

DR. SIMONE ROOS (Orcid ID : 0000-0002-7343-3283)

DR. CHRISTIAN MÖBS (Orcid ID : 0000-0002-5197-7669)

DR. BARBARA BOHLE (Orcid ID : 0000-0002-5105-7985)

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

Caterina Vizzardelli¹, Miriam Gindl¹, Simone Roos², Christian Möbs³, Birgit Nagl¹, Felix Zimmann¹, Veronika Sexl⁴, Lukas Kenner^{2,5,6}, Alina Neunkirchner⁷, Gerhard J. Zlabinger⁷, Winfried F. Pickl⁷, Wolfgang Pfützner³, and Barbara Bohle¹

¹Institute of Pathophysiology and Allergy Research, Medical University of Vienna, Vienna, Austria

²Unit of Laboratory Animal Pathology, University of Veterinary Medicine Vienna, Vienna, Austria

³Department of Dermatology and Allergology, Philipps University Marburg, Marburg, Germany

⁴Institute of Pharmacology and Toxicology, University of Veterinary Medicine Vienna, Austria

⁵Department of Experimental and Laboratory Animal Pathology, Medical University of Vienna, Austria

⁶Ludwig Boltzmann Institute for Cancer Research (LBI-CR), Vienna, Austria

⁷Institute of Immunology, Medical University of Vienna, Vienna, Austria

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Short title: AIT-induced blocking antibodies reduce allergy in humanized mice

Corresponding author

Barbara Bohle, PhD

Institute of Pathophysiology and Allergy Research,

Center of Pathophysiology, Infectiology & Immunology,

Medical University of Vienna

Waehringer Guertel 18-20,

1090 Vienna, Austria

Phone: +43 (1) 40400 51140

Fax: +43 (1) 40400 61880

Email: Barbara.bohle@meduniwien.ac.at

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 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 allergen or PBS. 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 post-AIT sera was assessed *in vitro*.

Results: Human cells were detected in cell suspensions of murine lungs and spleens indicating successful humanization. Humanized mice displayed a more pronounced AHR and bronchial inflammation when challenged with allergen compared to negative controls. Post-AIT sera exerted IgE-blocking activity. In contrast to pre-AIT sera, the presence of heterologous and autologous post-AIT sera significantly reduced the allergic airway inflammation and matched their IgE-blocking activity determined *in vitro*.

Conclusion: Our data demonstrate that post-AIT sera with IgE-blocking activity ameliorate allergic airway inflammation in a human/mouse chimeric model of respiratory allergy independently of AIT-induced cellular changes.

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 $\gamma c^{-/-}$; 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

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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 $\gamma c^{-/-}$ (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 BCATM 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_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 (2×10^5) 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, [³H]-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. Medium controls ranged from 750-18,820 cpm.

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). Cytokine levels were determined by bead array using the Luminex System 100 (Luminex, Austin, TX, USA). Allergen-specific IgE and IgG4 Ab were quantified by ImmunoCAP (Thermo Fisher Scientific). FAB, ELIFAB and BAT were performed as described (7, 17). IgE-facilitated antigen presentation (FAP) assays were performed as described (7). Briefly, Bet v 1-specific T cells were enriched by stimulating PBMC from a non-AIT-treated birch pollen-allergic individual with Bet v 1. rBet v 1 (0.1 µg/ml in 5 µl) was incubated with a serum (20 µl) containing >100 kU_A/L of birch pollen-specific IgE plus the different test sera (20 µl) for 2 h at 37°C. Autologous EBV-transformed B cells (5×10⁴/5 µl) were added and incubated for 1 h at 4°C. After washing twice with PBS, B cells were

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incubated in medium for 2 h at 37°C to allow allergen-uptake and processing, irradiated (60 Gy), and added to Bet v 1-specific T cells (5×10^4 cells/well) in duplicates. Proliferation was assessed after 48 h. T cells stimulated with Bet v 1 preincubated with pre-AIT sera ranged from 4120-4942 cpm and were set to 100%.

Depletion of IgG from post-AIT sera

Post-AIT sera from patients I-III were depleted from IgG Ab using Pierce™ Protein G agarose (ThermoFisher Waltham, MA, USA). Briefly, beads (123 µl of slurry) were equilibrated and incubated with post-AIT sera (400 µl) for 2 h at RT. After centrifugation, new beads were added to the supernatants. This procedure was repeated for six times. The content of Bet v 1-specific IgG antibodies was assessed by ELISA. 96-well plates (Nunc™ MaxiSorp, Nunc, Denmark) were coated with Bet v 1 (1 µg/ml). Sera were diluted 1:2 and incubated overnight at 4°C. HRP-conjugated mouse anti-human IgG (Southern Biotech, Birmingham, AL, USA) and ABTS (Sigma Aldrich) were used for detection. Absorbance was measured at 405 nm.

