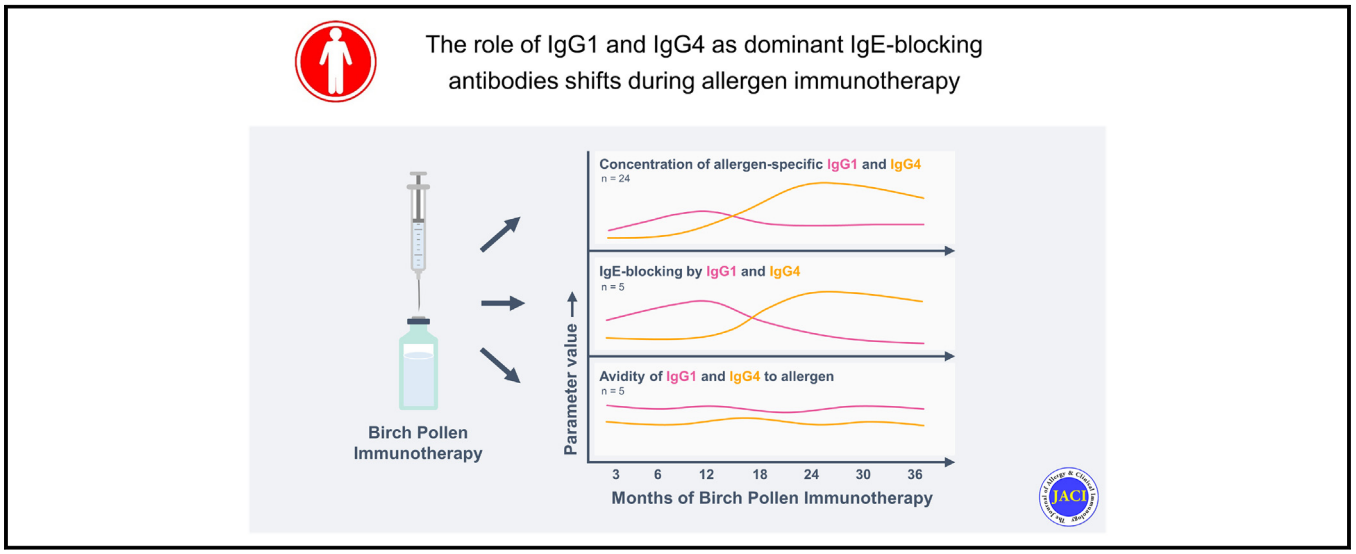
The role of IgG_1 and IgG_4 as dominant IgE-blocking antibodies shifts during allergen immunotherapy

Maria R. Strobl, DI,[a] Hilal Demir, MSc,[a] Gabriela Sánchez Acosta, PhD,[a] Anja Drescher, PhD,[b] Claudia Kitzmüller, PhD,[a] Christian Möbs, PhD,[c] Wolfgang Pfützner, MD,[c] and Barbara Bohle, PhD[a] *Vienna, Austria; and Freiburg and Marburg, Germany*

GRAPHICAL ABSTRACT



Background: The induction of allergen-specific IgE-blocking antibodies is a hallmark of allergen immunotherapy (AIT). The inhibitory bioactivity has largely been attributed to IgG_4; however, our recent studies indicated the dominance of IgG_1 early in AIT.

Objectives: Here, the IgE-blocking activity and avidity of allergen-specific IgG_1 and IgG_4 antibodies were monitored throughout 3 years of treatment.

Methods: Serum samples from 24 patients were collected before and regularly during AIT with birch pollen. Bet v 1–specific IgG_1 and IgG_4 levels were determined by ELISA and ImmunoCAP, respectively. Unmodified and IgG_1- or IgG_4-depleted samples were compared for their inhibition of Bet v 1–induced basophil activation. The stability of Bet v 1–antibody complexes was compared by ELISA and by surface plasmon resonance.

Results: Bet v 1–specific IgG_1 and IgG_4 levels peaked at 12 and 24 months of AIT, respectively. Serological IgE-blocking peaked at 6 months and remained high thereafter. In the first year of therapy, depletion of IgG_1 clearly diminished the inhibition of basophil activation while the absence of IgG_4 hardly reduced IgE-blocking. Then, IgG_4 became the main inhibitory isotype in most individuals. Both isotypes displayed high avidity to Bet v 1 *ab initio* of AIT, which

From [a]the Institute of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna; [b]Cytiva Europe GmbH, Freiburg; and [c]the Clinical and Experimental Allergology, Department of Dermatology and Allergology, Philipps-Universität Marburg.

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Corresponding author: Barbara Bohle, PhD, Institute of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria. E-mail: barbara.bohle@meduniwien.ac.at.

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did not increase during treatment. Bet v 1–IgG_1 complexes were enduringly more stable than Bet v 1–IgG_4 complexes were. Conclusions: In spite of the constant avidity of AIT-induced allergen-specific IgG_1 and IgG_4 antibodies, their dominance in IgE-blocking shifted in the course of treatment. The blocking activity of allergen-specific IgG_1 should not be underestimated, particularly early in AIT. (J Allergy Clin Immunol 2023;151:1371-8.)

Key words: *Allergen immunotherapy, antibodies, IgE-blocking, IgG_4, IgG_1, avidity, birch pollen, Bet v 1*

Allergic rhinoconjunctivitis symptoms can be effectively reduced by allergen immunotherapy (AIT).[1] As opposed to symptomatic treatment, AIT has the potential to modify the immunological response to the disease-eliciting allergen and counteract disease progression.[2] Subcutaneous AIT (SCIT) entails weekly injections of increasing doses of the respective allergen extract, followed by monthly injections of a maintenance dose that are continued for 3-5 years to achieve long-term tolerance and relief of allergic symptoms.[2,3]

It is well recognized that, along with modulated cellular immune responses, AIT induces allergen-specific IgG antibodies.[4-6] A fraction of these antibodies inhibit IgE-allergen interactions, thereby preventing crosslinking of IgE-loaded high-affinity FCεRI molecules and subsequent activation of effector cells, such as basophils and mast cells.[7] As a consequence, the release of inflammatory mediators is reduced, which may result in clinical improvement of allergen-induced symptoms.[8] This concept was recently confirmed in pilot studies with individuals allergic to cat and birch pollen who after receipt of IgE-blocking mAbs specific for the major allergens Fel d 1 and Bet v 1, respectively, developed fewer immediate reactions following allergen exposure.[9-11] While functional IgE-blocking has mostly been attributed to IgG_4 antibodies,[5,8,12] we have recently shown that after 16 weeks of sublingual AIT, allergen-specific IgG_1 antibodies blocked allergen-induced basophil activation more potently than IgG_4 antibodies did.[13] Previous studies have also shown that allergen-specific IgG_1 is the dominant isotype in the early immune response of AIT.[14-16] Hence, there is reason to assume that the IgE-blocking capacity of IgG_1 predominates initially before an increasing protective effect of IgG_4 sets in, which prevails in later phases of AIT. Nevertheless, others have shown that depletion of IgG_4 from sera collected after 2 years of AIT does not completely abrogate the IgE-inhibitory effect, suggesting a long-lasting protective role of IgG_1.[5,17]

Here, we analyzed plasma samples of individuals allergic to birch pollen taken before and at frequent time points during 36 months of SCIT with birch pollen (BP-SCIT) in terms of Bet v 1–specific IgG_1 and IgG_4 levels and their capacity to inhibit Bet v 1–induced basophil activation. To assess whether the latter depended on antibody avidity, we monitored the stability of Bet v 1–IgG_1 and Bet v 1–IgG_4 complexes during the course of treatment. Despite a constant high avidity of AIT-induced allergen-specific IgG_1 and IgG_4 antibodies, their dominance in IgE-blocking shifted in the course of treatment. This study expands our knowledge on the dynamics and avidity of allergen-specific IgG_1 and IgG_4 antibodies and their contribution to functional IgE-blocking in AIT.

