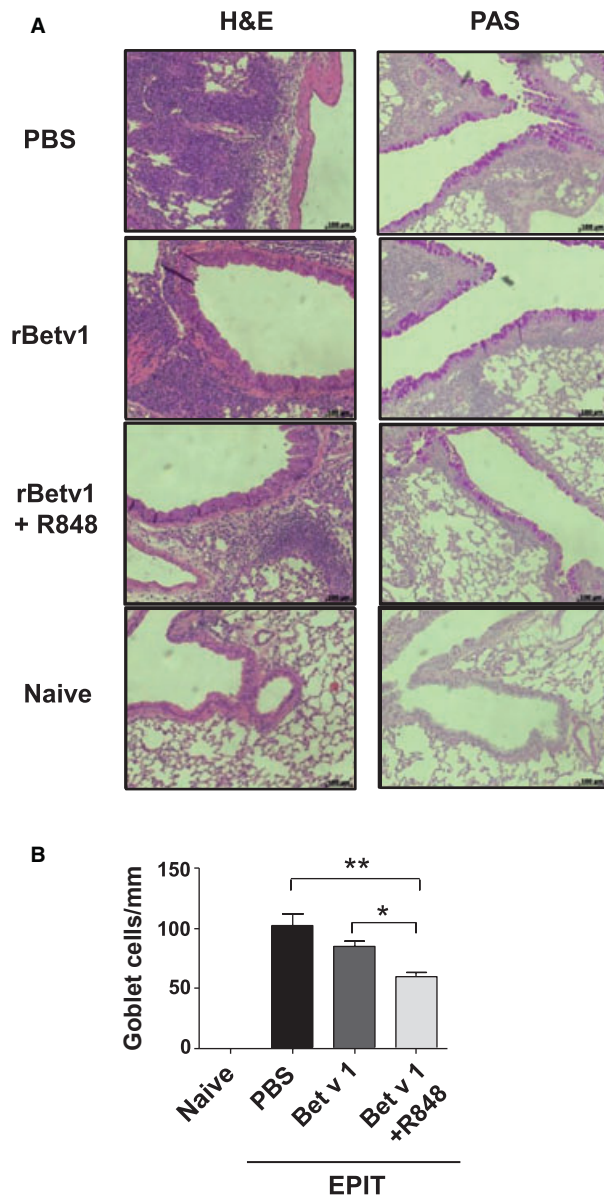


Figure 7 Effect of epicutaneous immunotherapy (EPIT) with rBet v 1 plus R848 on lung inflammation. After sensitization and challenge, mice received epicutaneous application of rBet v 1 alone, or in combination with R848, and rechallenged as shown in Fig. 1B. Two days after the final challenge, pulmonary tissues were harvested. (A) Tissue sections were stained with haematoxylin and eosin and periodic acid-Schiff (PAS). (B) The numbers of PAS-positive cells in 400–600 μ m in the sections were counted. The data are representative for two independent experiments with six to eight mice per group. * $P < 0.05$, ** $P < 0.01$.

IL-10 and CD11c⁺B220[−] cells on the suppression of established asthmatic features needs to be investigated.

In contrast to R848, poly(A:U) did not show adjuvant effects to induce Bet v 1-specific Th1 responses and could not prevent asthmatic features. This is consistent with recent

reports that poly(I:C), another TLR3 agonist, showed no adjuvant effect in dermal sensitization with allergen (36) nor in transcutaneous immunization by microneedles coated with influenza subunit vaccine (37), although intradermal injection with high amount of the agonist induced TLR3-mediated cutaneous inflammation (38). The lack of a response to poly(I:C) in transcutaneous immunization was partially explained by the induction of antimicrobial peptides inhibiting TLR3 expression due to the disruption of the skin barrier (39). However, poly(A:U) might not work in EPI simply because it did not penetrate epidermis effectively due to the size of the agonist. Poly(A:U) has a high molecular size, that is, approximately 34–252 kDa (1.0–8.5 Kbp), whereas R848 (0.314 kDa) is smaller. In murine cutaneous tissues, keratinocytes in the epidermis highly express TLR3 and have been suggested to initiate innate immune response against double-stranded RNA (40). The TLR3 agonist must pass through epidermis to access the cells, which might be obstacle due to the size of the agonist. The adjuvant activity of poly(A:U) might be obtained by methods, for example microneedles or laser microporation (41, 42), which can enhance the penetration of larger molecules in the epidermis.