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 (5×10^6) from a single patient plus BP extract (50 μ g) 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 isofluran (Abbott, Wiesbaden, Germany) and challenged by i.n. application of BP extract (20 μ g), PBS, rBet v 1 (5 μ g) or BSA (20 μ g). In some experiments, rBet v 1 (5 μ g in 5 μ l) was incubated with 15 μ l of pre-AIT or post-AIT sera from the same individual for 1 h at 37°C. As negative control, 30 μ l of the pre- and post-AIT sera from patients I-III were pooled (Σ =180 μ l), PBS (60 μ l) was added and 20 μ l 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 μ l 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-PerCP/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_A/L (median 6.3 kU_A/L) and their Bet v 1-specific IgE levels from 2.2-22.6 kU_A/L (median 5.7 kU_A/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

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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).

Post-AIT sera possess IgE-blocking activity

We employed pre- and post-AIT sera from patients no. I, II and III who had undergone successful AIT with BP (24). All post-AIT sera contained significantly enhanced levels of rBet v 1-specific IgG4 Ab compared to pre-AIT sera from the same individual (Table 2). In addition, all post-AIT sera inhibited rBet v 1-specific IgE activity in FAB assays and BAT (Table 2). To address their potency to block IgE-mediated T cell activation, Bet v 1-specific T cells were stimulated with autologous B cells pulsed with rBet v 1 that had been pre-incubated with a serum containing high levels of allergen-specific IgE in the presence of pre- and post-AIT sera, respectively. The presence of post-AIT sera reduced IgE-mediated proliferation of Bet v 1-specific T cells (Fig. 3A). To address the involvement of allergen-specific IgG Ab in the blocking activity we depleted the post-AIT sera from IgG. Post-AIT sera that were devoid of Bet v 1-specific IgG Ab (Fig. 3B) showed no activity to block IgE-mediated T cell activation (Fig. 3A).

Post-AIT sera 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-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 patients no. I, II and III. Mice challenged with a pool of these sera in PBS served as negative control (Fig. 4A). 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. 4B). 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. 4C). 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. 4D and E).

Post-AIT sera ameliorate allergic airway disease in NSG mice humanized with autologous PBMC

NSG mice were engrafted with cryopreserved PBMC from either patient 12C or 13C that had been collected before the onset of BP-AIT (Table 1). Due to limited cell numbers, patient 12C could not be tested in lymphoproliferation assays. I.n. challenges were performed with rBet v 1 plus the sera from either patient C12 or C13 collected before and after 12 months of AIT with BP (Fig. 5A). 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. 5B). Slightly reduced percentages of basophils, eosinophils and neutrophils were detected in BALF of mice challenged with rBet v 1 plus post-AIT sera (Fig. 5C), accompanied by reduced bronchial inflammation and reduced goblet cell hyperplasia in the lungs (Fig. 5D 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 demonstrating that an alteration of the allergen-specific T cell response may induce tolerance in AIT-treated patients who do not develop specific antibody responses (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 as well as cryopreserved PBMC from individuals with BP-induced rhinoconjunctivitis and low to moderate allergen-

specific IgE levels. 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).

We next 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 IgE-blocking capacity in *in vitro* assays and also 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.

NSG mice display only limited numbers of mast cells in the skin and intestinal mucosa and their FcεRI-bearing cells do not bind human IgE (29). 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, 30). Our PBMC samples contained allergen-reactive T cells as demonstrated by proliferative responses to stimulation with BP and rBet v 1. In addition to human T cells, we detected B cells and monocytes *in vivo* and consider them to be the most relevant APC in this model. Both, monocytes and B cells express the multifunctional FcεRII (CD23) on their surfaces (31, 32). However, CD23 on B cells predominantly binds allergen-IgE complexes which after internalization are directed to HLA class II-rich compartments (33). This process of FAP activates specific CD4⁺ T cells (33, 34) and contributes to allergic inflammation and disease severity in allergic individuals (35, 36). Blocking Ab are considered to reduce FAP by competing with IgE for allergen binding (10, 33, 37). Accordingly, the depletion of allergen-specific IgG from post-AIT sera diminished their activity to inhibit FAP to allergen-specific T cells *in vitro*. It is attractive to speculate that this blocking of IgE-facilitated allergen presentation to CD4⁺ T cells represents the major immune process reducing the allergic inflammation in the human/mouse chimeric model. To

