Blocking antibodies induced by allergen-specific immunotherapy ameliorate allergic airway disease in a human/mouse chimeric model

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ABSTRACT

Background: Allergen-specific immunotherapy (AIT) induces specific blocking antibodies (Ab) which are claimed to prevent IgE-mediated reactions to allergens. Additionally, AIT modulates cellular responses to allergens, e.g. by desensitizing effector cells, inducing regulatory T and B lymphocytes and immune deviation. It is still enigmatic which of these mechanisms mediate(s) clinical tolerance. We sought to address the role of AIT-induced blocking Ab separately from cellular responses in a chimeric human/mouse model of respiratory allergy.

Methods: Non-obese diabetic severe combined immunodeficient $\gamma_c^{-/-}$ (NSG) mice received intraperitoneally freshly isolated or cryopreserved allergen-reactive PBMC from birch pollen-allergic patients together with birch pollen extract and human IL-4. Engraftment was assessed by flow cytometry. Airway hyperresponsiveness (AHR) and bronchial inflammation were analyzed after intranasal challenges with birch pollen allergen, PBS or BSA. Sera collected from patients before and during AIT with birch pollen were added to the allergen prior to intranasal challenge. The IgE-blocking activity of AIT sera was demonstrated in facilitated antigen-binding assays and basophil activation tests.

Results: NSG mice were humanized with fresh and cryopreserved PBMC and displayed a more pronounced AHR and bronchial inflammation when challenged with allergen compared to negative controls. In contrast to pre-AIT sera, the presence of post-AIT sera reduced allergic inflammation in mice engrafted with heterologous and autologous PBMC.
Conclusion: Our data demonstrate that AIT-induced blocking Ab ameliorate allergic airway inflammation in a human/mouse chimeric model of respiratory allergy. The amelioration occurred independently from AIT-induced cellular changes and underlines the relevance of blocking Ab for effective treatment.

Key words: allergy, allergen-specific immunotherapy, blocking antibodies, chimeric human/mouse model, NSG mouse

Abbreviations
Ab, antibody; AHR, airway hyperresponsiveness; AIT, allergen-specific immunotherapy; APC, antigen-presenting cell; BALF, bronchoalveolar lavage fluid; BAT, basophil activation test; BP, birch pollen; BSA, bovine serum albumin; cpm, counts per minute; DC, dendritic cells; FAB, facilitated antigen-binding; FAP, facilitated antigen presentation; H&E, hematoxylin and eosin; i.n., intranasal; i.p., intraperitoneal; MCh, methacholine; NK, natural killer; NSG, non-obese diabetic severe combined immunodeficient γc<sup>−/−</sup>; PAS, periodic acid-Schiff; PBMC, peripheral blood mononuclear cells; r, recombinant; SEM, standard error of means; SI, stimulation index;
**INTRODUCTION**

The only treatment that improves IgE-mediated allergy with long-term clinical benefit is allergen-specific immunotherapy (AIT) (1). AIT results in manifold changes of the allergen-specific immune response in treated patients (2). At the cellular level, effective AIT is associated with the suppression of allergic inflammatory cells such as mast cells, eosinophils and basophils, a downregulation of the allergen-specific Th2-response, and the induction of regulatory T and B cells with suppressive capacity (3, 4). Furthermore, AIT induces high levels of allergen-specific IgG, in particular IgG4 antibodies (Ab) (5-7). These Ab are claimed to block IgE-binding to allergens and consequently prevent IgE-mediated processes, e.g. the activation of effector cells and histamine release (8, 9). Additionally, blocking Ab have been reported to reduce the activation of allergen-specific T cells through inhibition of receptor-mediated uptake of IgE-allergen complexes by antigen-presenting cells (APC) (7, 10-12).