We observed that EPI with rBet v 1 alone did not induce a significant IgE production and tended to suppress sensitization with rBet v 1 in ALUM. However, several studies showed that EPI would preferentially develop IgE production (43). The effect of EPI on IgE production might depend on dose, or types of allergen (e.g. native glycosylated, or recombinant form), purity of allergen (e.g. LPS contamination) and/or the immunization schedule. Further study is necessary to identify which factor crucially influences the effect of EPI on IgE production.

In conclusion, this study demonstrates for the first time that prophylactic and therapeutic epicutaneous treatment with the recombinant form of Bet v 1 in combination with R848 could prevent and suppress asthmatic features against the birch pollen allergen. Not only therapeutic, but also prophylactic applications of SIT could be of importance to prevent allergic sensitization, considering the high prevalence of allergic diseases. R848 could be a potential adjuvant for enhancing the prophylactic and therapeutic potential of EPIT for the treatment of birch pollen allergy.

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Author contributions

SuS established a murine model of birch pollen allergy, designed and performed the majority of EPI and EPIT experiments and was involved in preparing the manuscript. SR and CT supported immunological analysis of lung tissues and provided histological analysis. JM supported the measurement of PenH. AH performed lung histology. MK and AW were involved in the measurement of PenH and performed BAL

experiments and lung preparation. SW supported the preparation of the recombinant protein. StSchü was involved in animal works. SN supported to optimize epicutaneous application. SV and StSche co-supervised this project together with MT. MT designed and supervised the project and wrote the manuscript. All authors reviewed the manuscript.

Conflicts of interest

S. Vieths has received consultancy fees from the Food Allergy Resource and Research Program, Lincoln, NE, USA, the Institute for Product Quality, Berlin, Germany, and Fresenius Academy, Dortmund, Germany; has received an honorarium for evaluation of a PhD thesis from the Medical University of Vienna, Austria; has received lecture fees from the American Academy of Asthma, Allergy and Immunology, the Deutsche Dermatologische Gesellschaft, the Spanish Society of Allergy and Clinical Immunology, the Westdeutsche Arbeitsgemeinschaft für pädiatrische Pneumologie und Allergologie e.V., Köln, Germany, the Gesellschaft für pädiatrische Allergologie und Umweltmedizin, and the Ärztesverband Deutscher Allergologen; and has received royalties from Schattauer Allergologie Handbuch and Elsevier Nahrungsmittelallergien und Intoleranzen. His institution has received grants from Monsanto Company and Pioneer Hi-Bred International, Inc.; and has received travel/accommodations/meeting expenses from the German Research Foundation, the Federal Institute for

Risk Assessment, the Austrian Society for Allergology and Immunology, the French Society of Allergology, the European Directorate for the Quality of Medicines and Health Care, the European Academy of Allergy and Clinical Immunology, the World Allergy Organization, the Technical University of Munich, the Deutscher Allergie- und Asthmabund, the Association Monégasque pour le Perfectionnement des Connaissances des Médecins, the Federal Office of Consumer Protection and Food Safety, the German Chemical Society (GDCh), the Austrian Society for Dermatology and Venerology and AKM Allergiekongress. The other authors have no conflicts of interest to disclose.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effect of epicutaneous immunization with rBet v 1 plus R848 on induction of CD4⁺ T-cells producing IFN- γ .

Figure S2. Epicutaneous immunization with rBet v 1 plus R848 did not enhance the frequency of CD4⁺CD25⁺ Foxp3⁺ cells in pulmonary lymph nodes.

Figure S3. Epicutaneous immunization with rBet v 1 plus R848 increased CD11c⁺ B220⁺ cells in the lung upon challenge with birch pollen extract.

Data S1. Materials and methods.