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elucidate a possible contribution of cytokines we compared the levels of IL-5, IL-13, IFN- γ , TNF- α , IL-10, and TGF- β in the pre- and post-AIT sera. Except for TGF- β , the levels of all cytokines were below 15 pg/ml and similar before and after AIT (data not shown). Notably, post-AIT sera from all patients contained significantly lower levels of the immunosuppressive cytokine TGF- β (median value: 2.4 ng/ml, range 0.6-9.0 ng/ml) than the corresponding pre-AIT sera (median value: 6.5 ng/ml, range 2.9-12.0 ng/ml). From these findings we conclude that allergen-specific blocking Ab played a major role in the observed reduction of airway inflammation. Successful inhibition of the formation of IgE-allergen complexes by IgG Ab has been shown to require identical specificity and affinity as the corresponding IgE Ab by employing recombinant antibodies (38). 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 post-AIT sera ameliorate allergic airway responses in a human/mouse chimeric model. Our findings indicate that the *in vivo* amelioration was mediated by AIT-induced blocking Ab and occurred independently from AIT-induced cellular alterations. This autonomous functionality of AIT-induced blocking Ab supports their relevance for effective allergy therapy.

AUTHOR CONTRIBUTIONS

C.V. and B.B. designed the experiments; C.V., M.G., B.N., F.Z., and G.J.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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REFERENCES

1. Calderon MA, Demoly P, Casale T, Akdis CA, Bachert C, Bewick M, et al. Allergy immunotherapy across the life cycle to promote active and healthy ageing: from research to policies: An AIRWAYS Integrated Care Pathways (ICPs) programme item (Action Plan B3 of the European Innovation Partnership on active and healthy ageing) and the Global Alliance against Chronic Respiratory Diseases (GARD), a World Health Organization GARD research demonstration project. *Clin Transl Allergy* 2016;**6**:41.

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2. Kappen JH, Durham SR, Veen HI, Shamji MH. Applications and mechanisms of immunotherapy in allergic rhinitis and asthma. *Ther Adv Respir Dis* 2017;**11**:73-86.
 3. Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens. *J Allergy Clin Immunol* 2014;**133**:621-631.
 4. van de Veen W, Stanic B, Wirz OF, Jansen K, Globinska A, Akdis M. Role of regulatory B cells in immune tolerance to allergens and beyond. *J Allergy Clin Immunol* 2016;**138**:654-665.
 5. Djurup R, Osterballe O. IgG subclass antibody response in grass pollen-allergic patients undergoing specific immunotherapy. Prognostic value of serum IgG subclass antibody levels early in immunotherapy. *Allergy* 1984;**39**:433-441.
 6. Francis JN, James LK, Paraskevopoulos G, Wong C, Calderon MA, Durham SR, et al. Grass pollen immunotherapy: IL-10 induction and suppression of late responses precedes IgG4 inhibitory antibody activity. *J Allergy Clin Immunol* 2008;**121**:1120-1125 e1122.
 7. Subbarayal B, Schiller D, Mobs C, de Jong NW, Ebner C, Reider N, et al. Kinetics, cross-reactivity, and specificity of Bet v 1-specific IgG4 antibodies induced by immunotherapy with birch pollen. *Allergy* 2013;**68**:1377-1386.
 8. Wachholz PA, Soni NK, Till SJ, Durham SR. Inhibition of allergen-IgE binding to B cells by IgG antibodies after grass pollen immunotherapy. *J Allergy Clin Immunol* 2003;**112**:915-922.
 9. James LK, Till SJ. Potential Mechanisms for IgG4 Inhibition of Immediate Hypersensitivity Reactions. *Curr Allergy Asthma Rep* 2016;**16**:23-30.

