

Basophil expression of diamine oxidase: A novel biomarker of allergen immunotherapy response

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Background: Immunotherapy inhibits basophil histamine release, but the assay is cumbersome, and no one has studied the effects of immunotherapy withdrawal.

Objective: Intracellular fluorochrome-labeled diamine oxidase (DAO) was used as a novel functional readout of basophil histamine release after immunotherapy. Results were compared with conventional basophil surface expression of activation markers.

Methods: Subcutaneous immunotherapy (SCIT)-treated patients (n = 14), sublingual immunotherapy (SLIT)-treated patients (n = 12), participants who completed 3 years of treatment with grass pollen sublingual immunotherapy (the SLIT-TOL group; n = 6), patients with untreated seasonal allergic rhinitis (SAR; n = 24), and nonatopic control subjects (n = 12) were studied. Intracellularly labeled DAO⁺ and surface expression of CD203c^{bright}, CD63⁺, and CD107a⁺ on chemoattractant receptor-homologous molecule expressed on T_H2 lymphocytes (CRTh2)-positive basophils were measured by means of flow cytometry. Serum IgG₄ levels and serum inhibitory activity for IgE-allergen complex binding to B cells (IgE-FAB) and basophil histamine release were also determined. **Results:** Proportions of allergen-stimulated DAO⁺CRTh2⁺ basophils were higher in participants in the SCIT, SLIT, and SLIT-TOL groups (all *P* < .0001) compared with those in patients in the SAR group. Similarly, there were lower proportions of CRTh2⁺ basophils expressing surface

CD203c^{bright} (all *P* < .001), CD63 (all *P* < .001), and CD107a (all *P* < .01). Rhinitis symptoms were lower in the SCIT, SLIT, and SLIT-TOL groups (*P* < .001) compared with those in the SAR group. Serum inhibitory activity for IgE-FAB and basophil histamine release were also significantly greater in all immunotherapy groups (*P* < .05) compared with the SAR group. **Conclusion:** These results support long-term clinical and immunologic tolerance during and after grass pollen immunotherapy. Intracellularly labeled DAO expression by basophils merits further investigation as a surrogate biomarker for monitoring efficacy and tolerance after immunotherapy. (J Allergy Clin Immunol 2015;135:913-21.)

Key words: Diamine oxidase, basophils, allergen immunotherapy, sublingual immunotherapy, subcutaneous immunotherapy, histamine, basophil activation assay

In patients with severe hay fever with or without associated seasonal asthma, grass pollen subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) have been shown to be effective and induce long-term clinical and immunologic tolerance.¹⁻⁴ Immunologic changes after immunotherapy include suppression of allergen-specific T_H2 responses, induction of regulatory T cells (IL-10⁺CD4⁺CD25^{hi}, and CD4⁺CD25^{hi} forkhead box protein 3-positive cells), and the appearance of “protective” allergen-specific IgG antibodies, particularly of the IgG₄ subclass.^{3,5-8} Suppression of the cutaneous early allergic response after immunotherapy is temporally associated with increases in serum IgG-associated inhibitory activity, but the parallel effects of immunotherapy on *ex vivo* allergen-stimulated basophil reactivity have yet to be fully determined.

Basophils were first identified by Paul Ehrlich in 1879 and consist of less than 1% human leukocytes in peripheral blood.⁹ They contain cytoplasmic secretory granules, consisting of proteoglycans and histamine.¹⁰ Basophils express FcεRI, which can be cross-linked by allergen-specific IgE after allergen exposure, resulting in degranulation with release of histamine, leukotrienes, and other mediators of the allergic inflammatory response.^{11,12} Measurement of histamine release by basophils might be a better functional readout of basophil activation than expression of surface activation markers, such as CD63 (granule-associated tetraspan), CD203c (ectonucleotide pyrophosphatase/phosphodiesterase 3, a type II transmembrane ectoenzyme), and CD107a (lysosomal-associated membrane protein 1). However, the current functional immunoassays for measuring histamine release are complex, time-consuming, and poorly reproducible and require an indicator source of whole blood from an atopic subject to test basophil *ex vivo* allergen-induced histamine release. In the mid-1990s, an enzyme-affinity-gold method based on the affinity of diamine

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Abbreviations used

CRTh2:	Chemoattractant receptor-homologous molecule expressed on T _H 2 lymphocytes
DAO:	Diamine oxidase
NAC:	Nonatopic control subject
PE:	Phycoerythrin
RTSS:	Rhinoconjunctivitis total symptom score
SAR:	Seasonal allergic rhinoconjunctivitis
SCIT:	Subcutaneous immunotherapy
SLIT:	Sublingual immunotherapy
SLIT-TOL:	Participants who completed 3 years of treatment with grass pollen sublingual immunotherapy

oxidase (DAO) for its substrate histamine was used to localize intracellular histamine in mast cells.¹² Subsequently, a DAO-colloidal gold–based technique has also been used to localize histamine within basophils.¹³

We hypothesized that measurement of intracellular fluorochrome-labeled DAO in whole-blood basophils might be a simpler and immediately available functional readout of histamine release at the single-cell level.¹⁴ We postulated that the proportion of DAO⁺ chemoattractant receptor-homologous molecule expressed on T_H2 lymphocytes (CRTh2)–positive basophils would decrease after immediate *ex vivo* allergen stimulation of basophils in patients with grass pollen allergy compared with those in nonatopic control subjects (NACs). We further hypothesized that this decrease would be inhibited in immunotherapy-treated patients. Thus our aim was to both assess the utility of intracellular fluorochrome–labeled DAO compared with surface markers as an indicator of basophil activation in patients with allergic rhinitis and to explore the potential for labeled DAO as a functional biomarker of efficacy and tolerance after allergen immunotherapy. Basophil responsiveness was related to clinical response, serum IgG levels, IgG-associated serum inhibitory activity for IgE-dependent basophil activation, and IgE–allergen complex binding to B cells.

METHODS**Subjects**

Patients receiving grass pollen SCIT (*Phleum pratense*; Alutard, ALK-Abelló, Hørsholm, Denmark; n = 14) or grass pollen SLIT (Grazax, ALK-Abelló; n = 12) or participants who completed 3 years of treatment with grass pollen sublingual immunotherapy (the SLIT-TOL group, n = 6), patients with seasonal allergic rhinoconjunctivitis (SAR; n = 24), and NACs (n = 12) provided blood samples and answered symptom questionnaires (Table 1). Patients receiving SCIT and SLIT had been receiving immunotherapy for between 12 months and 3 years. Patients in the SLIT-TOL group had completed 3 years of treatment and discontinued immunotherapy 12 to 24 months previously. Blood samples were collected during the grass pollen season (May–July) in 2012 (patients with SAR, n = 12; patients receiving SCIT, n = 7; patients receiving SLIT, n = 6; SLIT-TOL patients, n = 4; and NACs, n = 12) and 2013 (patients with SAR, n = 12; patients with SCIT, n = 7; patients with SLIT, n = 6; and SLIT-TOL patients, n = 2). The study was approved by the South West London REC3 Research Ethics Committee and the Research Office of the Royal Brompton and Harefield NHS Foundation Trust.

Rhinoconjunctivitis symptom scores

Participants were asked to assess the severity of their allergic rhinitis symptoms for the relevant pollen season according to a retrospective symptom questionnaire. This included the 6 categories of runny nose, blocked nose,

itchy nose, sneezing, itchy eyes, and watery eyes, each with a rating of 0 (no problem) to 3 (severe problem), resulting in a possible total score of 0 to 18 (rhinoconjunctivitis total symptom score [RTSS]). Participants were specifically asked to evaluate the severity of their symptoms when their allergic rhinitis was at its most severe during the pollen season.

Serum total IgE, specific IgE, and IgG₄ measurements

Total IgE, timothy grass–specific IgE, and specific IgG₄ levels were quantified by using the CAP FEIA system, according to the recommendations of the manufacturer (Phadia, Uppsala, Sweden). Additionally, a multiple-allergen-component analysis was performed for 2 polysensitized patients by using the microarray technique (Immuno Solid Allergen Chip [ISAC]; Thermo Fisher Scientific, Loughborough, United Kingdom), according to the manufacturer's protocol.