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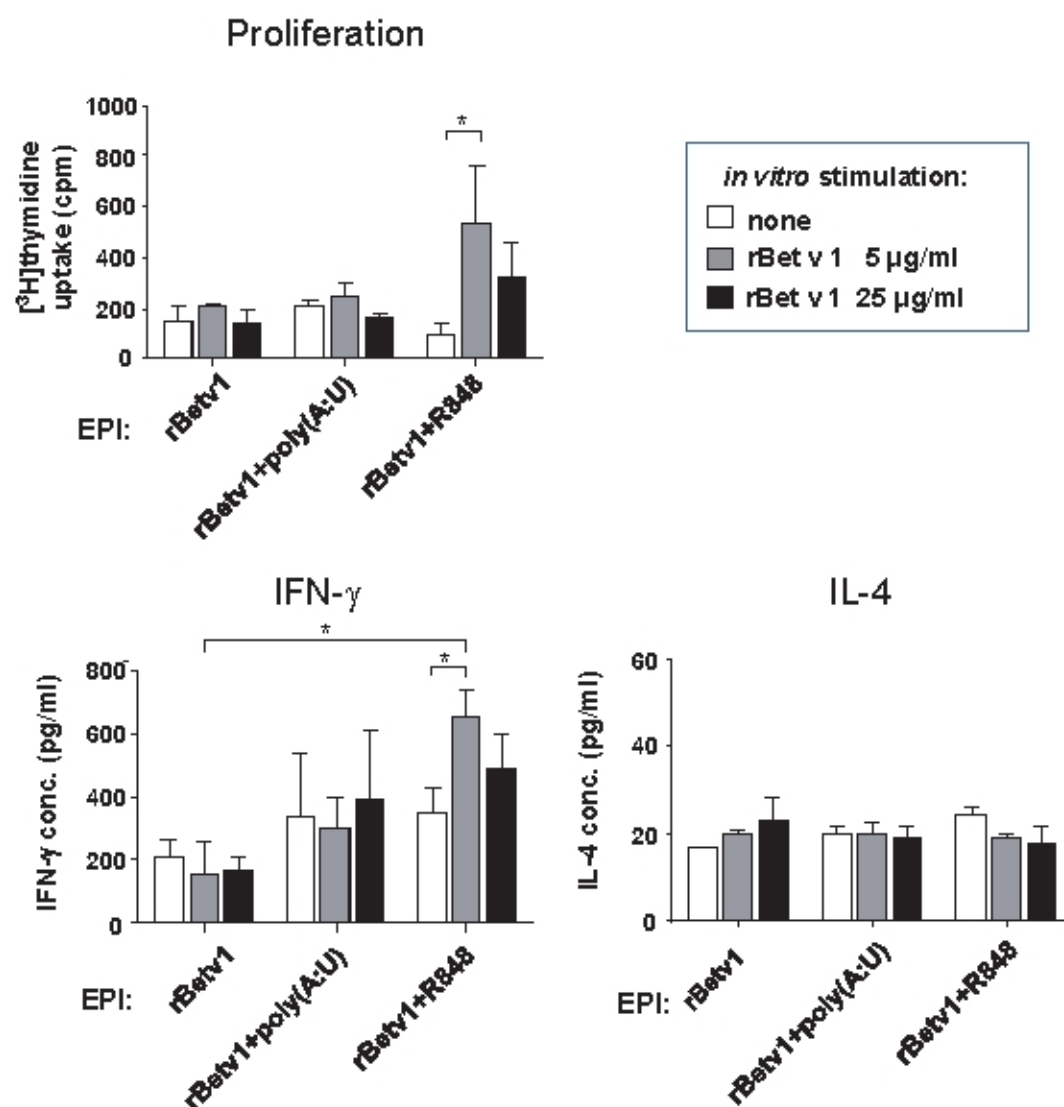


Fig. S1 Siebeneicher et al.