The inhibition of IgE activity in sera from patients undergoing AIT was reported to correlate better with clinical efficacy than the levels of allergen-specific IgG4 Ab (13-15). Consequently, robust and reproducible *in vitro* tests to detect allergen-specific blocking Ab have been established. In IgE-facilitated antigen-binding (FAB) assays, allergens are incubated with an indicator serum containing high levels of specific IgE Ab to form allergen-IgE-complexes. The latter then bind to the low affinity IgE-receptor CD23 either expressed on the surface of B cells or coated to a solid surface (16, 17). A reduction of CD23-bound allergen-IgE complexes detectable either by flow cytometry or ELISA in the presence of post-AIT as compared to pre-AIT sera indicates the presence of Ab which inhibit the formation of allergen-IgE complexes. In basophil activation tests (BAT)(18),
allergens are pre-incubated with pre- and post-AIT sera and added to allogenic basophils from allergic patients. The reduced expression of CD63 on the surface of basophils provides evidence for Ab that prevent cross-linking of already FcεRI-bound IgE by allergens (7, 19, 20). Usually, these in vitro assays do not assess the blocking of autologous allergen-specific IgE Ab. However, one study has demonstrated that stripped basophils re-sensitized with pre-AIT sera showed reduced histamine release when incubated with allergen plus post-AIT sera from the same individuals (12).

We wondered whether in an AIT-treated individual allergen-specific blocking Ab may reduce allergic symptoms independently from therapy-induced cellular alterations. To address this issue we chose a human/mouse chimeric model of birch pollen (BP) allergy that had previously been employed to investigate the function of regulatory CD4+ T cells in inhibiting allergic airway disease (21, 22). There, non-obese diabetic severe combined immunodeficient γc−/− (NSG) mice received PBMC from BP-allergic patients intraperitoneally (i.p.) and displayed airway hyperresponsiveness (AHR) and bronchial inflammation upon intranasal (i.n.) challenge with BP. We adapted this model to our needs, i.e. to engraft NSG mice with cryopreserved PBMC from BP-allergic patients collected before the onset of AIT. Subsequently, post-AIT sera from the same individuals that possessed IgE-inhibitory activity in FAB assays and BAT were assessed for their ability to reduce airway inflammation in response to i.n. allergen challenge in vivo.
METHODS

Allergens

BP extract was prepared from pollen purchased from Allergon (Thermo Fisher Scientific, Uppsala, Sweden) as described (23). Protein concentration was determined by BCA\textsuperscript{TM} Protein Assay kit (Thermo Fisher Pierce, Rockford, IL, USA). The presence of Bet v 1 was assessed by immunoblotting (data not shown). Recombinant Bet v 1.0101 (rBet v 1) was purchased from Biomay (Vienna, Austria) and bovine serum albumin (BSA) from Sigma-Aldrich (Vienna, Austria). The endotoxin levels of all proteins were below 25 EU/mg (LAL assay, Lonza, Basel, Switzerland).

Human PBMC

PBMC were isolated from 13 untreated BP-allergic patients with rhinoconjunctivitis in spring, positive skin prick reaction to BP (ALK-Abelló, Hørsholm, Denmark) and BP-specific IgE levels >0.35 kU\textsubscript{A}/L (ImmunoCAP, Thermo Fisher Scientific, Table 1). The study was approved by the local ethics committees and all patients provided written informed consent. After isolation, PBMC from some patients were cryopreserved in liquid nitrogen. To assess allergen-specific proliferative responses freshly isolated or thawed cryopreserved PBMC (2×10\textsuperscript{5}) were cultured in triplicates in 96-well plates at 37°C for 6 days with titrated concentrations of BP (6.25 to 50 µg/ml), rBet v 1 (3.125 to 25 µg/ml) or with human rIL-2 (10 U/ml, Roche Diagnostics GmbH, Vienna, Austria) as positive control. During the last 16 h of culture, \[^{3}\text{H}\]-thymidine was added and the incorporated radioactivity was measured by scintillation counting. The stimulation index
(SI) was calculated as ratio between counts per minute (cpm) obtained for PBMC plus allergen and PBMC in medium alone. Cpm in medium controls ranged from 750-18,820.

**Flow cytometry**

Human cells were stained with anti CD45-FITC (Biolegend, San Diego, CA) or CD45-eF450, CD14-PerCP (eBioscience, Vienna, Austria), CD3-BV 510, CD3/16+56 FITC/PE (BD Biosciences, Heidelberg Germany), CD4-BV 421 or CD4-Pe/Cy7, CD123-PerCP, CD19-Pe/Cy7 or CD19-APC, CD8-APC/Cy7 or CD8-APC/Cy7, CD56-FITC, CCR3-APC, BDCA3-APC, CD14-APC/Cy7 (Biolegend, San Diego, CA, USA), BDCA2-PE, BDCA1-APC (Miltenyi, Bergisch Gladbach, Germany), and analyzed on a FACSCanto II with FACSDiva (BD Biosciences) and FlowJo software (TreeStar, Ashland, Ore, USA).