- Accepted Article
10. van Neerven RJ, Wikborg T, Lund G, Jacobsen B, Brinch-Nielsen A, Arnved J, et al. Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4+ T cells by inhibiting serum-IgE-facilitated allergen presentation. *J Immunol* 1999;**163**:2944-2952.
 11. van Neerven RJ, Knol EF, Ejrnaes A, Wurtzen PA. IgE-mediated allergen presentation and blocking antibodies: regulation of T-cell activation in allergy. *Int Arch Allergy Immunol* 2006;**141**:119-129.
 12. Wurtzen PA, Lund G, Lund K, Arvidsson M, Rak S, Ipsen H. A double-blind placebo-controlled birch allergy vaccination study II: correlation between inhibition of IgE binding, histamine release and facilitated allergen presentation. *Clin Exp Allergy* 2008;**38**:1290-1301.
 13. James LK, Shamji MH, Walker SM, Wilson DR, Wachholz PA, Francis JN, et al. Long-term tolerance after allergen immunotherapy is accompanied by selective persistence of blocking antibodies. *J Allergy Clin Immunol* 2011;**127**:509-516 e501-505.
 14. Shamji MH, Ljorring C, Francis JN, Calderon MA, Larche M, Kimber I, et al. Functional rather than immunoreactive levels of IgG4 correlate closely with clinical response to grass pollen immunotherapy. *Allergy* 2012;**67**:217-226.
 15. Shamji MH, Kappen JH, Akdis M, Jensen-Jarolim E, Knol EF, Kleine-Tebbe J, et al. Biomarkers for Monitoring Clinical Efficacy of Allergen Immunotherapy for Allergic Rhinoconjunctivitis and Allergic Asthma: an EAACI Position Paper. *Allergy* 2017;**72**:1156-1173.
 16. Shamji MH, Wilcock LK, Wachholz PA, Dearman RJ, Kimber I, Wurtzen PA, et al. The IgE-facilitated allergen binding (FAB) assay: validation of a novel flow-

cytometric based method for the detection of inhibitory antibody responses. *J Immunol Methods* 2006;**317**:71-79.

17. Shamji MH, Francis JN, Wurtzen PA, Lund K, Durham SR, Till SJ. Cell-free detection of allergen-IgE cross-linking with immobilized phase CD23: inhibition by blocking antibody responses after immunotherapy. *J Allergy Clin Immunol* 2013;**132**:1003-1005 e1001-1004.
18. Hoffmann HJ, Santos AF, Mayorga C, Nopp A, Eberlein B, Ferrer M, et al. The clinical utility of basophil activation testing in diagnosis and monitoring of allergic disease. *Allergy* 2015;**70**:1393-1405.
19. Ejrnaes AM, Svenson M, Lund G, Larsen JN, Jacobi H. Inhibition of rBet v 1-induced basophil histamine release with specific immunotherapy-induced serum immunoglobulin G: no evidence that FcγRIIB signalling is important. *Clin Exp Allergy* 2006;**36**:273-282.
20. Dodev TS, Bowen H, Shamji MH, Bax HJ, Beavil AJ, McDonnell JM, et al. Inhibition of allergen-dependent IgE activity by antibodies of the same specificity but different class. *Allergy* 2015;**70**:720-724.
21. Martin H, Reuter S, Dehzad N, Heinz A, Bellinghausen I, Saloga J, et al. CD4-mediated regulatory T-cell activation inhibits the development of disease in a humanized mouse model of allergic airway disease. *J Allergy Clin Immunol* 2012;**129**:521-528, 528 e521-527.
22. Meyer-Martin H, Hahn SA, Beckert H, Belz C, Heinz A, Jonuleit H, et al. GARP inhibits allergic airway inflammation in a humanized mouse model. *Allergy* 2016;**71**:1274-1283.
23. Bohle B, Kinaciyan T, Gerstmayr M, Radakovics A, Jahn-Schmid B, Ebner C. Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-

specific T-cell tolerance, and immune deviation. *J Allergy Clin Immunol* 2007;**120**:707-713.

24. Mobs C, Slotosch C, Loffler H, Jakob T, Hertl M, Pfutzner W. Birch pollen immunotherapy leads to differential induction of regulatory T cells and delayed helper T cell immune deviation. *J Immunol* 2010;**184**:2194-2203.
25. Gomez E, Fernandez TD, Dona I, Rondon C, Campo P, Gomez F, et al. Initial immunological changes as predictors for house dust mite immunotherapy response. *Clin Exp Allergy* 2015;**45**:1542-1553.
26. Ziegelmayer P, Focke-Tejkl M, Schmutz R, Lemell P, Ziegelmayer R, Weber M, et al. Mechanisms, safety and efficacy of a B cell epitope-based vaccine for immunotherapy of grass pollen allergy. *EBioMedicine* 2016;**11**:43-57.
27. Couroux P, Patel D, Armstrong K, Larche M, Hafner RP. Fel d 1-derived synthetic peptide immuno-regulatory epitopes show a long-term treatment effect in cat allergic subjects. *Clin Exp Allergy* 2015;**45**:974-981.
28. Ellis AK, Frankish CW, O'Hehir RE, Armstrong K, Steacy L, Larche M, et al. Treatment with grass allergen peptides improves symptoms of grass pollen-induced allergic rhinoconjunctivitis. *J Allergy Clin Immunol* 2017;**140**:486-496.
29. Burton OT, Stranks AJ, Tamayo JM, Koleoglou KJ, Schwartz LB, Oettgen HC. A humanized mouse model of anaphylactic peanut allergy. *J Allergy Clin Immunol* 2017;**139**:314-322 e319.
30. Bellinghausen I, Saloga J. Analysis of allergic immune responses in humanized mice. *Cell Immunol* 2016;**308**:7-12.
31. Paul-Eugene N, Kolb JP, Abadie A, Gordon J, Delespesse G, Sarfati M, et al. Ligation of CD23 triggers cAMP generation and release of inflammatory mediators in human monocytes. *J Immunol* 1992;**149**:3066-3071.