Abbreviations used
AIT: Allergen immunotherapy
BP-SCIT: Birch pollen-SCIT
PBS-T: PBS/0.05% Tween 20
RUs: Response units
SCIT: Subcutaneous AIT
SPR: Surface plasmon resonance

METHODS

Plasma samples

Ample volumes (1.2 mL) of plasma samples (diluted 1:2 in PBS) collected at baseline and indicated time points during BP-SCIT were available from 24 patients (age range 10–57 years, median 35 years). All individuals received ALK Alutard SQ197 (ALK-Abello Ltd, Reading, UK), which is a mix of native extracts of pollen from early-flowering trees (ie, birch, alder, and hazel) adsorbed to aluminum hydroxide, and showed improvement of birch pollinosis. The cumulative dose of Bet v 1 was around 150 μg for 1 year and around 500 μg for 3 years of SCIT. Blood samples were collected at months 0, 1, 3, 12, 24, and 36 out of the birch pollen season and at months 6, 18, and 30 in the birch pollen season, respectively. The study was approved by the Ethics Committee of the Medical Faculty of the Philipps-Universität, Marburg, Germany (Az 128/05 and 24/13). All patients provided written informed consent to participate in the trial.

Quantification of allergen-specific IgG antibodies

Bet v 1–specific IgG_4 antibodies were measured by ImmunoCAP 100 (Thermo Fisher Scientific, Uppsala, Sweden). To measure allergen-specific IgG_1, microplates (Nunc MaxiSorp, Thermo Fisher Scientific, Waltham, Mass) were coated with recombinant Bet v 1 (1 μg/mL) produced in-house[13] in carbonate buffer (pH 9.6) overnight at 4°C. After saturation for 6 hours with PBS/0.05% Tween 20 (PBS-T) supplemented with 1% human serum albumin, plasma samples were incubated overnight at 4°C. After washing 5 times with PBS-T, bound IgG_1 antibodies were detected with a mouse anti-human IgG_1 (Thermo Fisher Scientific) followed by a horseradish peroxidase–conjugated sheep anti-mouse IgG antibody (Amersham, GE Healthcare, Pittsburgh, Pa). After addition of the respective substrates, OD was measured on a Tecan infinite F50 microplate reader (Tecan, Männedorf, Switzerland). All samples were used at serial dilutions of 1:40-1:200. Measurements of all time points were done simultaneously on frozen samples. Bet v 1–specific IgG_1 levels were quantified by using a human Bet v 1–specific IgG_1 mAb as in-house standard done in duplicates.

Depletion and elution of IgG_1 and IgG_4 antibodies

Plasma samples (200 μL) were incubated with either CaptureSelect IgG_1 or CaptureSelect IgG_4 human affinity matrices (120-150 μL, Thermo Fisher Scientific) for 1.5 hours at room temperature in constant rotation. After centrifugation, supernatants were added to the respective fresh CaptureSelect matrix. These cycles of depletion were repeated 3 times. The fourth incubation was done overnight at 4°C and successful depletion was confirmed by ELISA. Matrix-bound IgG_1 and IgG_4 were eluted using 100 mmol/L glycine-HCl, pH 3.0, and neutralized with 1 mol/L Tris-HCl, pH 8.2. Fractions containing IgG as measured by absorbance at 280 nm were pooled and dialyzed against PBS, pH 7.4.

Inhibition of basophil activation

Heparinized blood was collected from 7 birch pollen-allergic individuals who did not receive treatment and displayed Bet v 1–specific IgE levels of > 0.35 kU/L with informed consent and ethical clearance by the local ethics committee (EK1344/2018). Basophil inhibition tests were performed as described.[13] Briefly, Bet v 1 was diluted in HEPES calcium buffer (pH 7.4)

supplemented with BSA (1 mg/mL) and IL-3 (2 ng/mL). Plasma samples (15 μL) were added for 1 hour at 37°C prior to the addition of heparinized blood collected from 2-4 different donors. After 15 minutes at 37°C, the reaction was stopped by addition of HEPES/EDTA buffer (pH 7.4). Cells were stained with anti-CCR3-APC (allophycocyanin), anti-CD123-PerCP (peridinin-chlorophyll-protein), and anti-CD63-PE (phycoerythrin) antibodies (all from BioLegend, San Diego, Calif). Erythrocytes were lysed by incubation with an ammonium chloride buffer (pH 7.3). The cells were washed and acquired on a FACSCanto II using FACSDiva Software Version 6.1.3 (BD Biosciences, San Jose, Calif). Basophils were identified as $CD123^+CCR3^+$ cells with CD63 as marker of activation. Allergen concentrations inducing 15%-70% $CD63^+$ basophils in the presence of month 0 samples were used for analysis. This baseline activation was normalized to 100%, and the mean percentages of inhibition by samples collected during SCIT were individually calculated.

Avidity ELISA

We modified protocols based on chaotropic disruption of antigen-antibody complexes by using acidic solutions to measure the binding strength of IgG_1 and IgG_4.[18,19] Briefly, microplates (Nunc MaxiSorp, Thermo Fisher Scientific) were coated with Bet v 1 (1 μg/mL) and saturated with PBS-T supplemented with 1% human serum albumin. Sixteen replicates of each plasma sample were incubated overnight at 4°C. After washing 5 times, phosphate-citrate buffers with pH values of 7.4, 6.4, 6.0, 5.4, 5.0, 4.4, 4.0, or 3.4 were added in duplicate and incubated for exactly 2 hours at room temperature to dissociate Bet v 1–bound antibodies. Remaining IgG_1 antibodies were detected by a horseradish peroxidase–labeled mouse–anti-human IgG_1 antibody (Southern Biotech, Birmingham, Ala). IgG4 was detected by an AKP-labeled mouse–anti-human IgG4 (BD Pharmingen, San Diego, Calif). OD values obtained with a phosphate-citrate buffer of pH 7.4 were normalized to 100%, and the percentage of remaining antibodies was individually calculated. Then, the avidity index, defined as the pH value eluting 50% of antibodies, was individually calculated.