**Characterization of pre- and post-AIT sera**

Sera were derived from individuals who underwent conventional subcutaneous AIT with BP extract (ALK-depot SQ, ALK-Abelló). Allergen-specific IgE and IgG Ab were quantified by ImmunoCAP (Thermo Fisher Scientific). FAB or ELIFAB tests were performed as described (7, 17). The ability to prevent effector cell activation was measured by inhibition of basophil activation (7). Briefly, rBet v 1 (1 ng/ml) was incubated with the test sera (15 µl) in RPMI supplemented with human IL-3 (5 ng/ml; PeproTech, Rocky Hill, NJ, USA) for 1 h at 37°C prior to addition to heparinized blood from BP-allergic patients for 30 min at 37°C. Cells were stained with CD63-PE, CD123-FITC (both from Biolegend), CD203c-APC, CD45-PerCP (both from Miltenyi Biotec), or the respective isotype controls (all from BD Biosciences). After lysis of erythrocytes, the
percentage of CD63+ cells in the CD45+CD123+ population was assessed by flow
cytometry. All analyses were performed in duplicates.

Mice

NSG mice were kept in animal facilities of the Medical University of Vienna under
specific pathogen-free conditions. Six- to 8-week-old female mice were used. All animal
procedures were conducted in accordance with current institutional guidelines and
performed according to the Helsinki convention for the use and care of animals. All
experiments were reviewed by the Institutional Review Board of the Medical University
of Vienna and approved by the Ministry of Sciences (BMWF-66.009/0040-
WF/V/3b/2015).

Experimental protocol for mouse experiments

NSG mice (n=10-15) received one i.p. injection of PBMC (5 x 10^6) from a single patient
plus BP extract (50 µg) and human rIL-4 (1000 U, Miltenyi Biotec). The i.p.
administration of BP extract and rIL-4 was repeated on day 7. On days 20-22, mice were
anesthetized with isoflurane (Abbott, Wiesbaden, Germany) and challenged by i.n.
application of BP extract (20 µg), PBS, rBet v 1 (5 µg) or BSA (20 µg). In some
experiments, rBet v 1 (5 µg in 5 µl) was incubated with 15 µl of pre-AIT or post-AIT sera
from the same individual for 1 h at 37°C. As negative control, 30 µl of the pre- and post-
AIT sera from patients I-III were pooled (Σ=180 µl), PBS (60 µl) was added and 20 µl of
this serum pool were i.n. administered per mouse. On day 24, invasive measurement of
AHR was performed on anesthetized, intubated, and mechanically ventilated mice in
response to increasing doses of inhaled methacholine (MCh, 1.6, 3.1, 4.8, 6.3, 12.5
mg/ml) with FinePointe Series Resistance and Compliance (RC)(Buxco, Data Sciences
International, St. Paul, MN, USA). RC were measured every 15 seconds following
nebulization. The response to each single dose of MCh was analyzed until a plateau
phase was reached. The response to PBS was set to 100%. Untreated NSG mice
received neither human PBMC, BP and rIL-4, nor i.n. challenges.

**Bronchoalveolar lavage**

After lung function measurement, cold PBS (1-3 ml) was instilled through an
intratracheal tube and immediately recovered. Bronchoalveolar lavage fluid (BALF) was
centrifuged and cells were resuspended in 100 µl of PBS, counted and stained with
mouse Siglec-F-BV 421 (BD Biosciences), CCR3-FITC, F4/80-BV 510 (Biolegend) or
F4/80-FITC, CD45-PE, CD11b-PerCP/Cy5.5, CD11c-Pe/Cy7, CD123-APC, GR-1-APC-
eF780, and Ly-6G-APC (eBioscience) for flow cytometry analysis.

**Lung and spleen staining**

Lungs were digested with liberase DL for 20 min at 37°C (0.1 mg/ml, Roche Diagnostics
GmbH), followed by erythrocyte-lysis. Spleens were dispersed through 70 µm cell
strainer (Falcon® Cell Strainer Corning, Durham, USA) before erythrocyte-lysis.
Subsequently, human cells were analyzed by flow cytometry.

**Histology**
Lungs were fixed in 4% formalin for 24 h at room temperature, subsequently embedded in paraffin, cut into 4 µm sections and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS).

