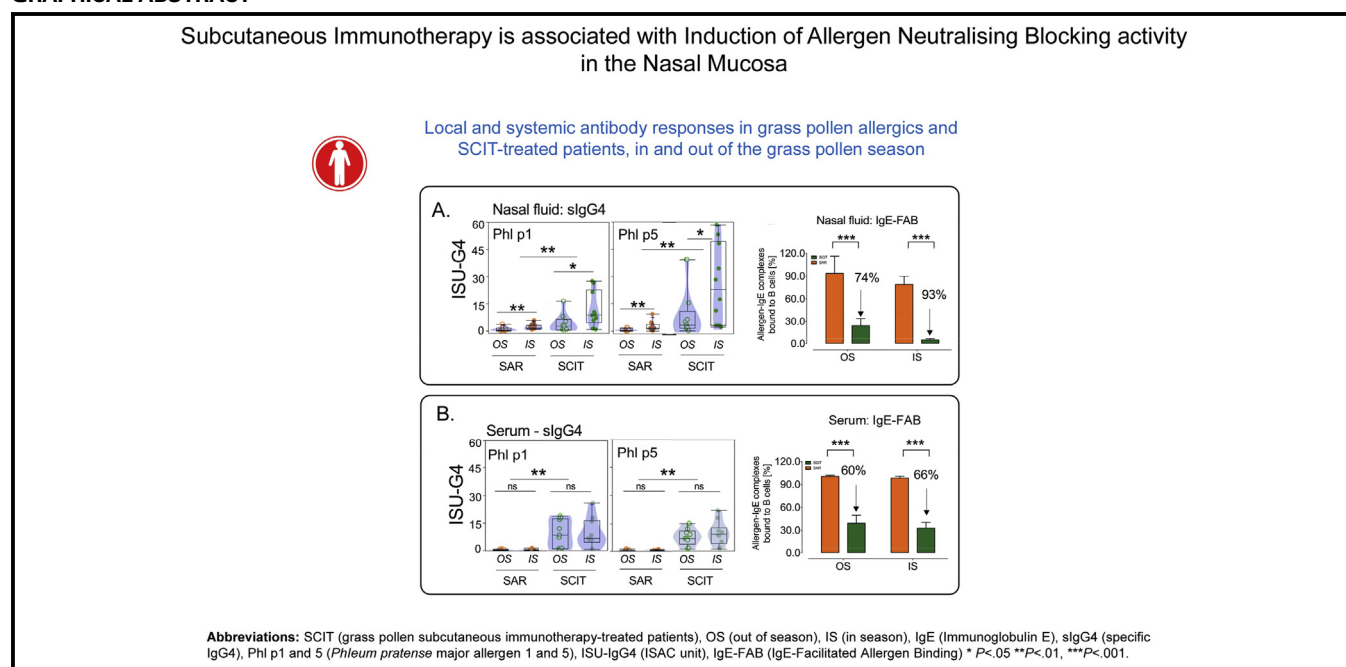


Nasal allergen-neutralizing IgG₄ antibodies block IgE-mediated responses: Novel biomarker of subcutaneous grass pollen immunotherapy



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GRAPHICAL ABSTRACT



Background: Grass pollen subcutaneous immunotherapy (SCIT) is associated with induction of serum IgG₄-associated inhibitory antibodies that prevent IgE-facilitated allergen binding to B cells.

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This study was funded by Imperial College Research Funds, United Kingdom, and Royal Brompton Hospital NHS Trust research funds, United Kingdom.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts interest.

Received for publication October 31, 2017; revised September 19, 2018; accepted for publication September 28, 2018.

Available online November 14, 2018.

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0091-6749/\$36.00

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<https://doi.org/10.1016/j.jaci.2018.09.039>

Objective: We sought to determine whether SCIT induces nasal allergen-specific IgG₄ antibodies with inhibitory activity that correlates closely with clinical response.

Methods: In a cross-sectional controlled study, nasal fluid and sera were collected during the grass pollen season from 10 SCIT-treated patients, 13 untreated allergic patients (with seasonal allergic rhinitis [SAR]), and 12 nonatopic control subjects. Nasal and serum IgE and IgG₄ levels to *Phleum pratense* components were measured by using the Immuno Solid Allergen Chip microarray. Inhibitory activity was measured by IgE-facilitated allergen binding assay. IL-10⁺ regulatory B cells were quantified in peripheral blood by using flow cytometry.

Results: Nasal and serum Phl p 1- and Phl p 5-specific IgE levels were increased in patients with SAR compared to nonatopic control subjects (all, $P < .001$) and SCIT-treated patients (nasal, $P < .001$; serum Phl p 5, $P = .073$). Nasal IgG₄ levels were increased in the SCIT group compared to those in the SAR group ($P < .001$) during the pollen season compared to out of season. IgG-associated inhibitory activity in nasal fluid and serum was significantly increased in the SCIT group compared to that in the SAR (both, $P < .01$). The magnitude of the inhibitory activity was 93% ($P < .001$) in nasal fluid

compared to 66% ($P < .001$) in serum and was reversed after depletion of IgG. Both nasal fluid ($r = -0.69$, $P = .0005$) and serum ($r = -0.552$, $P = .0095$) blocking activity correlated with global symptom improvement. IL-10⁺ regulatory B cells were increased in season compared to out of season in the SCIT group ($P < .01$).