Surface plasmon resonance

Surface plasmon resonance (SPR) experiments were performed on a Biacore T200 (Cytiva, Uppsala, Sweden). Bet v 1 diluted in 10 mmol/L sodium acetate, pH 4.6, was immobilized on flow cell 2 of a CM5 chip (Cytiva) to a level of approximately 2400 response units (RUs) using standard amine-coupling chemistry. The activated and deactivated flow cell 1 served as reference surface. A 10 mmol/L HEPES buffer containing 150 mmol/L NaCl and 0.05% Surfactant P20 (HBS-P+, Cytiva) was used as running buffer. All analyses were performed at 37°C and a flow rate of 10 μL/min. Purified IgG_1 and IgG_4 antibodies were injected undiluted or diluted in PBS over both flow cells for 240 seconds and dissociation was monitored for 900 seconds. If samples showed low binding levels, they were concentrated using centrifugal filters with a 30-kDa cutoff (Merck, Darmstadt, Germany). Thereafter, the Bet v 1 surface was regenerated by a 30-second pulse of 10 mmol/L glycine-HCl, pH 1.5. Samples that reached >15 RUs were considered positive. All IgG samples were analyzed on the same Bet v 1 sensor chip surface. The integrity of immobilized Bet v 1 was monitored by repeated injections of a murine Bet v 1–specific IgG_1 mAb. PBS injections served as blanks to correct for drift and bulk effects. The binding levels of all samples were normalized to 100 RUs to compare the dissociation behavior independently from the concentration. Original sensorgrams are shown in Fig E1 in this article's Online Repository at www.jacionline.org. Additionally, the sensorgrams were evaluated using the Biacore T200 evaluation tool "Stability", which mathematically fits the data to a kinetic model that considers only the concentration-independent dissociation phase and assumes 2 independent monovalent 1:1 dissociations. The data-fitting process calculates half-lives ($t_{1/2}$) for each of the 2 interactions and the collective half-life for each IgG sample was calculated by proportional addition of the 2 half-lives. Goodness of fit was confirmed by consistently low chi-square values (≤0.4 % of binding level).

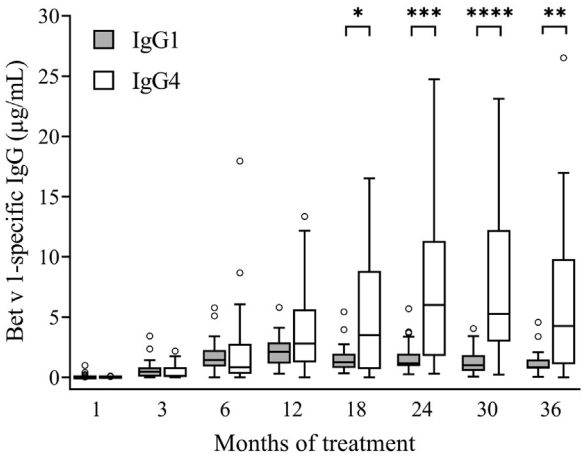

FIG 1. Bet v 1–specific IgG_1 and IgG_4 levels during AIT. Bet v 1–specific IgG_1 and IgG_4 levels were quantified at indicated time points during BP-SCIT (n = 24). Levels in pre-SCIT samples were individually subtracted from levels at later time points. Boxplots with Tukey *whiskers* are shown, and *dots* indicate outliers. *P < .05, **P < .01, ***P < .001, ****P < .0001, Mann-Whitney U test.

Statistics

Statistical analyses were performed using GraphPad Prism 9.3.1 (San Diego, Calif) and IBM SPSS 23.0 software (Armonk, NY). Between-group comparisons were done with Mann-Whitney U test. Intragroup analyses were done with the Wilcoxon signed-rank test. All tests were 2-tailed and differences were considered significant when $P \leq .05$.

RESULTS

Bet v 1–specific IgG_1 and IgG_4 levels during AIT

The levels of Bet v 1–specific IgE, IgG_1, and IgG_4 antibodies in samples from 24 individuals were determined in regular intervals during 36 months of BP-SCIT (see Tables E1-E3 in this article's Online Repository at www.jacionline.org). After 1 month of therapy, no significant increase of specific IgG_1 and IgG_4 antibodies was found; however, after 3 months, the levels started to be significantly higher than before treatment (P < .001 for all subsequent time points, Wilcoxon signed-rank test). To better compare the course of both isotypes during AIT, Bet v 1–specific IgG_1 and IgG_4 levels were summarized after individual subtraction of the levels in pre-SCIT samples (Fig 1). AIT-induced Bet v 1–specific IgG_1 levels peaked at month 12 and IgG_4 levels at month 24. Overall, Bet v 1–specific IgG_1 levels were slightly higher than IgG_4 levels during the first 6 months of therapy and equalized at month 12. Thereafter, Bet v 1–specific IgG_4 levels significantly outnumbered IgG_1 levels (Fig 1, and Table E2 and E3).

Serological IgE-blocking activity during AIT

By monitoring Bet v 1–specific IgG_1 and IgG_4 levels in 24 patients during BP-SCIT, we found 3 types of responders: 46% of the individuals showed higher allergen-specific IgG_1 levels until month 12 followed by higher levels of allergen-specific IgG_4 (group 1), 42% displayed continuously higher IgG_4 than IgG_1 levels (group 2), and 12% showed continuously higher IgG_1 than IgG_4 responses (group 3). Accordingly, we selected 6 representative subjects of group 1 (P2, P7, P19, P23, P27, P28), 4 of group 2 (P11, P13, P24, P38), and 2 of group 3 (P14, P16)