Statistical analysis

Student’s t-tests and Mann Whitney-U tests were applied to test the statistical significance of the results using the software SPSS10.01 (SPSS, Chicago, IL, USA). Differences were considered significant if P<0.05.
RESULTS

NSG mice engrafted with PBMC from BP-allergic patients show allergen-induced airway hyperresponsiveness

Freshly isolated PBMC from nine BP-allergic patients were used to engraft NSG mice (Fig. 1A). The patients’ BP-specific IgE values ranged from 3.5-27.6 kU/L (median 6.3 kU/L) and their Bet v 1-specific IgE levels from 2.2-22.6 kU/L (median 5.7 kU/L) (Table 1). PBMC proliferated when stimulated with BP, rBet v 1 or IL-2 (Table 1). Prior to injection, cell samples were characterized for their content of CD4+ and CD8+ T cells (CD3+), B cells (CD19+), monocytes (CD14+), natural killer (NK) cells (CD16+CD56+CD3+), NKT cells (CD16+CD56+CD3+), myeloid dendritic cells (DC) type 1 (BDCA-1+CD19+) and type 2 (BDCA-3+CD14+), plasmacytoid DC (BDCA-2+CD123+CCR3+), and basophils (CD123+CCR3+) and showed a composition characteristic for PBMC (Fig. 1B). On day 24, cell suspensions from lungs and spleens from mice challenged with either BP or PBS were stained for human CD45 (Fig. 1C). Lungs contained 32% (median value) versus 28% and spleens 39.3% versus 29.4% CD45+ cells, respectively. Some animals showed no engraftment which was donor-independent. When >1.5% of human CD45+ cells were detected, the percentages of the individual cell types mentioned above were analyzed. Lungs and spleens from both groups of mice contained comparable percentages of CD4+ and CD8+ T cells, monocytes, B cells and NK cells (Fig. 1C). NKT cells, DC and basophils were all below 1.0% (data not shown). In both groups, significantly higher percentages of human B cells were found in spleens than in lungs (Fig. 1C).
Engrafted mice challenged i.n. with BP showed a significantly increased AHR compared to engrafted mice challenged with PBS and untreated NSG mice (Fig. 1D). BALF from engrafted animals challenged with BP or PBS contained similar percentages of basophils, eosinophils and neutrophils (Fig. 1E). Similarly, the histopathology of lungs from engrafted mice challenged with either BP or PBS revealed no difference regarding inflammatory parameters (data not shown).

**NSG mice engrafted with cryopreserved PBMC from BP-allergic patients show allergen-induced airway disease**

PBMC from seven BP-allergic patients with BP-specific IgE ranging from 1.8-25.7 kU\(_A\)/L (median 4.9 kU\(_A\)/L) and Bet v 1-specific IgE ranging from 2.3-24.9 kU\(_A\)/L (median 4.7 kU\(_A\)/L) were cryopreserved (Table 1). After thawing, cells proliferated when stimulated with BP, rBet v 1 or IL-2 (Table 1) and showed a similar distribution of cell types as freshly isolated PBMC (Fig. 2B). On day 24 after engraftment, cell suspensions of lungs and spleens from mice challenged with BP or PBS contained 24.6% (median value) versus 35.6% and 33.2% versus 26.8% of CD45\(^+\) cells, respectively (Fig. 2C). Similar to mice injected with fresh PBMC, some animals showed no engraftment. In engrafted mice (>1.5% CD45\(^+\) cells), lungs and spleens contained comparable percentages of CD4\(^+\) and CD8\(^+\) T cells, monocytes, B cells and NK cells after challenge with BP or PBS. Again, spleens contained significantly higher numbers of B cells than lungs in both groups (Fig. 2C). In contrast to animals engrafted with freshly isolated PBMC, mice engrafted with cryopreserved PBMC contained a lower percentage of CD8\(^+\) T cells.
Humanized mice challenged i.n. with BP showed a significantly higher AHR than animals challenged with PBS and untreated mice, respectively (Fig. 2D). To further prove the allergen-specificity of our model we challenged mice i.n. with rBet v 1 or BSA as negative control. Exposure to the major BP allergen induced a significantly stronger AHR compared to BSA (Fig. 2E). BALF from BP-exposed animals contained higher percentages of basophils, eosinophils and neutrophils than PBS-challenged engrafted mice, however, the differences did not reach statistical significance (Fig. 2F). Histopathological analysis revealed that lungs of BP-challenged mice showed a higher degree of peribronchial inflammatory infiltrate than PBS-challenged mice (Fig. 2G). An increased goblet cell hyperplasia was present in BP-challenged compared to PBS challenged animals (Fig. 2H).