Measurements of basophil histamine release by using the DAO flow cytometry assay

Intracellular basophil histamine release after *ex vivo* allergen stimulation was measured by means of flow cytometry with DAO, as kindly provided by Dr Frans Nauwelaers and Dr Noel Drury (BD Biosciences, San Jose, Calif). Phycoerythrin (PE)–conjugated DAO was used to detect histamine release at the single-cell level. CRTh2⁺ basophils were also immunostained for the surface activation markers CD203c, CD63, and CD107a and acquired on the BD FACSCanto II flow cytometer (BD Biosciences; see the [Methods](#) section in this article's Online Repository at www.jacionline.org for further details).

Ex vivo basophil reactivity as measured by CD63, CD203c, and CD107a

Heparinized whole blood (100 μL) was incubated with 0, 0.1, 1, 10, 100, and 1000 ng/mL *P pratense* extract (ALK-Abelló) or anti-IgE (0.5 μg/mL) in a 37°C water bath for 15 minutes. Cells were immunostained with anti-human CD3, CD303, CD294 (CRTh2), CD203c, CD63, and CD107a (all from BD Biosciences). Erythrocytes from whole blood were lysed with BD lysing solution (BD Biosciences) for 10 minutes at room temperature in the dark, samples were centrifuged (for 5 minutes at 200g), and supernatants were discarded. The resulting cell pellets were washed in 3 mL of PBS (without Ca²⁺ and Mg²⁺) and resuspended in 450 μL of ice-cold fixative solution (CellFix, BD Biosciences) before acquisition on the BD FACSCanto II flow cytometer. Nonactivated and activated basophils were identified as CD203c^{dim}CRTh2⁺ and CD203c^{bright}CD3⁺CD303⁺CRTh2⁺ cells, respectively. Additionally, activated cells were also identified as CD63⁺ and CD107a⁺CD3⁺CD303⁺CRTh2⁺ basophils. Analyses were performed with BD FACSDiva V6.1.1 software (BD Biosciences).

Inhibition of ex vivo basophil reactivity by sera obtained after immunotherapy (SCIT and SLIT)

Heparinized whole blood (100 μL) was incubated with 0, 0.1, 1, 10, 100, and 1000 ng/mL *P pratense* extract (ALK-Abelló) with or without the presence of sera from patients receiving SCIT or those receiving SLIT (100 μL) at 37°C for 15 minutes. Cells were immunostained and acquired by means of flow cytometry, as above.

Basophil histamine release assay

Heparinized whole blood from a subject with grass pollen allergy (specific IgE, >100 kU/L) was incubated with 10 ng/mL *P pratense* extract (ALK-Abelló) in the presence of pre- and post–grass pollen SCIT sera (n = 6). Basophil histamine release into cell-free supernatants was determined by means of ELISA, according to the manufacturer's instructions (IBL, Hamburg, Germany).

TABLE I. Subjects' characteristics

	SCIT group (n = 14)	SLIT group (n = 12)	SLIT-TOL group (n = 6)	SAR group (n = 24)	Nonatopic control group (n = 12)
Sex (M/F)	8/6	2/10	2/4	14/10	7/5
Age (y), mean (range)	44 (19/70)	37 (19/58)	44 (32/65)	34 (20/55)	38 (22/56)
Allergen grass-specific IgE (kU/L), mean (SD)	48.7 ± 32.1	28.8 ± 23.8	19.8 ± 12.4	29.6 ± 35.4	0.0 ± 0.0*
Total IgE (kU/L), mean (SD)	230.6 ± 217.8	391.3 ± 446.0	454.5 ± 664.2	500.4 ± 538.1	26.4 ± 19.39*
Monosensitized	3/14	4/12	1/6	6/24	0/12
Polysensitized	11/14	8/12	5/6	18/24	0/12
House dust mite	4/12	2/12	2/6	9/24	0/12
Cat	6/14	5/12	3/6	7/24	0/12
Birch	9/14	4/12	3/6	11/24	0/12

Distribution of age, sex, specific IgE level, and sensitization profile.

F, Female; M, male.

* $P < .01$, comparison between the NAC and SAR groups. There were no significant differences for any other parameter among the 4 groups.

Inhibition of CD23-mediated IgE-facilitated allergen presentation

IgE-facilitated allergen binding to B cells was performed, as previously described.^{15,16} Briefly, serum from a grass pollen-sensitized donor (*P pratense*-specific IgE, >100 kU/L) was incubated with 1 µg/mL *P pratense* in the presence or absence of an equal volume of sera from study patients for 1 hour at 37°C. EBV-transformed B cells (100,000 cells per test) were added for 1 hour at 4°C and washed. Allergen-IgE complexes binding to B cells were detected by using a PE-labeled polyclonal anti-IgE antibody (0.8 µg/mL; DakoCytomation, Ely, United Kingdom). Cells were acquired by means of flow cytometry (BD FACSCanto II flow cytometer, BD Biosciences; see the [Methods](#) section in this article's Online Repository for further details).

Statistical analysis

Within-group comparisons were performed by using the Wilcoxon signed-rank test. Between-group comparisons were performed with the Mann-Whitney *U* test. Correlation coefficients were obtained by using the Spearman method. The statistical software package used was GraphPad Prism, version 6 (GraphPad Software, San Diego, Calif). *P* values of less than .05 were considered significant.

RESULTS

Rhinoconjunctivitis symptom scores

RTSSs were significantly less in the SCIT ($P < .0001$), SLIT ($P < .0009$), and SLIT-TOL ($P < .0001$) groups compared with untreated patients with SAR (Fig 1).

Ex vivo basophil responsiveness to allergen in allergic subjects and NACs

Ex vivo grass pollen-induced basophil reactivity was significantly increased in patients with SAR compared with that seen in NACs. An allergen dose-dependent increase of the surface activation markers CD63, CD203c^{bright}, and CD107a on CRTh2⁺ basophils was demonstrated in patients with SAR (see Fig E1 in this article's Online Repository at www.jacionline.org). No change in surface marker levels was observed for the NACs. Between groups, at the optimal allergen concentration (100 ng/mL), the proportion of CD63⁺, CD203c^{bright}, and CD107a⁺CRTh2⁺ basophils was significantly increased in patients with SAR (n = 12) compared with that seen in NACs (n = 12; $P < .001$, $P < .001$, and $P < .001$; Fig 2, B, and see Fig E1). The proportion of CD63⁺ and CD203c^{bright}CRTh2⁺ basophils in patients with SAR correlated with serum grass pollen-specific IgE levels at the optimum allergen concentration

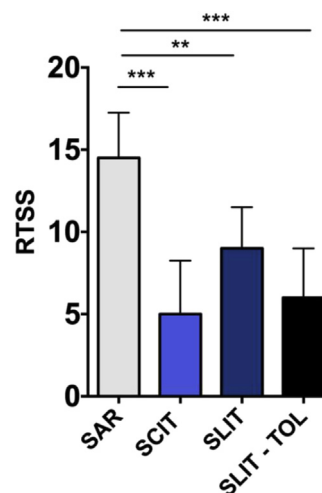


FIG 1. RTSSs: SAR group, n = 24; SCIT group, n = 14; SLIT group, n = 12; SLIT-TOL group, n = 6. Data are expressed as medians (interquartile ranges). Mann-Whitney *U* test: ** $P < .01$ and *** $P < .001$.

($r = 0.85$, $P < .0001$ and $r = 0.84$, $P < .0001$, respectively; see Fig E1). CD203c^{bright}, CD63⁺, and CD107a⁺ expression on CRTh2⁺ basophils after *ex vivo* allergen stimulation was optimally increased within 15 minutes and remained increased up to 120 minutes (see Fig E2 in this article's Online Repository at www.jacionline.org). All assays were performed within 2 hours of venesection. The viability of basophils was reduced if samples were kept for 6 hours or longer *ex vivo* (see Fig E3 in this article's Online Repository at www.jacionline.org).

Sera from 2 untreated polysensitized patients with grass pollen allergy were further investigated to assess the specificity of basophil reactivity. Subject 1 was sensitized to grass pollen (sIgE: rPhl p 5 = 1.3 ISU; rPhl p 4 = 1.3 ISU) and house dust mite (nDer p 1 = 8.5 ISU; nDer f 1 = 6.4 ISU; nDer p 2 = 7.2 ISU; see Fig E4 in this article's Online Repository at www.jacionline.org). Subject 2 was sensitized to grass pollen (rPhl p 5 = 0.7 ISU), birch (rBet v 1 = 5.1 ISU), and peanut (nAra h 2 = 0.3 ISU). Increased *ex vivo* allergen-induced basophil reactivity, as measured based on CD203c^{bright}, CD63, and CD107a on CRTh2⁺ basophils, was specific for the sensitizing allergens in each case (see Fig E4).