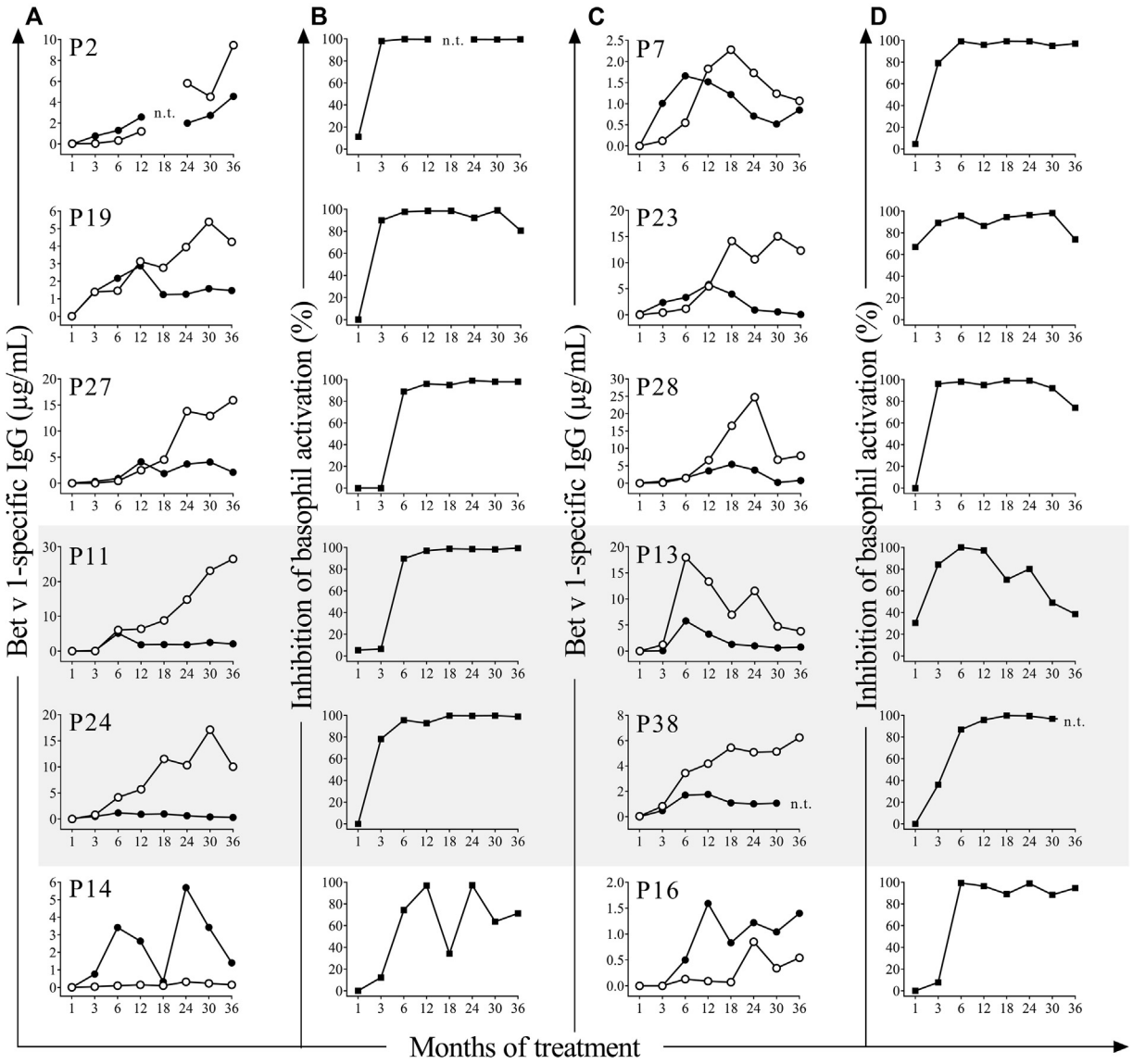

FIG 2. Allergen-specific IgG levels and IgE-blocking activity during AIT. The levels of Bet v 1–specific IgG_1 *(black circles)* and IgG_4 *(white circles)* **(A,C)** and the percentage of inhibition of Bet v 1–induced basophil activation at indicated time points **(B,D)** are shown. Levels in pre-SCIT samples were individually subtracted from levels at later time points. The *gray box* separates the 3 types of IgG responders described in the results section. *NT,* Not tested.

to assess the IgE-blocking activity at months 1, 3, 6, 12, 18, 24, 30, and 36 of AIT. Fig 2 depicts individual allergen-specific IgG_1 and IgG_4 levels together with serological IgE-blocking activity. At month 1, all subjects hardly reduced Bet v 1–induced basophil activation, which accorded with not yet increased IgG levels. All individuals developed an IgE-blocking activity of >90%, however, at different time points of AIT. Two individuals started at month 3, 6 at month 6, and 4 individuals at month 12. In 7 individuals, full IgE-blocking persisted thereafter. In 1 individual (P13), the IgE-blocking activity began to decline after month 12 and in 3 individuals (P19, P23, P28) after month 30. One patient (P14) showed a fluctuating IgE-blocking activity throughout therapy.

Contribution of IgG_1 and IgG_4 antibodies to IgE-blocking during AIT

To evaluate the contribution of IgG_1 and IgG_4 to IgE-blocking in the course of therapy, we selected 5 individuals who had developed both isotypes and depleted either antibody class from the samples collected at months 3, 6, 12, 18, 24, 30, and 36, respectively. Then, the inhibition of Bet v 1–induced basophil activation by complete samples was compared to those devoid of either IgG_1 or IgG_4 (Fig 3). In all individuals, the depletion of IgG_1 resulted in a prominent reduction of IgE-blocking activity at months 3, 6, and 12 of AIT, whereas the absence of IgG_4 caused a moderate reduction of inhibitory capacity. After month 12, the depletion of IgG_4 more potently reduced IgE-blocking than the absence of IgG_1 in 4

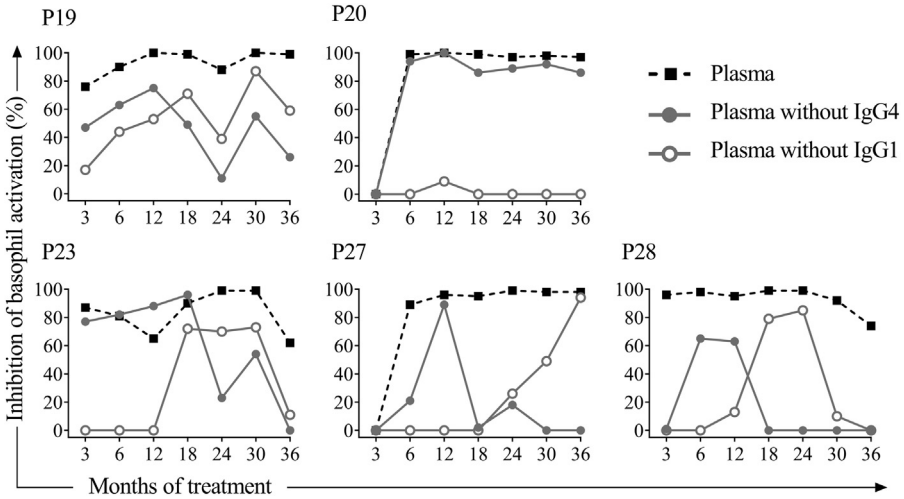

FIG 3. The contribution of Bet v 1–specific IgG_1 and IgG_4 to IgE-blocking during AIT. Basophils were activated with Bet v 1 after incubation with plasma or with plasma devoid of either IgG_1 or IgG_4. The percentage of inhibition of basophil activation was calculated in relation to month 0.

TABLE I. Avidity index* of Bet v 1–specific IgG_1 and IgG_4 antibodies during AIT

	Months of treatment					
	12		24		36	
Patient	IgG_1	IgG_4	IgG_1	IgG_4	IgG_1	IgG_4
P19	3.85*	4.11	3.75	4.40	3.68	4.26
P20	4.02	4.68	3.90	4.70	3.71	4.52
P23	3.89	4.82	3.76	5.39	3.79	5.57
P27	3.73	4.78	<3.4	4.79	<3.4	4.65
P28	3.85	4.83	<3.4	4.75	<3.4	4.48
Median	3.85	4.78	3.75	4.75	3.68	4.52

*Avidity indexes were defined as the pH value eluting 50% of antibodies.

of 5 individuals. Notably, in 1 individual (P20) allergen-specific IgG_4 was irrelevant for IgE-blocking at every time point.