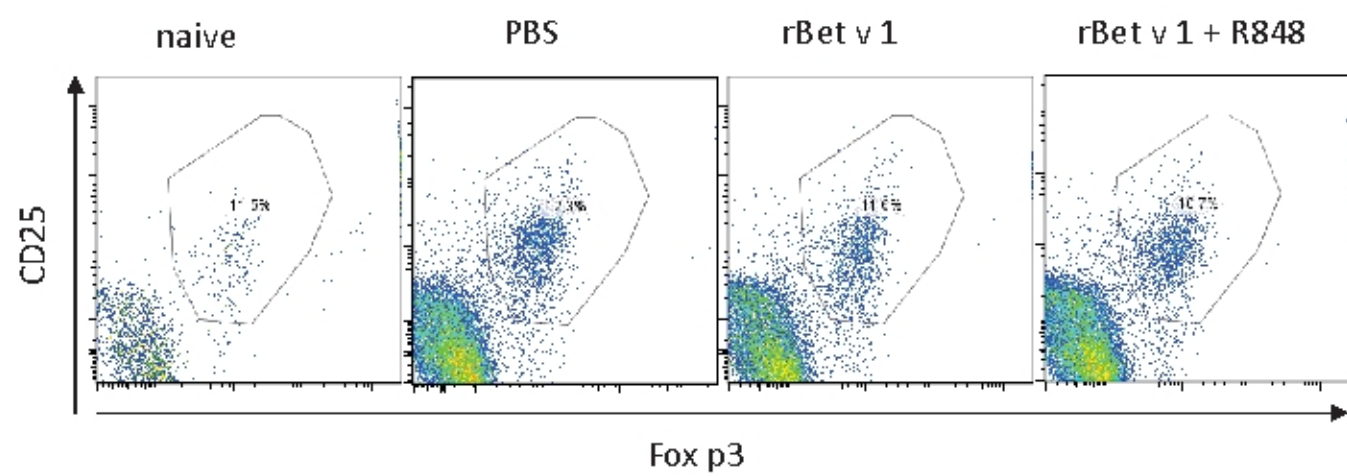


Fig. S2 Siebeneicher et al.

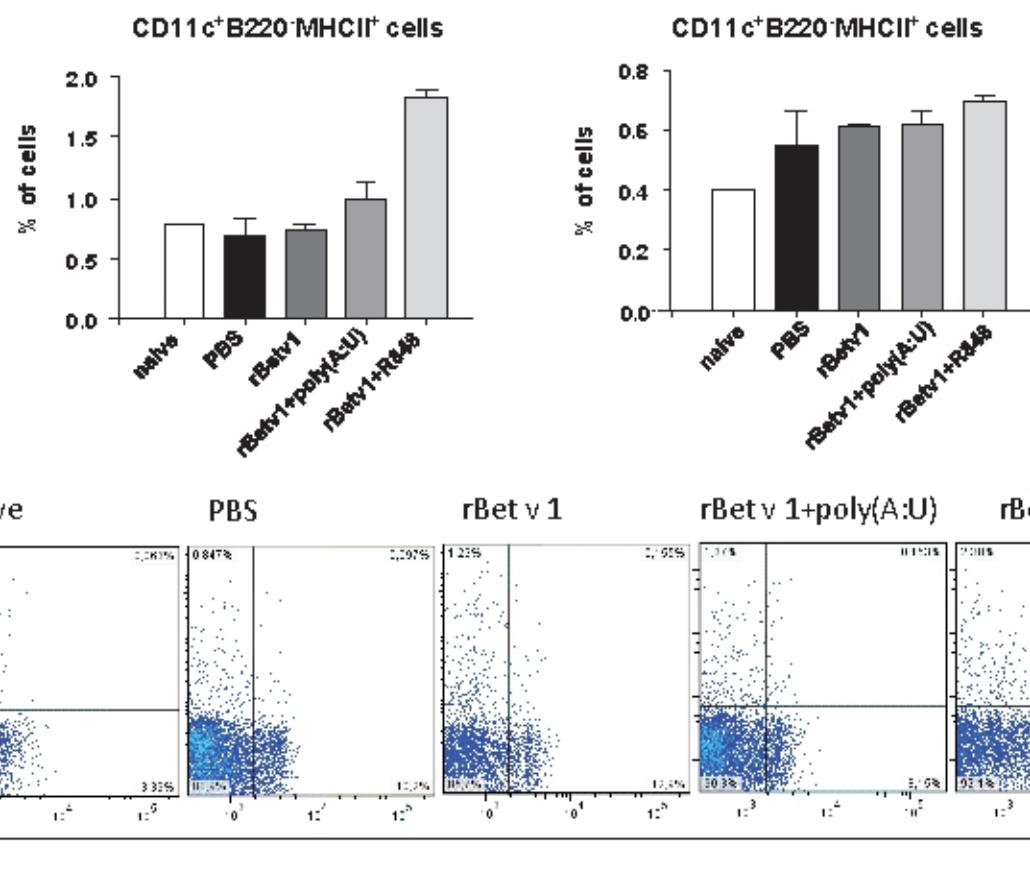


Fig. S3 Siebeneicher et al.