DAO was conjugated to PE and used to detect intracellular histamine before and after basophil *ex vivo* allergen stimulation.

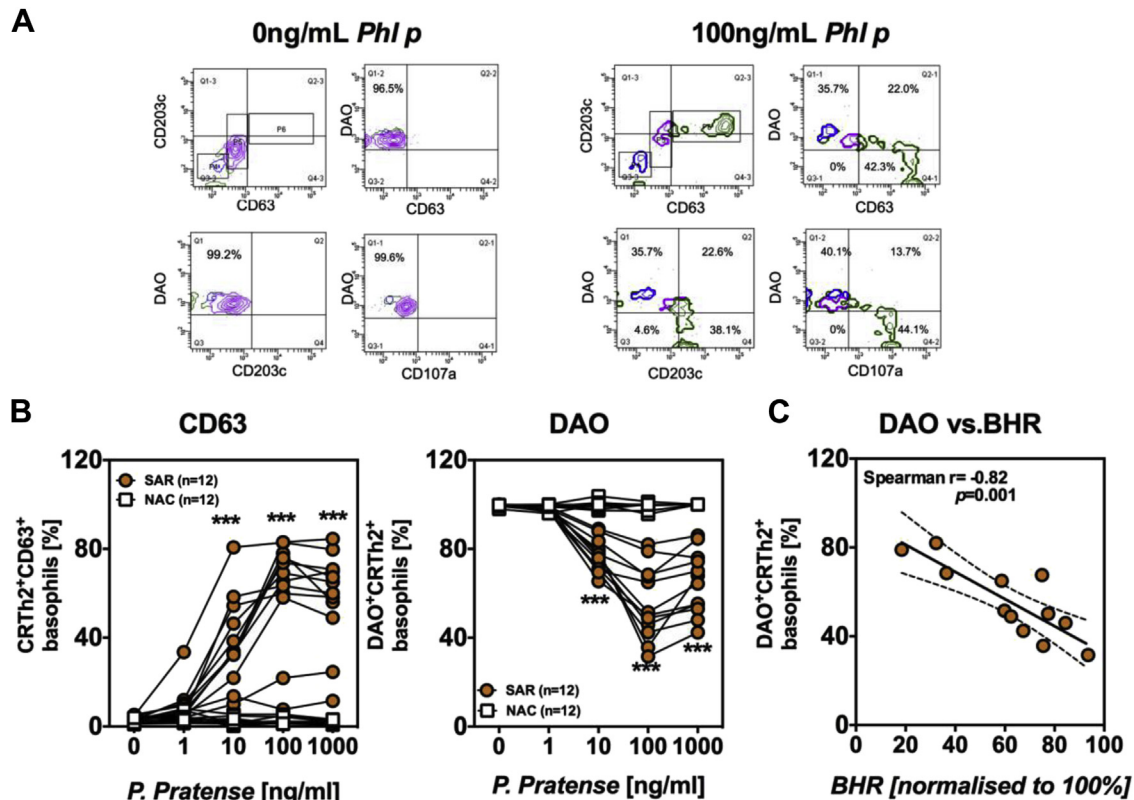


FIG 2. DAO as a measure of intracellular histamine in CRTh2⁺ basophils. **A**, Representative intracellular fluorochrome-labeled DAO immunostaining on CD63⁺CRTh2⁺ basophils of 1 allergic subject after *ex vivo* timothy grass pollen allergen stimulation. **B**, Percentage of CD63⁺CRTh2⁺ and DAO⁺CRTh2⁺ basophils after *ex vivo* allergen stimulation in patients with SAR (n = 12) and NACs (n = 12). **C**, DAO⁺CRTh2⁺ basophils measured in patients with SAR were correlated with basophil histamine release (BHR). Mann-Whitney U test: ***P < .001. Correlation was determined by using the Spearman rank method.

The proportion of DAO⁺CRTh2⁺ basophils was significantly decreased after *ex vivo* allergen stimulation in participants with SAR ($P < .001$) and remained unchanged in the nonallergic control subjects (Fig 2, A and B). The observed decreases in DAO⁺CRTh2⁺ basophil numbers correlated inversely with basophil histamine release ($r = -0.82$, $P = .0001$; Fig 2, C) and the increased proportion of CD203c^{bright}, CD63⁺, and CD107a⁺CRTh2⁺ basophils (CD203c: $r = -0.77$, $P < .0001$; CD63: $r = -0.80$, $P < .0001$; CD107a: $r = -0.79$, $P < .0001$; data not shown).

Effects of SLIT and SCIT on *ex vivo* allergen-induced basophil reactivity

The SLIT- and SCIT-treated groups had significantly lower proportions of CD63⁺ ($P = .0003$ and $P = .0002$), CD203c^{bright} ($P = .0002$ and $P = .0007$), and CD107a⁺CRTh2⁺ ($P = .0002$ and $P = .0005$) basophils after *in vitro* allergen stimulation compared with the untreated allergic group (Fig 3). A shift in the allergen dose response was observed in which 10- to 100-fold more allergen was required to elicit optimal basophil activation (Fig 3, B-D). This effect was allergen specific because cross-linking of FcεRI by anti-human IgE resulted in an increased proportion of CD63⁺ ($P < .0001$) and CD203c⁺ ($P < .0004$) basophils and a decrease in DAO⁺CRTh2⁺ basophil numbers in both types of immunotherapy-treated participants (see Fig E5 in

this article's Online Repository at www.jacionline.org). Although both treated groups exhibited allergen-specific impaired basophil responsiveness, there was no difference observed between the effects of SLIT and SCIT treatment for CD63, CD203c^{bright}, and CD107a expression. These findings were confirmed by means of flow cytometry for intracellularly labeled DAO. In contrast to findings in patients with untreated allergic rhinitis (SAR group), proportions of DAO⁺CRTh2⁺ basophils were not decreased in response to *ex vivo* allergen stimulation after treatment with both SLIT ($P < .0001$) and SCIT ($P < .0001$, Fig 4). These analyses for CD63, CD203c, CD107a, and DAO were performed on samples from 2 consecutive years for which pollen counts varied (see Fig E6, A, in this article's Online Repository at www.jacionline.org). Nonetheless, the suppression of basophil activation by immunotherapy (see Fig E6, B) was consistent despite year-on-year variation in pollen exposure.

Ex vivo basophil hyporesponsiveness after discontinuation of SLIT

Allergen-induced basophil reactivity, as measured based on CD203c^{bright}, CD63, and CD107a expression on CRTh2⁺ basophils, was recorded in 6 patients who received SLIT for 3 years and then discontinued treatment for 12 to 24 months. Persistent *ex vivo* basophil hyporesponsiveness to allergen was observed. The level of suppression (compared with that in the