**AIT-induced allergen-specific blocking antibodies ameliorate allergic airway disease in NSG mice humanized with heterologous PBMC**

For proof of principle of the *in vivo* blocking capacity of AIT-induced allergen-specific Ab we employed pre- and post-AIT sera from patients no. I, II and III (Table 2) who all underwent successful AIT with BP (24). All post-AIT sera contained higher levels of Bet v 1-specific IgG4 Ab and inhibited rBet v 1-specific IgE activity in FAB assays and BAT (Table 2). NSG mice were engrafted with cryopreserved PBMC from patient C3 (Table 1) and challenged i.n. with rBet v 1 plus pre- or post-AIT sera from the same treated individual. Mice challenged with a pool of these sera in PBS served as negative control (Fig. 3A). Animals challenged i.n. with rBet v 1 plus pre-AIT sera showed a significantly higher AHR than mice challenged with rBet v 1 plus the corresponding post-AIT sera.
(Fig. 3B). The latter group showed an AHR comparable to the negative control. However, no notable differences in the percentages of basophils, eosinophils and neutrophils were detected in BALF from the different groups (Fig. 3C). In contrast, lung histology showed reduced bronchial inflammation and goblet cell hyperplasia when NSG mice were exposed to rBet v 1 plus post-AIT sera (Fig. 3D and E).

**AIT-induced allergen-specific blocking antibodies ameliorate allergic airway disease in NSG mice humanized with autologous PBMC**

NSG mice were engrafted with cryopreserved PBMC from either patient C6 or C7 that had been collected before the onset of BP-AIT (Table 1). Due to limited cell numbers, patient C6 could not be tested in lymphoproliferation assays. I.n. challenges were performed with rBet v 1 plus the sera from either patient C6 or C7 collected before and after 12 months of AIT with BP. Clinical improvement was apparent in both patients by significantly diminished clinical scores regarding their rhinoconjunctivitis. Both individuals had developed enhanced levels of Bet v 1-specific IgG4 Ab and their post-AIT sera displayed blocking activity in ELIFAB and BAT (Table 2). Mice challenged i.n. with rBet v 1 plus post-AIT sera displayed a significantly reduced AHR as compared to animals challenged with rBet v 1 plus the corresponding pre-AIT sera (Fig. 4C). Slightly reduced percentages of basophils, eosinophils and neutrophils were detected in BALF of mice challenged with rBet v 1 plus post-AIT sera, accompanied by reduced bronchial inflammation and reduced goblet cell hyperplasia in the lungs (Fig. 4D and E).
**DISCUSSION**

It is one of our major goals to unravel which AIT-induced immune mechanism(s) contribute(s) to clinical tolerance to allergen exposure. The induction of allergen-specific non-IgE Ab that prevent IgE-binding to allergens is a hallmark of AIT, however, a direct correlation of their appearance with clinical improvement has rarely been demonstrated (25). A recent clinical trial with B cell-derived peptides that induced blocking Ab without altering the allergen-specific T cell response supported their relevance for clinical improvement (26). On the other hand, clinical trials employing peptides that target allergen-specific T cells but are too short to induce Ab responses have also shown clinical efficacy (27, 28).

We sought to set up an *in vivo* model of respiratory allergy to study the involvement of AIT-induced humoral changes in clinical tolerance to allergens separately from AIT-induced cellular alterations. We adapted a human/mouse chimeric model of BP allergy previously described with freshly isolated PBMC from patients with BP-induced asthma and BP-specific IgE levels of >50 kU/L (21, 22). First, we evaluated whether this model worked with freshly isolated and cryopreserved PBMC from individuals with BP-induced rhinoconjunctivitis and low to moderate allergen-specific IgE levels (Table 1). Injection of both cell preparations engrafted the majority of mice, and except for CD8+ T cells no major differences regarding the different human cell types were found *in vivo.* Furthermore, NSG mice humanized with both fresh and cryopreserved PBMC displayed a significantly increased AHR upon allergen challenge when compared to engrafted mice challenged with PBS or the control allergen BSA and to untreated animals. Notably, NSG mice humanized with freshly isolated PBMC and exposed to allergen or
PBS showed no differences of inflammation in BALF and lung histopathology. The latter revealed a perivascular inflammation in both groups indicative of a Graft versus Host disease and the overlapping of perivascular and peribronchial inflammatory infiltrates complicated histopathological analyses. This xenogenic response of human immune cells was less pronounced in mice humanized with cryopreserved PBMC and accompanied by lower in vivo percentages of CD8+ cells. Lung sections of these animals indicated an allergen-induced peribronchial inflammation and mucus production. In accordance with previous results (21), NSG mice engrafted with PBMC from non-allergic donors showed no significant signs of airway inflammation upon challenge with BP allergen (data not shown).