Supporting information

Materials and Methods

Expression and purification of recombinant Bet v 1

The sequence corresponding to the major birch pollen allergen Bet v 1a (GenBank accession no. P15494) was cloned into the pET15b expression vector (Novagen, Darmstadt, Germany). Recombinant Bet v 1a was expressed in *E. coli* BL21(DE3) (Novagen, Darmstadt, Germany) (1). Protein purification was performed with immobilized metal affinity chromatography (IMAC) using Ni-NTA resin (Quiagen, Hilden, Germany) and subsequently submitted to size exclusion chromatography (SEC) on a FPLC-Device (Äkta, GE Healthcare, Solingen, Germany) with a HiLoad 26/60 Superdex 75 prep grade column (GE Healthcare). Purified proteins were analyzed by denaturing SDS-PAGE and visualized by coomassie staining. Protein concentrations were determined using BCA protein assay (Fisher scientific, Rockford, USA) and circular dichroism measurements were performed at 20°C to demonstrate the structural integrity of the protein. Endotoxin level in the recombinant allergen was below 0.05 pg/μg protein.

Measurement of levels of Betv1-specific antibodies in sera

Microtiter plates (Greiner, Germany) were coated with 1.0 μg/ml rBet v 1 in 50 mM sodium carbonate buffer (pH 9.8) at 4°C overnight. After blocking with 10%FCS, serum samples were applied to the wells. IgE binding to Bet v 1 was detected by biotin-conjugated rat anti-mouse IgE antibodies (R35-72, BD Bioscience, Heidelberg, Germany) in combination with horseradish peroxidase (HPRP)-conjugated streptavidin (BD Bioscience). IgG1, or IgG2a binding to Bet v 1 were detected by HRPO-conjugated rabbit anti-mouse IgG1, or IgG2a antibodies (Invitrogen, Darmstadt, Germany). A substrate, 3, 3', 5, 5'-tetramethylbenzidine (BD Bioscience) was used for HRPO, and the absorbance was read at 405 nm. The levels of

Bet v 1-specific IgE and IgG1 antibodies were determined using in house generated Bet v 1 specific mAbs (mP33 for IgE and mP6 for IgG1) as standards (2).

Biological activities of Bet v 1-specific IgE antibodies in sera

Biological activities of Bet v 1-specific IgE antibodies in sera were assessed using rat basophil leukemia (RBL)-2H3 cells (3). Briefly, cells were incubated with mouse sera to allow IgE to bind to the FcεRI, washed, and stimulated with 1 µg/ml rBet v 1. Secretion of β-hexosaminidase from the cells was measured using *p*-nitrophenyl *N*-acetyl-β-d-glucosamide (Sigma-Aldrich, Steinheim, Germany) as a substrate.

T-cell proliferation and cytokine production assay

CD4⁺ T-cells were isolated from axillary and inguinal lymph nodes of mice after the eighth EPI using an isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). T cells (5.0x10⁵ cells/ml) were then co-cultured with Mitomycin-treated syngenic splenocytes (2.0x10⁶ cells/ml) as antigen presenting cells in the absence, or presence of 5.0 or 25 µg/ml of rBet v 1 in a 96 well plate for 72 h. Supernatants of cell cultures were then harvested to determine concentrations of IFN-γ and IL-4 by means of ELISA (eBioscience, Frankfurt, Germany). To measure T-cell proliferation, 1.0 µCi/ml of [³H] methyl thymidine (Perkin Elmer, Rodgau, Germany) was added to the wells. Incorporation of [³H] methyl thymidine into the cells for a further 20 h was measured by a liquid scintillation counter (Perkin Elmer).