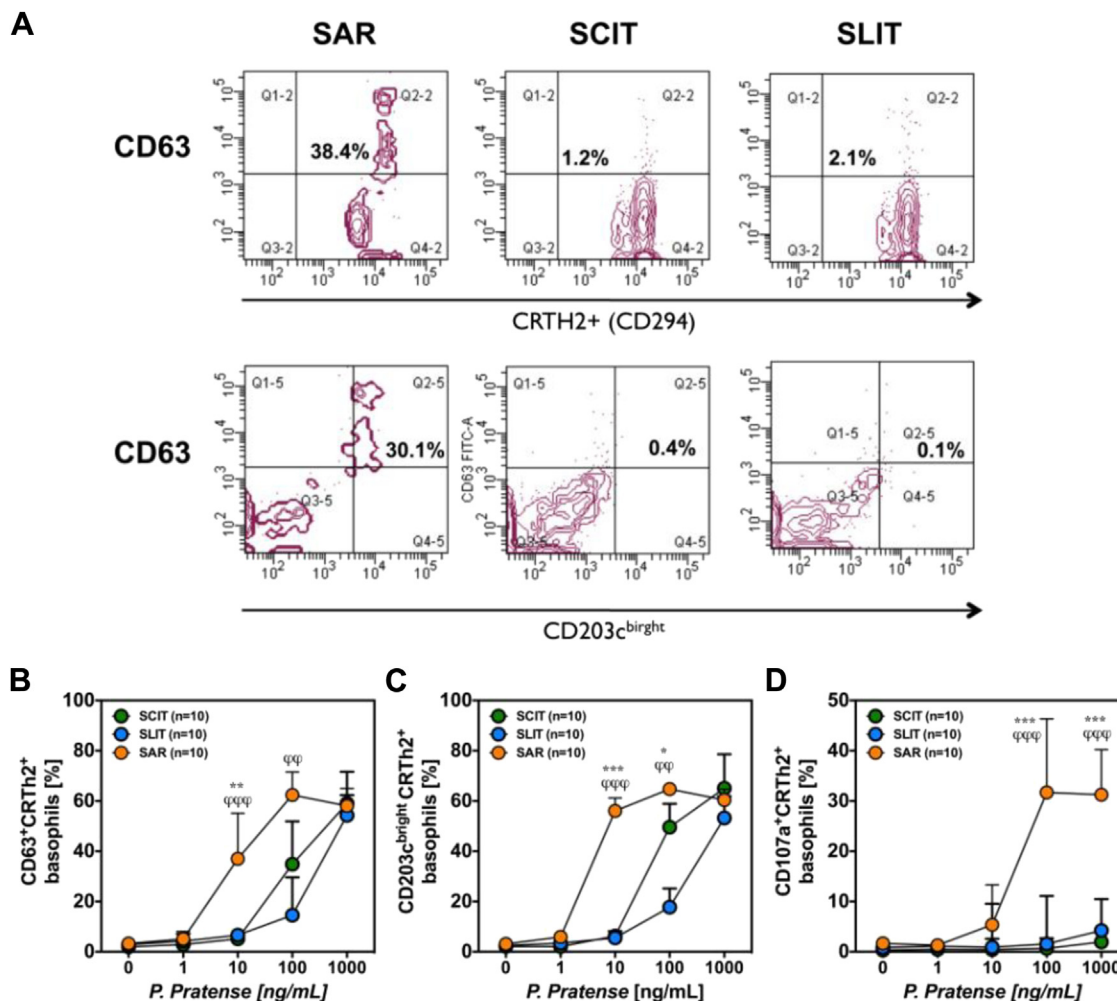


FIG 3. Grass pollen-specific immunotherapy: *ex vivo* allergen-induced basophil activation. **A**, Representative flow cytometric analysis from 3 subjects showing single (CD63) and dual expression of CD63 and CD203c^{bright} on CRTh2⁺ basophils. Whole-blood basophils obtained from SLIT-treated patients (n = 10), SCIT-treated patients (n = 10), and untreated patients with SAR (n = 10) were stimulated with varying concentrations of *P. pratense*. **B-D**, *Ex vivo* allergen-induced basophil responsiveness was measured based on expression of CD203c^{bright} (Fig 3, B), CD63 (Fig 3, C), and CD107a (Fig 3, D) on CRTh2⁺ basophils. Green circle, SCIT; blue circle, SLIT; orange circle, SAR. Data are expressed as medians (interquartile ranges). Mann-Whitney *U* test: statistical difference between patients receiving SCIT and patients with SAR (**P* < .05) and patients receiving SLIT and patients with SAR (φ*P* < .05). ***P* < .01 and ****P* < .001.

untreated allergic control group) was no different from that observed in subjects currently receiving immunotherapy (Fig 4). Similarly, intracellular histamine release in DAO⁺CRTh2⁺ basophils from patients in the SLIT-TOL group did not suppress after *ex vivo* allergen stimulation, which is consistent with findings in those who continued immunotherapy (Fig 4).

Ex vivo allergen-induced basophil reactivity correlates with clinical response to treatment

The relationship between *ex vivo* allergen-induced basophil reactivity and RTSSs was assessed in combined data from all 4 allergic groups of participants, namely patients with SAR, patients receiving SCIT, patients receiving SLIT, and the SLIT-TOL group. A significant correlation was revealed between RTSSs and the proportion of intracellularly labeled DAO⁺ and CD63⁺CRTh2⁺ basophils (*r* = -0.65, *P* = .0001

and *r* = -0.54, *P* < .0001), respectively (see Fig E7 in this article's Online Repository at www.jacionline.org).

Effects of SLIT and SCIT on serum grass pollen-specific IgG₄ antibodies with inhibitory activity for FcεRI- and CD23-mediated responses

Subjects treated with SLIT and SCIT had increased concentrations of grass pollen-specific IgG₄ antibodies compared with those seen in untreated allergic control subjects (*P* < .001 and *P* < .001). SCIT-treated subjects had higher levels of specific IgG₄ compared with the SLIT-treated group (*P* < .0001; Fig 5, A). Cooperative binding of IgE-allergen complexes to CD23 on the surfaces of B cells was significantly decreased in the SCIT (*P* < .0001), SLIT (*P* < .0001), and SLIT-TOL (*P* = .0007) groups (Fig 5, B). Similarly, basophil histamine release was significantly reduced after *ex vivo* grass

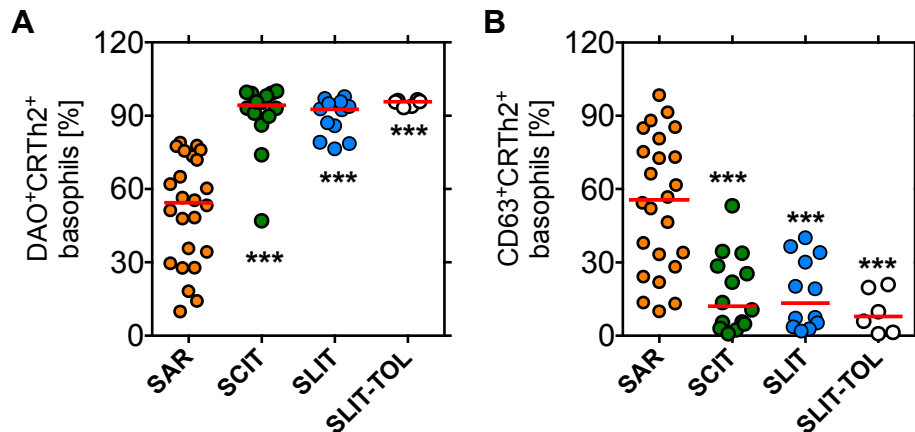


FIG 4. Persistence of *ex vivo* allergen-induced basophil hyporeactivity after withdrawal of sublingual immunotherapy. DAO⁺CRTh2⁺ (A) and CD63⁺CRTh2⁺ (B) basophils were measured in the SAR (n = 24), SCIT (n = 14), SLIT (n = 12), and SLIT-TOL (n = 6) groups. Bars represent medians. ****P* < .001, Mann-Whitney *U* test.

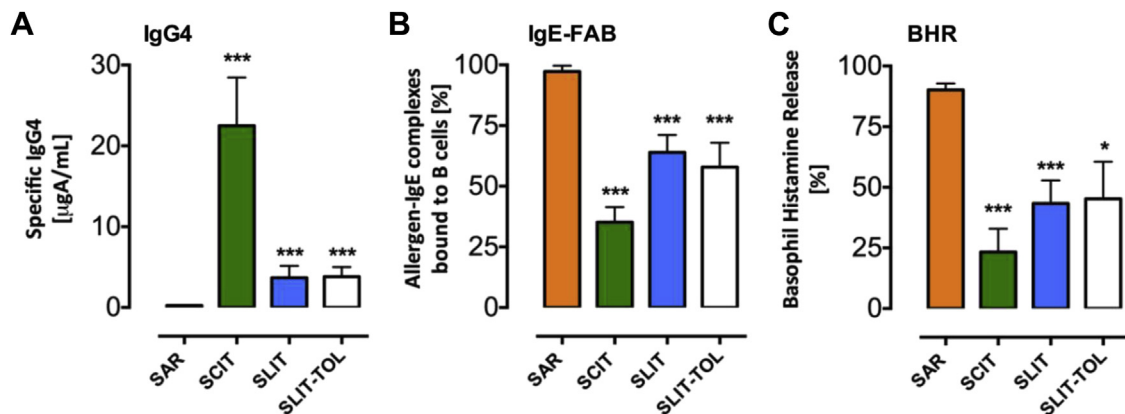


FIG 5. Induction of protective and functional IgG₄ antibodies after immunotherapy. A, Serum specific IgG₄ levels are represented for all 4 groups: SAR, SCIT, SLIT, and SLIT-TOL. B and C, The ability of IgG-containing serum obtained after immunotherapy to inhibit both CD23- and FcεRI-dependent IgE-mediated events is represented for all 3 immunotherapy-treated groups by the consistently lower percentages of IgE-FAB binding to B cells (Fig 5, B) and the lower percentages of basophil histamine release (Fig 5, C), respectively, compared with those in the patients with SAR. Data are expressed as medians (interquartile ranges). **P* < .05 and ****P* < .001, Mann-Whitney *U* test.

pollen allergen stimulation in the SCIT (*P* = .0003), SLIT (*P* = .0007), and SLIT-TOL (*P* = .01) groups compared with that seen in patients with untreated allergy (Fig 5, C). Basophil histamine release was also measured before and after immunotherapy in 6 patients. Significant suppression of basophil histamine release was shown after both SCIT and SLIT compared with pretreatment values (*P* = .03, see Fig E8 in this article's Online Repository at www.jacionline.org).