To establish the experimental protocol we first engrafted NSG mice with cryopreserved PBMC from one untreated BP-allergic individual and tested the in vivo blocking capacity of sera from three different patients collected after 6, 18 and 24 months of AIT with BP, respectively (24). Compared to the corresponding pre-AIT sera, all post-AIT sera displayed blocking capacity in in vitro assays and significantly ameliorated allergen-induced airway inflammation in animals that had been humanized with heterologous PBMC. Subsequently, we engrafted NSG mice with PBMC collected and cryopreserved from two BP-allergic individuals before they started AIT and compared the response to intranasal challenge with rBet v 1 in the presence of their own pre- and post-AIT sera, respectively. Also in this autologous set-up, the presence of post-AIT sera significantly reduced allergen-induced airway inflammation, matching their blocking capacity determined in vitro. The amelioration of the disease-eliciting immune response before
AIT occurred without the contribution of autologous AIT-induced regulatory T cells, regulatory B cells or desensitized effector cells as these were absent in the mice.

The allergic response in humanized NSG mice has been shown to depend on CD4$^+$ T cells as their depletion from PBMC prior to engraftment abolished allergen-induced lung inflammation (21, 29). Our PBMC samples contained allergen-reactive T cells as demonstrated by proliferative responses to stimulation with BP and rBet v 1 (Table 1). In addition to human T cells, we detected B cells and monocytes in vivo and considered them to be the most relevant APC in this model. Both, monocytes and B cells express the multifunctional FcεRII (CD23) on their surfaces (30, 31). However, CD23 on B cells predominantly binds allergen-IgE complexes which after internalization are directed to HLA class II-rich compartments (32). This process of facilitated antigen presentation (FAP) then activates specific CD4$^+$ T cells (32, 33) and contributes to allergic inflammation and disease severity in allergic individuals (34, 35). Blocking Ab are considered to reduce FAP by competing with IgE for allergen binding (10, 32, 36). It is attractive to speculate that this represents the major immune process reducing the allergic inflammation in the human/mouse chimeric model. When employing recombinant antibodies a successful inhibition of the formation of IgE-allergen complexes IgG Ab requires the identical specificity and affinity as the corresponding IgE Ab (37). Our data indicate that functional AIT-induced blocking Ab exhibit these characteristics. Previously, we provided evidence that AIT-induced allergen-specific IgG4 Ab recognize similar surface areas as autologous IgE Ab (7). Here, we complement these observations by providing evidence that the affinity of AIT-induced Ab is sufficient to form complexes that remain stable in vivo.
In summary, we demonstrate that AIT-induced blocking Ab ameliorate allergic airway responses in a human/mouse chimeric model. The in vivo amelioration matched the results from FAB assays and BAT and thereby supports their reliability for the detection of AIT-induced blocking Ab. Furthermore, our results indicate that blocking Ab may cause clinical tolerance to allergens independently from AIT-induced cellular alterations. This autonomous functionality of AIT-induced blocking Ab supports their relevance for effective therapy and supports the concept that they represent biomarkers for successful allergy treatment.
AUTHOR CONTRIBUTIONS

C.V. and B.B. designed the experiments; C.V., M.G., B.N., and F.Z. performed the experiments and analysed the data; S.R. and L.K. analysed the tissue sections; C.M. and W.P. provided patients’ samples and performed ELIFAB; V.S. provided NSG mice; A.N. and W.F.P. helped with the RC analyses, C.V. and B.B. wrote the manuscript.

CONFLICT-OF-INTEREST STATEMENT:

The authors declare no competing financial interests.