- Accepted Article
32. Dugas B, Paul-Eugene N, Cairns J, Gordon J, Calenda A, Mencia-Huerta JM, et al. Leukotriene B4 potentiates the expression and release of Fc epsilon RII/CD23, and proliferation and differentiation of human B lymphocytes induced by IL-4. *J Immunol* 1990;**145**:3406-3411.
 33. Peng W, Grobe W, Walgenbach-Brunagel G, Flicker S, Yu C, Sylvester M, et al. Distinct Expression and Function of FcepsilonRII in Human B Cells and Monocytes. *J Immunol* 2017;**198**:3033-3044.
 34. van der Heijden FL, Joost van Neerven RJ, van Katwijk M, Bos JD, Kapsenberg ML. Serum-IgE-facilitated allergen presentation in atopic disease. *J Immunol* 1993;**150**:3643-3650.
 35. Mudde GC, Reischul IG, Corvaia N, Hren A, Poellabauer EM. Antigen presentation in allergic sensitization. *Immunol Cell Biol* 1996;**74**:167-173.
 36. Selb R, Eckl-Dorna J, Neunkirchner A, Schmetterer K, Marth K, Gamper J, et al. CD23 surface density on B cells is associated with IgE levels and determines IgE-facilitated allergen uptake, as well as activation of allergen-specific T cells. *J Allergy Clin Immunol* 2017;**139**:290-299 e294.
 37. Francis JN. The facilitated antigen binding (FAB) assay-a protocol to measure allergen-specific inhibitory antibody activity. *Methods Mol Med* 2008;**138**:255-261.
 38. Holm J, Willumsen N, Wurtzen PA, Christensen LH, Lund K. Facilitated antigen presentation and its inhibition by blocking IgG antibodies depends on IgE repertoire complexity. *J Allergy Clin Immunol* 2011;**127**:1029-1037.

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

patient		IgE [kU _A /L] specific for		Lymphoproliferation [SI] to		
No.	Sex	Birch	Bet v 1	BP	rBet v 1	IL-2
1	M ^{a)}	12.8	n.t.	2.1 ^{b)}	3.4	n.t.
2	F	4.3	3.8	11.0	4.5	12.3
3	M	4.9	4.2	n.t.	n.t.	n.t.
4	F	27.6	22.6	n.t.	n.t.	n.t.
5	F	15.8	14.7	4.0	2.4	11.0
6	F	3.5	2.2	9.9	2.9	20.1
7	M	6.3	5.4	3.4	1.1	26.4
8	F	6.3	5.9	4.0	2.4	11.0
9	M	6.7	9.1	64.3	20.7	51.3
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)

Patient	Bet v 1-specific IgE [kU _A /L]		Bet v 1-specific IgG ₄ [μg/ml]		Blocking activity [%] of post-AIT sera in	
	Pre-AIT	Post-AIT	Pre-AIT	Post-AIT	FAB	BAT
I [°]	20.9	23.9	0.00	2.49	98	79
II ^{°°}	22.0	58.8	0.28	26.8	85	75
III ^{°°°}	71.8	49.2	1.22	2.41	89	64
12C*	24.9	19.2	1.56	7.17	89**	93
13C*	10.0	13.5	0.18	2.45	84**	76

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. NK, natural killer cells; 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. NK, natural killer cells; 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. Blocking of IgE-mediated activation of allergen-specific T cells by post-AIT sera depends on IgG Ab. (A) Proliferation of Bet v 1-specific T cells in response to autologous B cells pulsed with rBet v 1 pre-incubated with an indicator serum plus pre-AIT (white bars), post-AIT (gray bars) or IgG-depleted post-AIT (black bars) sera; (B) rBet v 1-specific IgG Ab in non-depleted (gray bars) and IgG-depleted (black bars) post-AIT sera as tested by ELISA, O.D. optical density.

Figure 4. 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 (E) PAS.

Figure 5. 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.