To explore the mechanism of the observed decrease in basophil responsiveness after immunotherapy, we assessed the ability of autologous postimmunotherapy (SLIT and SCIT) sera to inhibit *ex vivo* allergen-induced basophil responsiveness. SLIT and SCIT sera significantly inhibited the proportion of CD63⁺CRTh2⁺ basophils (SCIT, *P* = .01; SLIT, *P* = .01) after allergen stimulation versus that seen in control serum alone. This inhibitory effect of SLIT and SCIT sera was partially blocked after pretreatment of basophils with anti-human CD32 blocking antibodies that recognize 2 receptor isoforms: FcγRIIA and FcγRIIB (Fig 6).

DISCUSSION

In this cross-sectional study *ex vivo* grass pollen allergen-induced basophil activation was significantly higher in patients with seasonal allergic rhinitis, specific for the sensitizing allergen, and inhibited by allergen immunotherapy. Proportions of CD203c^{bright}CRTh2⁺, CD63⁺CRTh2⁺, and CD107a⁺CRTh2⁺ basophils were significantly lower after both SCIT and SLIT, when 10- to 100-fold more allergen was required to elicit optimal basophil activation. Clinical tolerance (persistence of benefit after immunotherapy withdrawal) was also associated with reduced basophil responsiveness, as shown in the SLIT-TOL group. The effect was confirmed by the use of flow cytometry for intracellularly labeled DAO, a novel marker of basophil histamine release.¹⁴ Suppression of basophil responsiveness and histamine release significantly correlated with lower allergic rhinitis symptom scores. In particular, the magnitude of this correlation was greater for intracellularly labeled DAO⁺CRTh2⁺ than CD63⁺CRTh2⁺ basophils (*r* = -0.65,

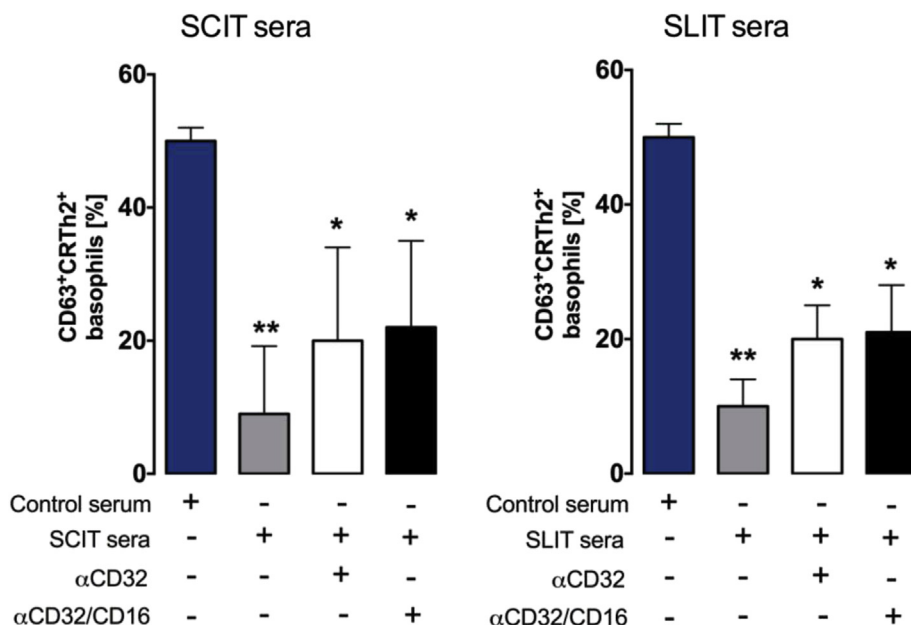


FIG 6. Suppression of *ex vivo* allergen-induced basophil reactivity after SCIT and SLIT is serum and FcγRIIb dependent. SCIT (n = 6), SLIT (n = 6), or control serum from a nonatopic subject were incubated with whole blood in the presence of grass pollen allergen (10 ng/mL). Suppression of *ex vivo* basophil allergen-induced reactivity was measured with or without αCD32 and αCD32/CD16. Data are expressed as medians (interquartile ranges). *P < .05 and **P < .01, Mann-Whitney U test.

P = .0001 and r = -0.54, P < .0001), respectively. Levels of specific IgG₄- and IgG-associated serum inhibitory activity for basophil activation were increased after SCIT and SLIT. Our data support the detection of basophil hyporesponsiveness to allergen, including through use of intracellularly labeled DAO as a useful biomarker for monitoring efficacy and induction of allergen-specific tolerance during immunotherapy.

Several strategies for gating basophils for flow cytometry have been reported previously.^{14,17} One approach is selection based on the level of bound IgE. FcεRI expression on basophils correlates closely with serum IgE levels.^{18,19} However, in patients with low serum IgE levels, only a small proportion of basophils express IgE, resulting in difficulty in gating and in enumeration of basophil numbers.²⁰ An alternative approach involves a gating strategy using the IL-3 receptor (CD123) and HLA-DR. The IL-3 receptor is also expressed on plasmacytoid dendritic cells. Basophils express low HLA-DR intensity and can therefore be distinguished from CD123⁺HLA-DR^{high} plasmacytoid dendritic cells. The IL-3 receptor and HLA-DR can be influenced by thymic stromal lymphopoietin²¹ and inflammatory conditions,²² respectively. We gated basophils on the basis that they were CD3⁻CD303⁻CRTh2⁺. This gating strategy ensured that we could reproducibly enumerate the proportion of basophils to assess function after *ex vivo* allergen stimulation. Additionally, we used this multiparametric gating strategy to measure intracellularly labeled DAO at the single-cell level. Basophil histamine release assays are complex, laborious, and costly; require a technically competent operator; and are dependent on assessment of the response using 1 allergic donor with high basophil repeatability. The multiparametric combined labeled DAO and CD markers provide not only activation status at the single-cell level but also functional allergen-specific

basophil readout. The assay is robust, highly reproducible, and less expensive than conventional basophil histamine release.

The availability of multiparametric flow cytometry permits rapid detection of basophil responsiveness in whole blood before and after immunotherapy, a particular advantage in young children because only a small volume (1 mL) of blood is required, making the assay potentially applicable in clinical practice, including pediatric practice.

Allergen-specific immunotherapy is associated with decreased frequency of allergen-specific T_H2 effector cells²³ and induction of regulatory T cells.^{5,24-26} Regulatory T cells have been shown to suppress basophils and mast cells in OX40/OX40 ligand-dependent mechanisms.²⁷ Our data confirm reduced basophil responsiveness to grass pollen but not to anti-IgE stimulation (0.5 μg/mL) after immunotherapy. It would be of interest to assess whether this FcεRI-mediated suppression was due to the effects of induced IL-10 or regulatory T cells after immunotherapy. Critically, allergen-specific suppression of basophils was detectable for at least 12 to 24 months after immunotherapy withdrawal, implying that the induced basophil anergy might be a long-term phenomenon, which is consistent with the known long-term effects of allergen immunotherapy through both the subcutaneous and sublingual routes.^{1,4}

Kepley et al²⁸ previously showed suppression of basophil histamine release after immunotherapy. Lalek et al²⁹ demonstrated decreased expression of the basophil surface activation marker CD63 in patients after birch pollen immunotherapy. Furthermore, autologous postimmunotherapy serum was able to inhibit *ex vivo* allergen-stimulated basophils. This inhibitory activity was lost after depletion of IgG from serum. These findings are consistent with our previous demonstration of post-grass pollen immunotherapy IgG-associated serum inhibitory activity for allergen-IgE binding to B cells.^{16,25}

The present study confirms and extends the findings of Lalek et al²⁹ to include 3 basophil surface markers and to explore in parallel the use of intracellular DAO as a functional readout of basophil activation, being a surrogate for histamine release. For the first time, we used these combined techniques to assess the effects of both SCIT and SLIT on basophil activation. We have shown that the inhibitory effects on basophils persist after completion of immunotherapy, which is consistent with tolerance induction. There was a modest correlation observed between allergen-induced basophil activation *in vitro* and seasonal rhinoconjunctivitis symptom scores *in vivo* (see Fig E7). However, in view of the small numbers and cross-sectional study design, the clinical relevance of this association is questionable and requires further exploration in a large prospective randomized controlled immunotherapy trial.