Avidity of Bet v 1–specific IgG_1 and IgG_4 antibodies during AIT

We took 2 approaches with selected samples from 5 patients (P19, P20, P23, P27, P28) to investigate the avidity of Bet v 1–specific IgG_1 and IgG_4 antibodies in the course of AIT. First, the binding of either IgG subclass in plasma samples to plate-bound Bet v 1 was challenged by acidic conditions. As shown in Table I, the pH value eluting 50% of antibodies (avidity index) of IgG_1 was significantly lower than the avidity index of IgG_4 ($P < .05$ for each time point, Wilcoxon signed-rank test) indicating a higher avidity of IgG_1 antibodies to Bet v 1.

Second, IgG_1 and IgG_4 antibodies eluted from isotype-specific affinity matrices were injected over a Bet v 1–loaded SPR sensor chip surface. Of a total of 70 IgG samples, 1 IgG_1 sample and 5 IgG_4 samples from plasma collected at months 3 or 6 of AIT showed no association to Bet v 1 due to too low antibody concentrations. The stability of allergen-antibody complexes was compared by overlaying the dissociation phase of normalized sensorgrams (Fig 4, *A*). The dissociation of IgG_1 and IgG_4 antibodies varied markedly among the individuals. In 3 of 5 individuals (P20, P23, P27) allergen-IgG_1 complexes were more stable than allergen-IgG_4 complexes as indicated by a less pronounced decrease of RUs during the dissociation phase. We found no correlation between complex stability and therapy duration. To allow a quantitative comparison of IgG_1 and IgG_4, hypothetical half-lives ($t_{1/2}$) of allergen-antibody complexes were calculated for all samples from 1 individual (Fig 4, *B*). Median $t_{1/2}$ of Bet v 1–IgG_1 complexes were significantly higher than those of Bet v 1–IgG_4 complexes in 4 of 5 individuals (Fig 4, *B*).

DISCUSSION

The increase of allergen-specific IgG antibodies by AIT is a consistent immunological finding; however, only their IgE-blocking activity has been associated with therapeutic efficacy.[7] We monitored the serological IgE-blocking activity as well as the quantity and avidity of allergen-specific IgG_1 and IgG_4 antibodies during 36 months of BP-SCIT. We hypothesized on an alternating dominance of the 2 isotypes. Our close monitoring of their quantity confirmed an overall trend of higher Bet v 1–specific IgG_1 levels in the first year of therapy. Thereafter, allergen-specific IgG_4 significantly outnumbered IgG_1 antibodies. Still, a quantitative predominance of 1 subclass throughout therapy was detected in several patients and highlighted the individual diversity of humoral responses to AIT.

IgE-blocking activity was assessed by inhibition of allergen-induced basophil activation with samples collected at 8 time

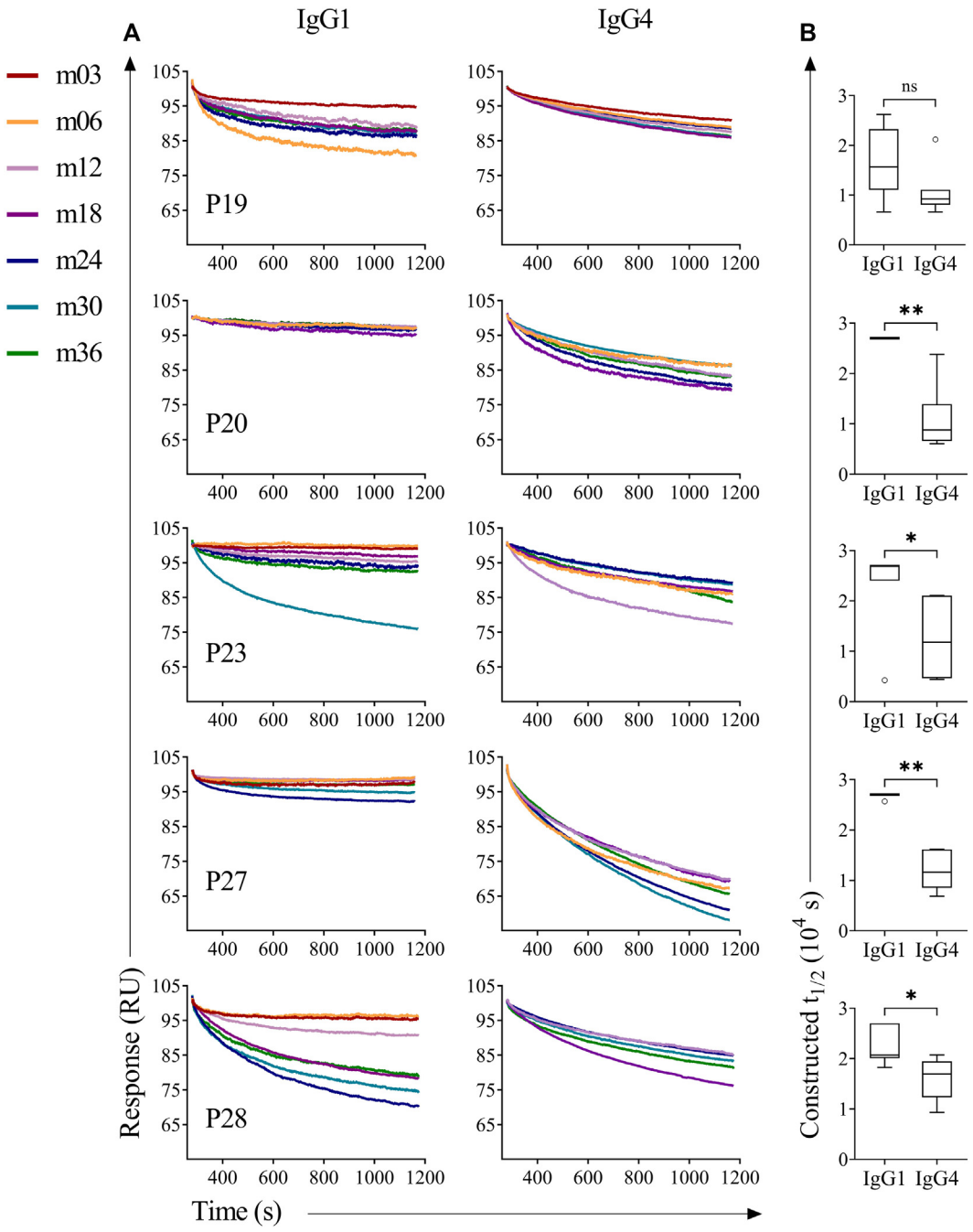

FIG 4. Stability of Bet v 1–IgG complexes during AIT assessed by SPR. **(A)** The dissociation of IgG_1 and IgG_4 antibodies purified from plasma collected after 3, 6, 12, 18, 24, 30, and 36 months (*m*) of BP-SCIT from Bet v 1 was monitored for 5 patients. The binding levels were normalized to 100 RUs and a zoom-in on the dissociation phase is shown. **(B)** Boxplots of hypothetical $t_{1/2}$ of complexes of all time points are shown with Tukey *whiskers*, and *dots* indicate outliers. *$P < .05$, **$P < .01$; Mann-Whitney *U* test.