Analysis of bronchoalveolar lavage (BAL)

Two days after the final challenge, mice were sacrificed and lungs were washed with 1 ml cold RPMI1640 to prepare BAL. The number of leucocytes and the concentrations of cytokines in the Monocytes, eosinophils and neutrophils in BAL were stained using Microscopy Hemacolor Set (Merck, Darmstadt, Germany), and counted. IFN-γ, IL-4, IL-5

and IL-10 concentrations in BAL were determined by OptEIA ELISA (BD Biosciences), whereas IL-13 concentration in the fluid was determined by ELISA Ready-SET-Go! (eBioscience).

Analysis of regulatory T cells in pulmonary lymph nodes

Two days after the final challenge, pulmonary lymph nodes were harvested, and disrupted mechanically. The single cells were then incubated with Fc-receptor blocking mAb (anti-CD16/CD32; ebioscience), and stained with FITC-labeled anti-CD4, PE-labeled anti-CD25 and PE-Cy5.5 anti-Foxp3 mAbs (ebioscience) following a manufacturer's protocol. The cells positive for CD4 expression were gated to analyse CD25 and Foxp3 expression. FACS measurement was performed on a LSR SORP II (BD Biosciences). Data were analysed using FlowJo V. 7 (Treestar Inc., Ashland, Ore).

Analysis of dendritic cell population in pulmonary tissues

Two days after the final challenge, lungs were harvested, and single cell suspension were prepared. The tissues were disrupted mechanically and enzymatically digested by incubation in collagenase type I (0.5 mg/ml PBS) at 37°C for 45 min. Single-cell suspensions were prepared by resuspending the digested tissue through a 0.9 × 40 mm cannula and red blood cells were removed by osmotic lysis. The single cells were then incubated with Fc-receptor blocking mAb (anti-CD16/CD32), and stained with FITC-labeled anti-MHC class II, Pacific Blue labeled anti-B220 and PE-Cy5.5 anti-CD11c mAbs (ebioscience). The cells positive for MHC class II expression were gated to analyse CD11c and B220 expression. FACS measurement was performed on a LSR SORP II.

Analysis of airway hyperreactivity

One day after the final challenge, airway responsiveness to β -methacholine was measured using non-invasive whole-body plethysmography (Buxco Electronics, Birmingham, U.K.). Mice were placed in chambers, and treated with a saline aerosol followed by increasing concentrations of β -methacholine aerosol (3.125, 6.25, 12.5, and 25 mg/ml; Sigma-Aldrich). This system measured peak expiratory and peak inspiratory pressures, expiratory time, and relaxation time (time of the pressure in the chamber decay to 36% of the peak expiratory pressure) of the mice. The enhanced pause (PenH that directly correlates with airway resistance) was calculated: $[\text{PenH} = \text{peak expiratory pressure} / \text{peak inspiratory pressure} \times (\text{expiratory time} - \text{relaxation time}) / \text{relaxation time}]$.

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Figure legends

Fig. S1: Effect of EPI with rBet v 1 plus R848 on induction of CD4⁺ T-cells producing IFN- γ . After eighth EPI with rBet v 1 alone, or plus poly(A:U), or R848, CD4⁺ T-cells were isolated from lymph nodes and co-cultured with mitomycin-treated syngenic splenocytes in the absence or presence of rBet v 1. (A) T-cell proliferation was assessed by incorporation of [³H] thymidine into the cells. (B) Concentrations of cytokines IFN- γ and IL-4 in culture supernatants were measured by ELISA. Data are representative for two independent experiments with three mice per group. *P < 0.05

Fig. S2: EPI with rBet v 1 plus R848 did not enhance the frequency of CD4⁺CD25⁺Foxp3⁺ cells in pulmonary lymph nodes. Mice received EPI with rBet v 1 alone, or plus R848. After eighth EPI, mice were i.p. sensitized with rBet v 1 plus ALUM and i.n. challenged with birch pollen. Two days after the final challenge, pulmonary lymph nodes were harvested, and disrupted mechanically. The frequency of CD4⁺ CD25⁺ Foxp3⁺ cells in the lymph node cells were measured by flow cytometry.

Fig. S3: EPI with rBet v 1 plus R848 increased CD11c⁺ B220⁻ cells in the lung upon challenge with birch pollen extract. Two days after the final challenge, lungs were harvested, and single cell suspension was prepared. The cells were stained for CD11c, B220, and MHC class II molecules. The cells positive for MHC class II expression were gated to analyse CD11c and B220 expression by flow cytometry.