In subjects treated with SCIT and SLIT, we demonstrated increases in allergen-specific IgG₄ levels. We also showed induction of IgG-associated serum inhibitory activity for both FcεRI-dependent basophil activation and CD23 (FcγRII)-dependent allergen-IgE binding to B cells in SCIT- and SLIT-treated patients and after SLIT withdrawal. The “blocking activity” of postimmunotherapy serum on IgE-dependent events is likely in part due to effective competition of IgG with IgE for allergen binding. However, our observation, in agreement with Kepley et al,²⁸ that the inclusion of an anti-CD32 blocking antibody (directed against the inhibitory FcγRII receptor present on basophils) partially reverses the inhibitory activity of SCIT/SLIT serum implies that in addition to simple competition IgG₄ might act, at least in part, through FcγRII to promote receptor-mediated inhibition of intracellular FcεRI-triggered basophil activation. That SCIT and SLIT should achieve similar levels of inhibition of basophil allergen-induced activation *in vitro*, despite the fact that SCIT results in about a 10-fold greater induction of specific IgG₄, is of interest. This might imply that only a relatively modest increase in IgG₄ levels is needed to reach a threshold required for inhibitory activity on basophils (and on B cell-facilitated allergen binding) that is reached in patients undergoing SLIT and greatly surpassed in those undergoing SCIT. Alternatively, the mean affinity or avidity of antibody produced during SLIT might be higher than that found in patients receiving SCIT, although this would have to be tested.

The 10- to 100-fold increased threshold in allergen concentration required to activate basophils *ex vivo* after immunotherapy might apply *in vivo* for basophils exposed to allergen at the nasal mucosal surface during the pollen season. Such an effect could potentially apply to local IgE-dependent triggering of both mucosal mast cells and basophils, as reflected in the reduction of the early- and late-phase allergic nasal response after successful immunotherapy.²⁹ Thus 10- to 100-fold more allergen might be required in the lining of the nasal mucosa to elicit basophil effector cell activation and release proallergic mediators.

In summary, using a novel method with intracellularly labeled DAO, as well as surface expression of CD203c, CD63, and CD107a, on basophils, we have demonstrated that basophil responsiveness *ex vivo* is significantly reduced after both SCIT and SLIT. This suppression is likely in part due to the action of IgG blocking antibodies, acting both through simple competition for allergen and through basophil surface FcγRIIb. In addition to casting light on the mechanisms of immunotherapy, these markers of basophil activation might be useful for monitoring the clinical

response to immunotherapy, although this remains to be tested in an appropriate, large, randomized controlled trial setting.

Key messages

- DAO is a simple novel functional readout of *ex vivo* basophil histamine release with potential to monitor response to immunotherapy.
- Grass pollen SLIT and SCIT induced similar levels of basophil suppression.
- Basophil suppression 12 to 24 months after stopping SLIT was consistent with long-term immune tolerance.

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METHODS

Basophil activation assay using flow cytometry

Assessment of *ex vivo* allergen-induced basophil reactivity by means of flow cytometry was performed on heparinized whole blood. One hundred microliters of whole blood was incubated with 20 μ L of *P pratense* extract (ALK-Abelló) at 0, 1, 10, 100, and 1000 ng/mL at 37°C for 15 minutes in the dark. Surface immunostaining for basophil activation markers or DAO intracellular staining was then performed. To quality control the assay, 20 μ L of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS (Invitrogen, Carlsbad, Calif) or 20 μ L of α -IgE D epsilon 2 (Beckman Coulter, Hialeah, Fla) at a final concentration of 100 ng/mL was added to 100 μ L of whole blood, and these were used as negative or positive controls, respectively.

Surface staining

Cells were immunostained with fluorescently labeled antibodies according to the manufacturers' recommendations: CD203c PerCP-Cy5.5 (5 μ L per test, 324608; BioLegend, London, United Kingdom), CD107a Pacific Blue (2 μ L per test, 328624; BioLegend), CD63 fluorescein isothiocyanate (20 μ L per test, 312004; BioLegend), CD3 PE-Cy7 (5 μ L per test, 557749; BD), CD294 PE (10 μ L per test, 130-091-238; Miltenyi Biotec, Bergisch Gladbach, Germany), and CD303 allophycocyanin (APC; 10 μ L per test, 130-090-905; Miltenyi Biotec). Erythrocytes from whole blood were lysed for 10 minutes with 2 mL of BD lysing solution (349202; BD) in the dark. Samples were then centrifuged at 200g for 5 minutes to remove supernatant and washed with 3 mL of PBS. Cells were then fixed with 450 μ L of ice-cold CellFix Solution (340181; BD) before analysis by using the flow cytometer.

DAO intracellular staining

Cells were immunostained with CD203c PerCP-Cy5.5 (5 μ L per test, 324608; BioLegend), CD63 fluorescein isothiocyanate (20 μ L per test, 312004; BioLegend), CD107a APC-H7 (5 μ L per test, 561343; BD), CD294 V450 (5 μ L per test, 561661; BD), CD3 PE-Cy7 (5 μ L per test, 557749; BD), and CD303 APC (10 μ L per test, 130-090-905; Miltenyi Biotec), according to the manufacturers' recommendations. Erythrocytes from whole blood were eliminated by incubating for 10 minutes with 2 mL of BD lysing solution (349202; BD) in the dark. Samples were centrifuged at 200g for 5 minutes to remove supernatant and washed with 1 mL of Perm/Wash buffer (554723; BD) at 1500 rpm for 5 minutes at 4°C. Cells were resuspended and incubated with 250 μ L of Cytofix/Cytoperm solution for 20 minutes at 4°C. Cells were then washed twice with 1 mL of Perm/Wash buffer, and intracellular staining was carried out by resuspending the cells in 50 μ L of Perm/Wash buffer and incubating with 10 μ L of DAO PE (10 μ L per test; BD) for 30 minutes at 4°C away from light. Cells were then washed twice with 1 mL of Perm/Wash buffer and fixed with 450 μ L of ice-cold CellFix solution (340181; BD) before analysis by using the flow cytometer. After staining, samples were then analyzed on a flow cytometer (BD FACSCanto II), which was calibrated daily with Cytometer Setup Beads (CST beads; BD). Analysis was performed with BD FACSDiva software, and basophil populations were determined by gating for $\text{CD3}^- \text{CD303}^- \text{CRTh2}^+$ cells. Results were expressed as percentage activation

of the actual marker and as mean fluorescence intensity related to entire basophil populations.

Basophil histamine release

The basophil histamine release assay was performed on fresh heparinized whole blood from a grass pollen-sensitized donor. Two hundred microliters of whole blood was incubated with 200 μ L of test sera, 200 μ L of human AB serum (Sigma, St Louis, Mo), 200 μ L of anti-IgE (IBL, Hamburg, Germany), or 200 μ L of release buffer alone. Five microliters of *P pratense* allergen was diluted in release buffer to a final concentration of 10 ng/mL. Five microliters of release buffer was added in place of allergen to determine spontaneous histamine release. Nine hundred fifty microliters of hypotonic medium was added to 50 μ L of whole blood separately in glass tubes to determine the total histamine content of the cells.

The plate was incubated at 37°C in a water bath for 1 hour, followed by 10 minutes of incubation in an ice bath. The plate was then centrifuged for 10 minutes at 700g and 4°C, and 100 μ L of supernatant was transferred to Eppendorf tubes. Histamine content of the supernatants was determined by using ELISA (IBL), according to the manufacturer's instructions (see below).

Acylation

Samples and standards were acylated before use because the detection antibody in the ELISA kit recognizes acylated histamine. One hundred microliters of indicator buffer (IBL) was added to 100 μ L of each histamine standard and sample in tubes. Twenty microliters of acylation reagent (IBL) was added to each tube and mixed immediately by vortexing to ensure homogenous mixture. Tubes were incubated at room temperature for 30 minutes, followed by addition of 750 μ L of diluted assay buffer (IBL).