points during AIT from a total of 13 individuals. Overall, the IgE-blocking activity reached its maximum within the first year of therapy. Although it is impossible to translate these results into clinical relevance, they accord with the evaluation that 2-4 months of AIT are required for onset of efficacy and 12-24 months for maximal clinical effects.[3] Three years of AIT have been proposed as necessary to achieve long-term remission of disease,[20,21] which has been associated with the persistence of IgE-blocking antibodies.[17] Interestingly, we noted a decline of IgE-blocking activity in some individuals already in the course of AIT. Collectively, these observations demonstrate that the bioactivity of protective IgG antibodies varies longitudinally and individually in the course of AIT.

To examine the contribution of IgG_1 and IgG_4 to the serologic inhibitory activity in the course of AIT, we performed basophil inhibition assays with samples devoid of either isotype. In all studied individuals, IgG_1 dominated IgE-blocking during the first year followed by a dominant role of IgG_4 thereafter in 4 of 5

individuals. Notably, in 1 individual, solely IgG_1 was protective in the entire course of treatment despite the fact that the patient developed comparable levels of allergen-specific IgG_1 and IgG_4 antibodies until month 12 and higher IgG_4 levels thereafter (see Tables E2 and E3). This example together with the individuals who displayed continued serological IgE-blocking during AIT in the absence of remarkable IgG_4 levels (P14 and P16) (Fig 2) revealed that IgG_1 have long-lasting relevance as protective antibodies. Furthermore, these findings indicate that IgG_4 is no reliable biomarker for clinical success in certain individuals.[22]

To elucidate a possible link between avidity and IgE-blocking activity, we investigated the binding strength of IgG_1 and IgG_4 to solid-phase bound Bet v 1 by an ELISA-based end-point measurement and by real-time monitoring of antibody dissociation in SPR. Both approaches with samples from several time points during AIT unanimously revealed that the avidity of either subclass did not substantially increase during treatment. Along these lines, Huber et al[23] detected no difference in the avidity of Bet v 1–specific IgG in sera from 5 patients obtained before and after 4 and 12 months and Svenson et al[24] in sera from 6 patients collected before and after 5 years of BP-SCIT, respectively. We tested a mouse anti-Bet v 1 mAb with an affinity constant (K_D) of 750 pmol/L under similar conditions as the IgG samples in SPR and obtained a comparable sensorgram (see Fig E2 in this article's Online Repository at www.jacionline.org). From this we conclude that the avidity of AIT-induced IgG_1 and IgG_4 antibodies was high right from the start of therapy and roughly corresponded to the 3 Bet v 1–specific mAbs that successfully prevented allergic reactions of patients to nasal allergen challenge with birch pollen.[10,11] The high initial avidity may result from sequential class switching required for the generation of high-affinity anaphylactic IgE antibodies.[25,26] This concept implies that a larger fraction of IgE derives from antigen-experienced IgG^+ B cells and a smaller fraction directly from naïve B cells expressing low-affinity IgM or IgD.[27] Thus, it is conceivable that such intermediate IgG^+ memory B cells accounted for the prompt production of high-affinity IgG_1 and IgG_4 antibodies when restimulated with allergen in AIT. In fact, single IgG1+, IgG2+, and IgG4+ memory B cells expressing allergen-specific antibodies and sharing common clonal origin with IgE+ B cells were recently detected in individuals undergoing sublingual AIT.[28] Finally, the comparable affinity of Bet v 1–specific IgG antibodies isolated from sera of untreated and BP-SCIT–treated individuals further supports this concept.[29]

Another major finding of this study is that during AIT allergen-IgG_1 complexes were enduringly more stable than allergen-IgG_4 complexes. An asset of SPR as a real-time measurement lies in the possibility to discriminate between different binding behaviors by studying the curvature of sensorgrams. By doing so, a less pronounced bivalent dissociation behavior became apparent in IgG_4 samples. This observation is in accordance with IgG_4 being described as a hetero-bivalent antibody.[30-33] Considering that a substantial proportion of allergen-specific IgG_4 antibodies in human serum may be functionally monovalent, it is plausible that bivalent IgG_1 antibodies form more stable immune complexes with Bet v 1. Moreover, preliminary data from our laboratory indicate that the epitope repertoire recognized by AIT-induced IgG_1 antibodies is broader than that of IgG_4 antibodies, which may also contribute to the formation of more stable allergen-antibody complexes.

For the first time to our knowledge, the protective role and avidity of AIT-induced IgG_1 and IgG_4 antibodies were compared regularly during 3 years of treatment. Although derived from a limited number of subjects, our results provide evidence for very individual humoral responses to AIT and challenge the dogma that IgE-blocking is mainly attributed to IgG_4 antibodies. The contribution of allergen-specific IgG_1 antibodies should not be underestimated, particularly in the first year of AIT.

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Key messages

- **Allergen immunotherapy induces highly affine allergen-specific IgG_1 and IgG_4 antibodies with functional IgE-blocking activity.**
- **IgG_1 dominates IgE-blocking in the first year of therapy.**
- **IgG_4 dominates IgE-blocking after the first year of therapy.**