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Table 1. Characterization of birch pollen-allergic patients and their PBMC

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Birch IgE [kU/L]</th>
<th>Bet v 1 BP</th>
<th>rBet v 1 IL-2</th>
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<tr>
<td>1</td>
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<td>12.8</td>
<td>n.t.</td>
<td>2.1 (^b)</td>
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<td>2</td>
<td>F</td>
<td>4.3</td>
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<tr>
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<td>F</td>
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<td>F</td>
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<td>9</td>
<td>M</td>
<td>6.7</td>
<td>9.1</td>
<td>64.3</td>
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1C\(^c\) M | 12.8 | n.t. | 21.3 | 2.2 | 14.9 |
2C F | 4.3 | 3.8 | 12.5 | 10.1 | 20.3 |
3C M | 4.9 | 4.2 | 1.2 | 2.5 | 9.5 |
10C M 1.8 | 2.3 | n.t. | 3.8 | 15.1 |
11C M 4.0 | 5.1 | 4.7 | 4.7 | 5.2 |
12C M 25.7 | 24.9 | n.t. | n.t. | n.t. |
13C M 8.6 | 10.0 | 2.1 | 2.6 | 7.0 |

\(^a\)M, male; F, female; \(^b\)stimulation index (SI) to the optimum concentration of allergen for each individual; \(^c\)C, cryopreserved, 1C-3C are cryopreserved PBMC from patients 1-3.
Table 2. Characterization of sera from patients treated with birch pollen immunotherapy (AIT)

<table>
<thead>
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<th>Patient</th>
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<th>Post-AIT</th>
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<th>Post-AIT [µg/ml]</th>
<th>Blocking activity [%] of post-AIT sera in FAB</th>
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<td>12C*</td>
<td>24.9</td>
<td>19.2</td>
<td>1.56</td>
<td>7.17</td>
<td>89**</td>
<td>93</td>
</tr>
<tr>
<td>13C*</td>
<td>10.0</td>
<td>13.5</td>
<td>0.18</td>
<td>2.45</td>
<td>84**</td>
<td>76</td>
</tr>
</tbody>
</table>

Samples collected after °6, °°18, °°°24, and *12 months of BP-AIT; FAB, facilitated antigen-binding assays; ** determined in ELI-FAB; BAT, basophil activation assays,
FIGURE LEGENDS

Figure 1. Humanization and allergic response of NSG mice engrafted with freshly isolated PBMC. (A) Experimental design; (B) Cell types in PBMC from nine patients; (C) percentage of human CD45$^+$ cells in lungs and spleens after intranasal (i.n.) challenge with BP (n=43, gray dots) or PBS (n=41, white dots) and cell type distribution; (D) Airway resistance (RI, means±SEM) in untreated (n=12) and engrafted mice after i.n. challenge with BP (n=28) or PBS (n=26); (E) Percentage of basophils (B), eosinophils (E), neutrophils (N) and alveolar macrophages (AM) in BALF of engrafted mice after i.n. challenge with BP or PBS. NKT, natural killer T cells; mDC, myeloid dendritic cells; pDC, plasmacytoid DC; *P<0.05, **P<0.01, Mann Whitney-U test, black stars indicate differences between engrafted mice challenged with BP or PBS, gray stars between engrafted BP-challenged mice and untreated mice;

Figure 2. Humanization and allergic response of NSG mice engrafted with cryopreserved PBMC. (A) Experimental design; (B) Cell types in cryopreserved PBMC from seven patients; (C) percentage of human CD45$^+$ cells in lungs and spleens after intranasal (i.n.) challenge with BP or rBet v 1 (n=35, gray dots) or PBS (n=28, white dots) and cell type distribution; (D) Airway resistance (RI, means±SEM) in untreated (n=12) and engrafted mice after i.n. challenge with BP (n=13) or PBS (n=9); (E) Airway resistance in engrafted mice after i.n. challenge with rBet v 1 (n=9) or BSA (n=8); (F) Percentage of basophils (B), eosinophils (E), neutrophils (N) and alveolar macrophages (AM) in BALF of engrafted mice after i.n. challenge with BP or PBS; (G) Pulmonary tissue sections stained with H&E and (H) PAS; NKT, natural killer T cells; mDC, myeloid
dendritic cells; pDC, plasmacytoid DC; *P<0.05, **P<0.01, Mann Whitney-U test, black stars indicate differences between engrafted mice challenged with BP or PBS, gray stars between engrafted BP-challenged mice and untreated mice;