Histamine ELISA

Fifty microliters of each acylated standard, control, and sample were added to the wells of 96-well precoated microtiter plates (IBL). Fifty microliters of freshly prepared enzyme conjugate (IBL) and 50 μ L of histamine antiserum (IBL) were added to each corresponding well. The plates were sealed with adhesive foil and incubated for 3 hours at room temperature on an orbital shaker at 500 rpm.

Plates were washed 4 times with 250 μ L of diluted wash buffer (IBL) to remove unbound molecules and lysed cell debris. One hundred microliters of TMB Substrate Solution (IBL) was added to each well and incubated for 40 minutes at room temperature in the dark on an orbital shaker at 500 rpm. The reaction was stopped with 100 μ L of TMB Stop Solution (IBL). OD was measured with an ELISA Microplate Reader (Molecular Devices, Sunnyvale, Calif) at 450 nm (reference wavelength, 600-650 nm). Histamine concentration was determined from the standard curve by using SoftMax Pro software (Version 4.3.1, Molecular Devices) within 15 minutes of stopping the TMB reaction. Background (spontaneous) histamine release was subtracted from each test value. The percentage of histamine release was determined from the total histamine content of the cells. Percentage inhibition was then calculated as the percentage reduction in histamine release compared with control values (release buffer only).

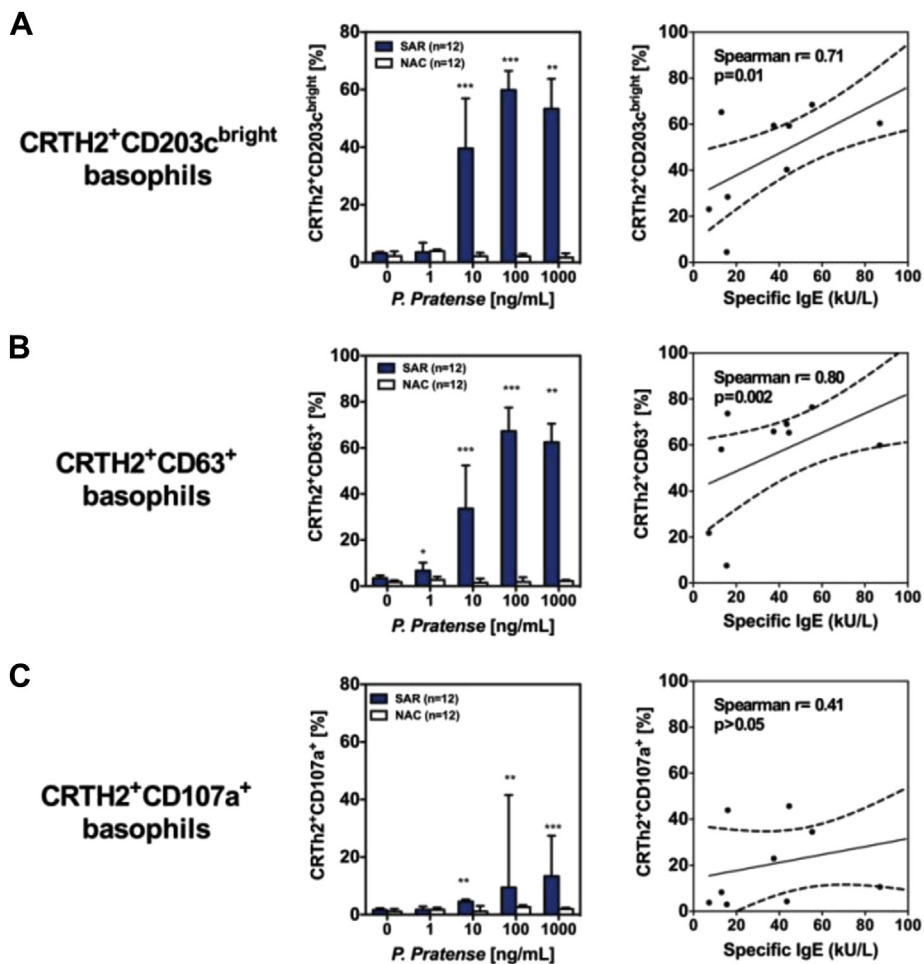


FIG E1. *Ex vivo* allergen-induced basophil reactivity is increased in patients with SAR compared with that seen in NACs. Whole-blood basophils obtained from patients with SAR ($n = 12$) and NAC ($n = 12$) volunteers were incubated with varying concentrations of *P. pratense* extract. Proportions of CD203c^{bright} (A), CD63⁺ (B), and CD107a⁺ (C) CRTh2⁺ basophils and their relationship with circulating sIgE levels in patients with SAR were assessed. Data are expressed as medians (interquartile ranges). Significance between groups was assessed by using the Mann-Whitney *U* test as follows: * $P < .05$, ** $P < .01$, and *** $P < .001$, respectively. Correlations were obtained by using the Spearman rank method.

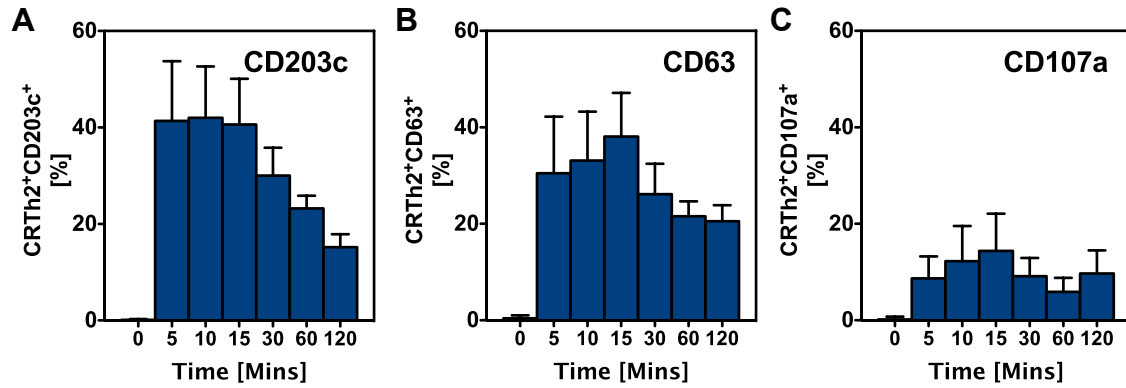


FIG E2. Time course of CD203c^{bright}, CD63, and CD107a surface activation markers on CRTh2⁺ basophils after *ex vivo* *P. pratense* stimulation. Basophils were stimulated with 100 ng/mL *P. pratense* and incubated for 0, 5, 10, 15, 30, 60, and 120 minutes. Expression of CD203c, CD63, and CD107a on CRTh2⁺ basophils was measured. Data are shown as median (interquartile range) percentages of CD203c^{bright}CRTh2⁺, CD63⁺CRTh2⁺, and CD107a⁺CRTh2⁺ basophils.

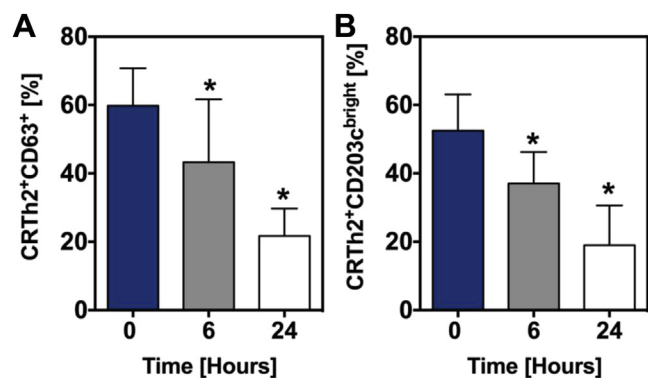


FIG E3. *Ex vivo* allergen-induced basophil activation is diminished if the cells are stimulated 6 and 24 hours after blood collection. Proportions of CD63⁺CRTh2⁺ and CD203c^{bright}CRTh2⁺ basophils were assessed by using blood at 0, 6, and 24 hours after venesection. Data are expressed as medians (interquartile ranges). Significance was assessed by using the Mann-Whitney *U* test. **P* < .05.