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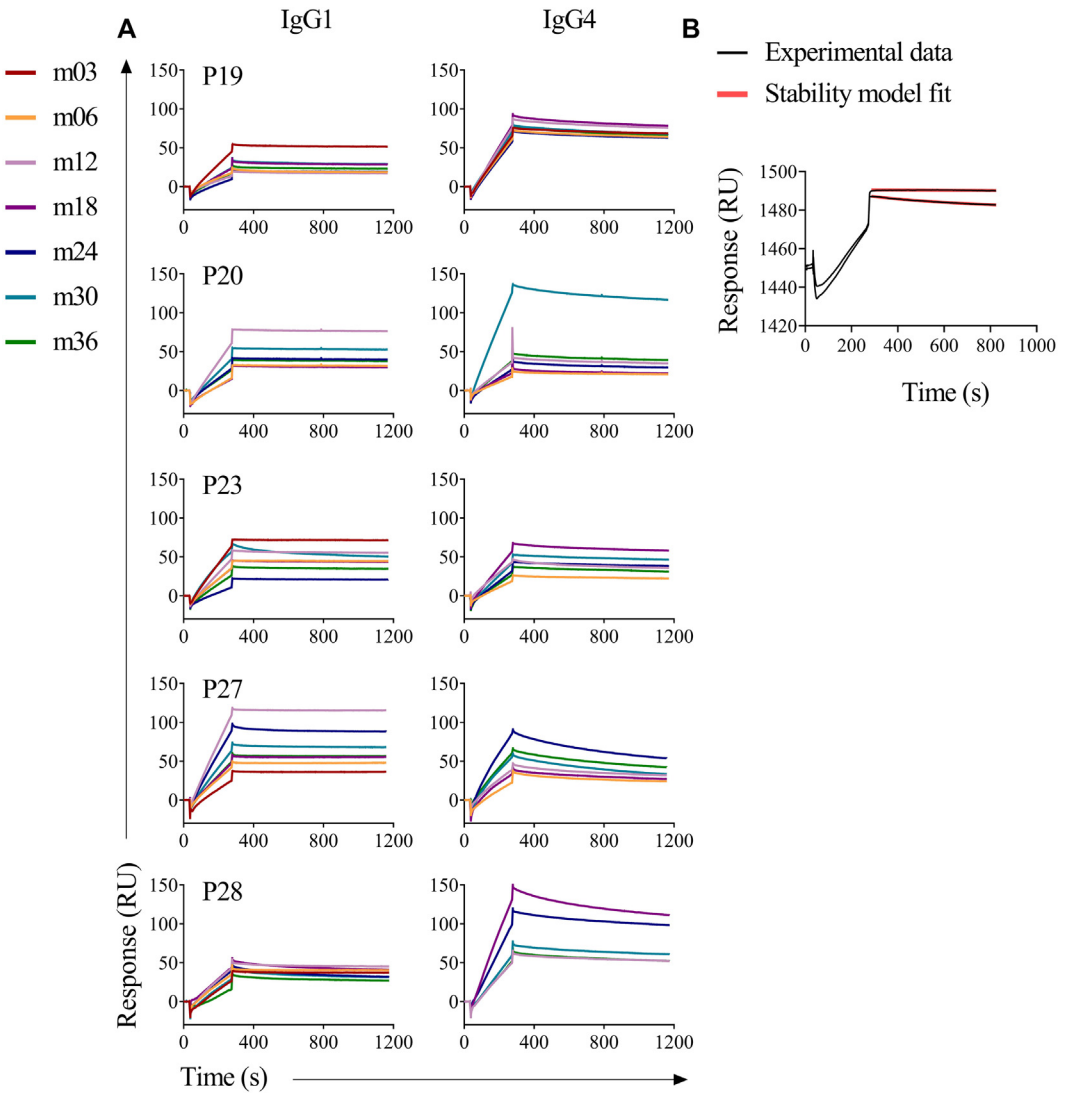

FIG E1. Sensorgrams of antibodies binding to immobilized Bet v 1. **(A)** IgG1 and IgG4 antibodies purified from samples collected at months 3, 6, 12, 18, 24, 30, and 36 of BP-SCIT were injected over a Bet v 1–immobilized SPR sensor chip and dissociation was monitored. **(B)** The Stability model fit *(red line)* considers the concentration-independent dissociation phase of IgG *(black line)* to calculate hypothetical complex half-lives.

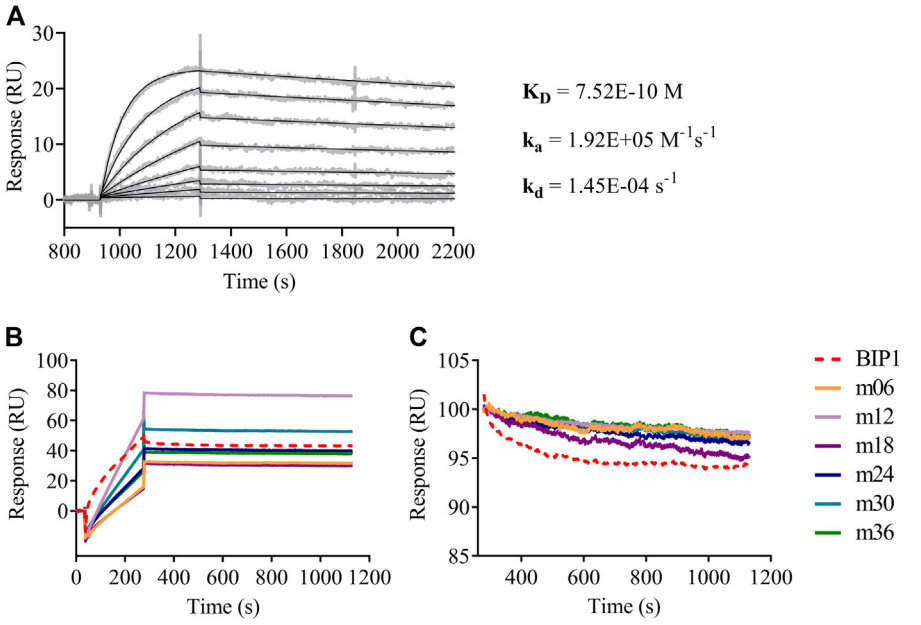

FIG E2. Affinity of the Bet v 1–specific murine BIP1 mAb and comparison with patient-derived IgG1. **(A)** Kinetic parameters of BIP1-binding to Bet v 1 measured by Biacore 3000 (Cytiva). BIP1 was captured by an anti-mouse IgG antibody immobilized to a CM5 sensor chip by amine-coupling chemistry in flow cell (*Fc*) 2. The activated and deactivated Fc1 served as reference. Then, Bet v 1 (64-0.5 nmol/L, *gray lines*) was injected over both Fcs, which were regenerated by a 30-second pulse of glycine, pH 1.5. Kinetics were evaluated using a 1:1 Langmuir binding model *(black lines)* in BIAevaluation software 4.1 (Cytiva). **(B)** BIP1 and IgG1 antibodies purified from samples of P20 collected at months 6, 12, 18, 24, 30, and 36 of BP-SCIT were injected over a Bet v 1–immobilized SPR sensor chip and dissociation was monitored. **(C)** The binding levels were normalized to 100 RUs and a zoom-in on the dissociation phase is shown.