**Figure 3. AIT-induced blocking Ab reduce allergic responses in NSG mice humanized with an unrelated BP-allergic patient.** (A) Experimental design; (B) Airway resistance (RI, means±SEM) in mice after intranasal (i.n.) challenge with rBet v 1 plus either pre-AIT (n=11) or post-AIT sera (n=13), or with a serum pool (n=10); ***P<0.001 indicates differences between challenge with rBet v 1 plus pre- or post-AIT sera; *P<0.05, **P<0.01 indicates differences between challenge with rBet v 1 plus pre-sera and the serum pool (Mann Whitney-U test); (C) Percentage of basophils (B), eosinophils (E), neutrophils (N) and alveolar macrophages (AM) in BALF; (D) Pulmonary tissue sections stained with H&E and (D) PAS.

**Figure 4. Autologous AIT-induced blocking Ab reduce allergic responses in humanized NSG mice.** (A) Experimental design; (B) Airway resistance (RI, means±SEM) in mice intranasally (i.n.) challenged with rBet v 1 plus pre-AIT sera (n=10) or post-AIT sera (n=9), *P<0.05, Mann Whitney-U test; (C) Percentage of basophils (B), eosinophils (E), neutrophils (N) and alveolar macrophages (AM) in BALF; (C) Pulmonary tissue sections stained with H&E and (D) PAS.
Figure 1

A

Day 0 i.p.
5x10^6 PBMNCs
50 μg of BP +
1000 U/rhIL-4

Day 7 i.p.
50 μg of BP +
1000 U/rhIL-4

Day 20-22 i.n. challenge
with BP [20 μg] or PBS

Day 24

Lung function
Bronchoalveolar lavage fluid (BALF)
Detection of human cells in murine organs
Histopathology of lungs

B

% of CD45+ cells in BAL

C

% of CD25+ cells in lungs

D

PI (% change from PBS)

E

Cells in BAL (%)

PBS 1.56 3.12 4.75 6.25 12.5

Methacholine [μg/ml]

Figure 1

152x122mm (300 x 300 DPI)
Figure 2

A

Day 0 i.p.  
5x10^5 PBMC +  
50 µg of BP +  
1000 U rhIL-4

Day 7 i.p.  

Day 30-22 i.n. challenge with  
BP [20 µg] or rBet v 1 [5 µg]  
or PBS or BSA [20 µg]

Day 24

B

C

D

E

F

G

H

BP PBS  

PBS  

Methacholine [mg/ml]

Methacholine [mg/ml]

rBet v 1  

rBet v 1  

H&E PBS  

PAS PBS

197x201mm (300 x 300 DPI)
Figure 3

A

<table>
<thead>
<tr>
<th>Day 0 i.p.</th>
<th>Day 7 i.p.</th>
<th>Day 20-22 i.n. challenge with rBet y 1 [5 μg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3] PBMC + 50 μg of BP + 1000 U/mL</td>
<td>50 μg of BP + 1000 U/mL</td>
<td>+ sera pre-AIT or post-AIT or serum pool</td>
</tr>
</tbody>
</table>

B

Day 24

Lung function
Bronchoalveolar lavage fluid (BALF)
Detection of human cells in murine organs
Histopathology of lungs

C

D

H&E

rtBet v 1+sera pre-AIT

H&E

rtBet v 1+sera post-AIT

E

PAS

rtBet v 1+sera pre-AIT

PAS

rtBet v 1+sera post-AIT

146x114mm (300 x 300 DPI)
Figure 4

A

Day 6 i.p.
5x10⁶ PBMC +
50 μg of BP +
1000 U rHL-4
Day 7 i.p.
Day 20-25 i.a. challenge with rBet v 1 (5 μg)
+ sera pre-AIT or post-AIT
from the same individual
Day 24
Long function
Bronchoalveolar lavage fluid (BALF)
Detection of human cells in murine organs
Histopathology of lungs

B

C

D

E

H&E
rBet v 1+ sera pre-AIT
H&E
rBet v 1+ sera post-AIT
PAS
rBet v 1+ sera pre-AIT
PAS
rBet v 1+ sera post-AIT

Figure 4

137x101mm (300 x 300 DPI)