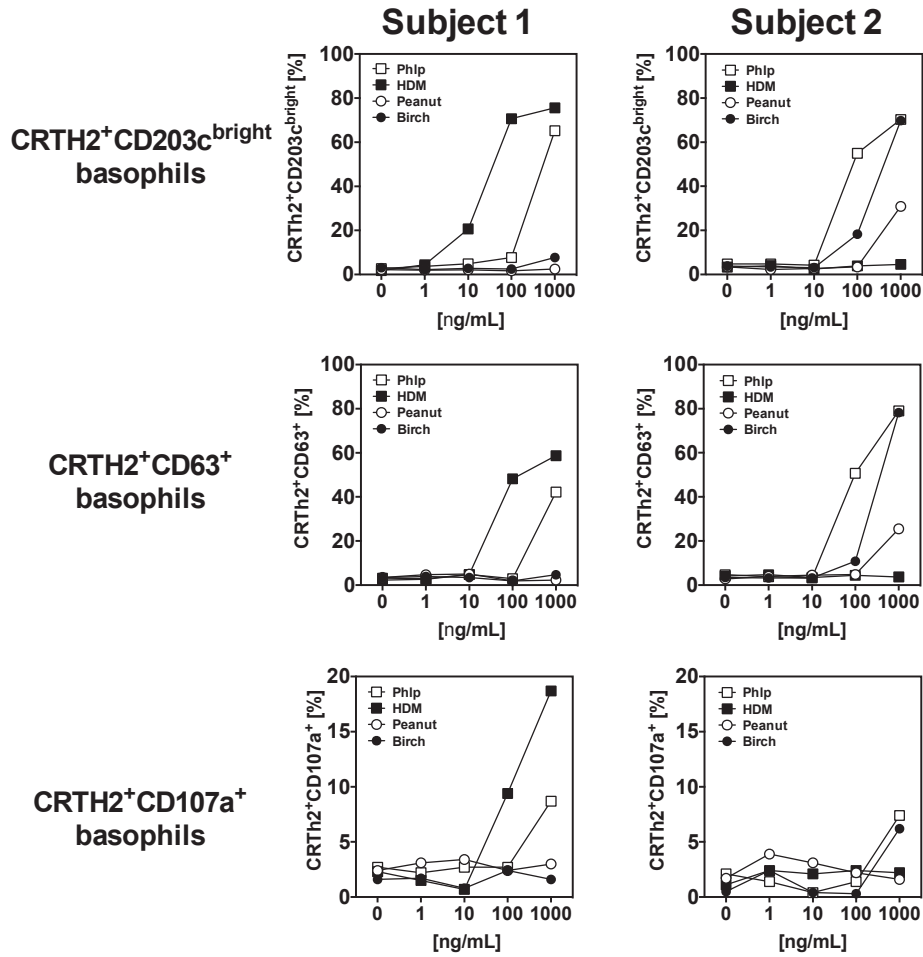


FIG E4. Increased expression of CD203^{bright}, CD63, and CD107a on CRTh2⁺ basophils is specific for the sensitizing allergen. Subject 1 is allergic and sensitized to *P pratense* and house dust mite (*HDM*). Subject 2 was sensitized to *P pratense* and birch and weakly sensitized to peanut. *Ex vivo* allergen-specific basophil activation was measured.

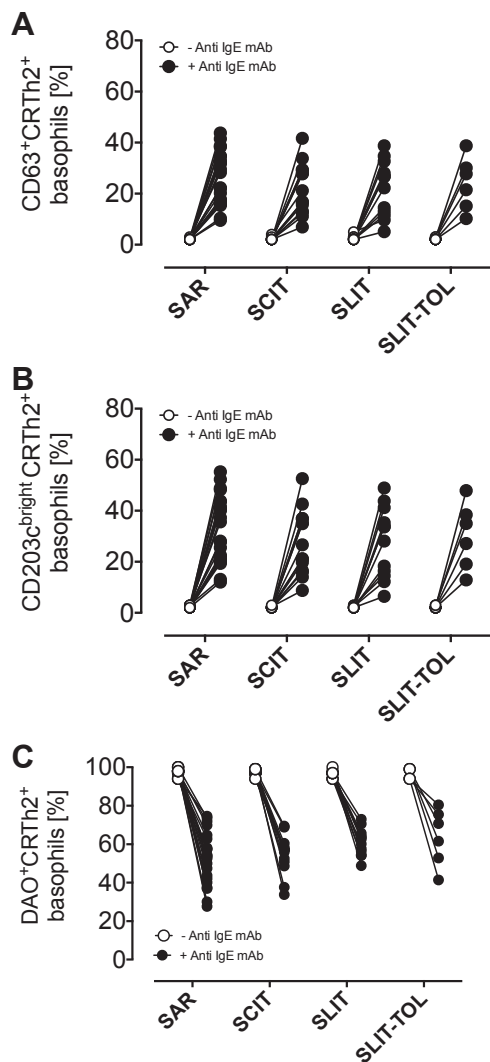


FIG E5. Basophil responsiveness after Fc ϵ RI cross-linking by anti-IgE mAb. DAO⁺CRTh2⁺ and CD63⁺CRTh2⁺ basophil numbers were measured in the SAR (n = 24), SCIT (n = 14), SLIT (n = 12), and SLIT-TOL (n = 6) groups. Results for CD63⁺CRTh2⁺ (**A**), CD203c^{bright}CRTh2⁺ (**B**), and DAO⁺CRTh2⁺ (**C**) basophils after anti-IgE stimulation (0.5 μ g/mL) are shown.

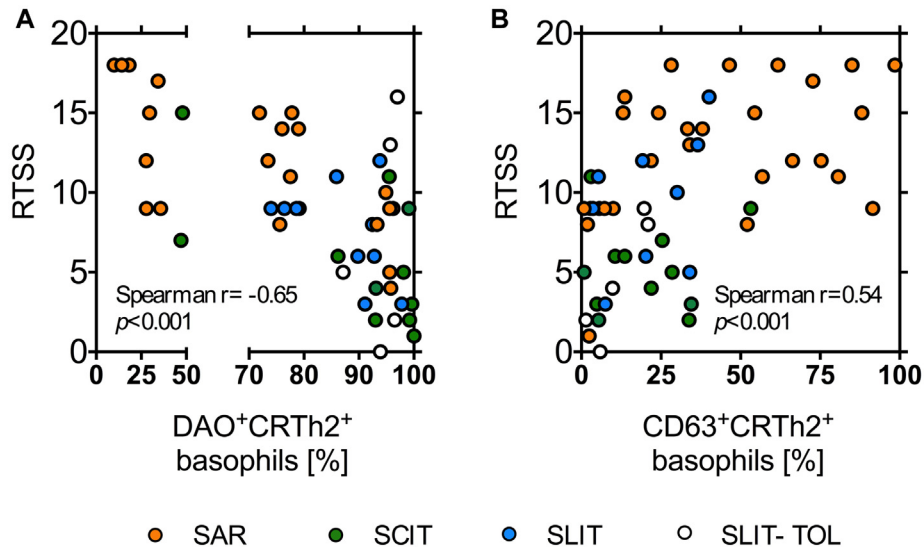


FIG E7. Association between *ex vivo* allergen-induced basophil responsiveness and RTSSs. DAO⁺CRTh2⁺ and CD63⁺CRTh2⁺ basophil numbers were measured in the SAR (n = 24, orange circles), SCIT (n = 14, green circles), SLIT (n = 12, blue circles), and SLIT-TOL (n = 6, white circles) groups. DAO⁺CRTh2⁺ (A) and CD63⁺CRTh2⁺ (B) basophils were correlated with RTSSs by using the Spearman rank method.

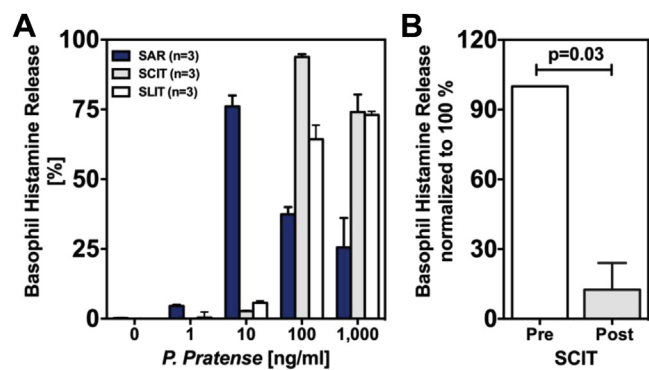


FIG E8. Ten- to 100-fold more allergen was required to induce basophil histamine release in patients treated with SCIT and SLIT. **A**, *Ex vivo* allergen-induced basophil histamine release was assessed in patients treated with SCIT or SLIT and in patients with SAR (each $n = 3$). **B**, Basophil histamine release was also measured before and after SCIT in 6 patients. Data are expressed as medians (interquartile ranges). Significance was assessed by using the Mann-Whitney U test.