TABLE E1. Bet v 1–specific IgE (kUA/L) levels in 24 individuals during 36 months of BP-SCIT

IgE (kU_A/L)	Month of treatment								
Patient	**0**	**1**	**3**	**6**	**12**	**18**	**24**	**30**	**36**
P2	0.48	1.25	2.43	1.74	1.39	NT	2.17	1.03	1.07
P5	2.66	2.53	2.95	6.64	4.26	5.78	3.40	2.44	2.60
P6	23.65	22.00	25.30	29.60	45.60	58.45	38.50	29.90	29.40
P7	6.28	5.68	7.06	10.10	5.71	15.05	7.00	5.66	4.45
P10	0.80	0.64	0.50	0.55	0.38	0.35	0.35	0.24	0.40
P11	60.70	46.50	62.00	36.60	58.70	53.90	60.40	40.40	36.90
P12	21.20	23.35	16.00	63.20	30.10	32.40	12.70	27.80	43.50
P13	0.73	0.56	0.58	0.38	0.76	NT	0.64	1.10	NT
P14	42.90	42.70	41.90	36.90	47.30	32.10	33.00	27.70	32.80
P16	16.35	17.70	16.90	20.10	10.70	15.80	12.00	17.30	13.40
P18	5.45	5.65	5.24	5.54	3.50	3.87	2.95	3.78	3.69
P19	5.08	6.51	8.16	7.74	7.08	5.78	4.16	5.49	4.18
P20	3.99	4.29	3.82	4.85	4.01	4.58	2.79	5.18	3.49
P21	5.68	6.56	6.81	4.47	2.41	2.11	8.55	4.10	2.27
P22	11.50	11.80	10.70	7.74	7.95	NT	8.29	7.17	4.86
P23	5.11	5.63	5.85	6.09	5.58	9.34	4.41	4.42	3.51
P24	14.00	9.13	8.43	17.40	5.98	8.37	3.34	2.99	1.99
P25	8.42	6.84	7.12	8.17	7.23	6.43	6.03	4.25	4.48
P27	55.10	38.30	36.20	44.10	37.30	21.70	23.60	22.10	22.90
P28	5.20	4.34	5.40	5.38	3.58	2.38	3.56	2.88	2.23
P34	30.80	32.10	NT	35.10	22.10	19.40	27.50	32.80	24.80
P35	44.15	42.00	40.70	26.90	37.90	32.90	29.90	21.60	19.00
P36	57.00	66.30	63.70	48.30	49.20	76.45	73.00	40.30	43.80
P38	22.60	29.00	35.30	23.00	26.00	14.10	9.71	16.80	17.70

Month 0 is before BP-SCIT.

TABLE E2. Bet v 1–specific IgG1 (μg/mL) levels in 24 individuals during 36 months of BP-SCIT

IgG_1 (μg/mL)	Month of treatment								
Patient	**0**	**1**	**3**	**6**	**12**	**18**	**24**	**30**	**36**
P2	0.55	0.48	1.31	1.86	3.13	NT	2.55	3.29	5.11
P5	0.0	0.0	0.29	1.11	2.17	0.56	0.93	0.69	1.15
P6	0.0	0.98	3.43	3.29	3.47	1.83	1.0	2.81	3.39
P7	0.21	0.15	1.22	1.87	1.72	1.43	0.92	0.73	1.06
P10	0.15	0.07	0.10	0.0	0.55	2.23	2.21	1.18	0.21
P11	0.0	0.0	0.19	5.10	1.83	1.89	1.82	2.49	2.08
P12	0.0	0.0	0.17	0.23	0.30	NT	0.28	0.47	NT
P13	0.36	0.22	0.41	6.13	3.61	1.66	1.36	0.99	1.11
P14	0.86	0.49	1.62	4.28	3.50	1.19	6.56	4.29	2.26
P16	0.0	0.0	0.0	0.50	1.59	0.83	1.22	1.04	1.4
P18	0.54	0.43	1.03	1.55	1.55	1.13	0.80	0.61	NT
P19	0.0	0.0	1.42	2.17	2.88	1.24	1.26	1.58	1.47
P20	0.29	0.25	0.23	1.23	2.86	1.04	1.50	2.15	1.63
P21	0.88	0.46	2.10	1.60	1.46	1.52	2.11	1.64	1.24
P22	0.15	0.16	0.0	0.33	2.27	NT	1.15	0.47	0.73
P23	0.40	0.63	2.75	3.73	6.20	4.37	1.32	0.95	0.45
P24	0.0	0.13	0.45	1.17	0.90	0.96	0.63	0.40	0.30
P25	1.05	0.85	NT	2.21	2.62	2.73	2.07	2.28	1.78
P27	0.84	0.47	1.16	1.74	4.94	2.70	4.51	4.89	2.92
P28	0.42	0.33	1.00	1.97	3.94	5.85	4.17	0.65	1.17
P34	0.0	0.0	NT	1.71	2.11	0.99	1.15	1.00	NT
P35	0.88	NT	0.73	2.45	1.58	2.88	2.10	1.76	1.69
P36	0.66	1.06	1.19	2.96	3.57	3.41	4.04	2.45	NT
P38	0.0	0.0	0.47	1.70	1.76	1.09	1.01	1.06	NT

Month 0 is before BP-SCIT.

TABLE E3. Bet v 1–specific IgG4 (μg/mL) levels in 24 individuals during 36 months of BP-SCIT

IgG_4 (μg/mL)	Month of treatment								
Patient	**0**	**1**	**3**	**6**	**12**	**18**	**24**	**30**	**36**
P2	0.01	0.04	0.06	0.34	1.22	NT	5.83	4.53	9.46
P5	0.02	0.04	0.07	0.3	1.14	0.47	1.25	1.14	2.05
P6	0.0	0.0	0.23	0.72	1.35	0.68	0.85	2.69	2.49
P7	0.03	0.02	0.15	0.58	1.86	2.31	1.76	1.27	1.10
P10	1.22	1.12	2.07	1.67	3.00	3.88	7.40	11.80	2.41
P11	0.28	0.25	0.31	6.34	6.67	9.10	15.10	23.40	26.80
P12	0.0	0.0	0.0	0.0	0.14	NT	0.12	0.55	NT
P13	0.55	0.55	1.77	18.50	13.90	8.52	12.10	5.29	4.37
P14	0.0	0.01	0.04	0.1	0.15	0.10	0.32	0.23	0.15
P16	0.02	0.0	0.0	0.15	0.11	0.09	0.87	0.36	0.56
P18	0.47	0.57	2.65	3.00	3.89	3.96	5.20	5.44	7.41
P19	0.11	0.12	1.50	1.57	3.24	2.88	4.06	5.50	4.36
P20	0.13	0.11	0.18	1.09	2.59	1.51	2.02	3.95	2.87
P21	0.28	0.27	1.15	1.54	3.90	5.55	12.00	11.10	NT
P22	0.29	0.36	0.37	0.38	5.06	NT	7.65	6.08	NT
P23	0.14	0.14	0.57	1.29	5.60	14.30	10.80	15.20	12.45
P24	0.47	0.36	1.28	4.61	6.16	12.00	10.80	17.60	10.50
P25	2.43	1.98	4.18	11.10	14.60	15.80	13.10	18.10	19.40
P27	0.29	0.23	0.22	0.72	2.77	4.79	14.10	13.20	16.20
P28	0.17	0.13	0.28	1.65	6.80	16.70	24.90	6.90	8.06
P34	0.16	0.25	NT	3.01	6.10	11.70	13.60	12.85	10.10
P35	1.30	1.40	0.92	0.69	0.95	2.67	4.57	6.01	5.56
P36	0.21	0.20	0.29	0.85	2.00	3.97	7.29	6.47	5.43
P38	0.05	0.08	0.87	3.49	4.23	5.50	5.14	5.19	6.30

Month 0 is before BP-SCIT.