Conclusion: For the first time, we show that nasal IgG₄-associated inhibitory activity correlates closely with the clinical response to allergen immunotherapy in patients with allergic rhinitis with or without asthma. (J Allergy Clin Immunol 2019;143:1067-76.)

Key words: Biomarkers of allergen immunotherapy, subcutaneous immunotherapy, IgE-facilitated allergen binding, blocking antibodies, IgG₄, IgA₁, IgA₂, allergic rhinoconjunctivitis with and without asthma

Grass pollen injection allergen immunotherapy (AIT) is an effective treatment for seasonal allergic rhinitis (SAR), an IgE-mediated disease that causes chronic inflammation of the nasal mucosa.¹⁻⁵ AIT is associated with a reduction in symptoms,^{4,6,7} less use of rescue medications, and improvement in quality of life.⁸⁻¹² It induces clinical and immunologic allergen-specific immune tolerance and thus confers a long-lasting clinical benefit.^{1-4,13,14} However, a minority of the patients do not respond optimally to AIT,^{13,15} indicating a need for biomarkers as surrogate end points both for early identification of responders and as for end points of clinical trials.¹⁵ Therefore biomarkers have an important role in AIT personalized medicine.^{15,16}

It is widely accepted that IgG antibodies, especially IgG₄, play a central role in the mechanism of AIT. Immediately after the start of AIT, an increase in the range of 10- to 100-fold in the concentrations of IgG₁ and particularly that of IgG₄ is observed.^{17,18} These antibodies have been shown to be produced by regulatory B (Breg) cells that predominantly secrete IL-10, favoring antibody class-switching from IgM or IgE to IgG₄.¹⁹ The correlation between serum IgG₄ levels and clinical outcomes is highly variable, and results from several clinical studies have been contradictory.^{20,21}

IgG₄ has 2 main effects in the mechanism of AIT, leading to tolerance. First, IgG₄ acts as a neutralizing antibody that competes with IgE for allergen binding and thus acts as a blocking antibody that prevents the activation and degranulation of effector cells.^{22,23} Second, IgG₄ blocks IgE-facilitated binding of allergen-IgE complexes to B cells, a surrogate and rate-limiting step for IgE-facilitated T-cell activation. In allergic patients allergen-IgE complexes bind to the low-affinity IgE receptor FcεRII (CD23) on the surfaces of B cells. Subsequently, activated B cells present processed allergen peptides on the surfaces of HLA class II molecules to the relevant peptide-specific T-cell clones along with costimulation, thereby resulting in T-cell activation, proliferation, and cytokine production. Thus sera obtained from subjects receiving birch and grass pollen immunotherapy inhibited IgE-facilitated presentation of allergen by B cells to allergen-specific T-cell clones.²²⁻²⁴ The majority of this inhibitory activity for IgE-FAB present in postimmunotherapy serum resides within the IgG₄ subclass, as shown by affinity chromatography.^{25,26} We and others have shown in several clinical studies that serum inhibitory activity for IgE-FAB can correlate with the clinical response to grass and birch AIT up to 2 years after discontinuation of AIT.^{2,23,26} Little is known on local functional

Abbreviations used

AIT:	Allergen immunotherapy
Breg:	Regulatory B
IgE-FAB:	IgE-facilitated allergen binding
ISAC:	Immuno Solid Allergen Chip
NA:	Nonatopic control
RTSS:	Rhinoconjunctivitis Total Symptom Score
SAR:	Seasonal allergic rhinitis
SCIT:	Subcutaneous immunotherapy
sIgE:	Specific IgE
sIgG ₄ :	Specific IgG ₄
VAS:	Visual analog scale

IgG₄ antibody responses and their relationship with clinical outcome. In addition to the induction of allergen-specific IgG (sIgG) antibodies, studies have also revealed the induction of IgA₁ and IgA₂ after AIT,²⁷ although its functional role in the mechanism for immunotherapy remains unclear.

We hypothesized that immunotherapy efficacy is associated with induction of blocking antibody production locally in the nasal mucosa. First, we demonstrate the specificity and function of nasal and serum grass pollen specific IgE and specific IgG₄ in and out of the grass pollen season in immunotherapy-treated patients (AIT), untreated allergic patients (SAR group), and nonatopic control (NA) subjects. Second, we report the relationship between IL-10⁺ Breg cells and peripheral and nasal IgG₄. Finally, we report that local functional antibody responses correlate with clinical response to injection immunotherapy.

METHODS

Subjects

A cross-sectional controlled study of grass pollen subcutaneous immunotherapy (SCIT) was conducted outside (September-February) and during (June-August) the grass pollen season. Ten patients with moderate-severe SAR treated with SCIT (*Phleum pratense* [Phl p]; Alutard SQ, ALK-Abelló, Hørsholm, Denmark) were recruited, along with 13 immunotherapy-naïve (untreated) patients with SAR and 12 NA subjects (Table 1). Inclusion and exclusion criteria can be found in the Methods section in this article's Online Repository at www.jacionline.org. All enrolled study participants completed the study. Blood samples were collected outside (September-February) and during (May-July) the grass pollen season.

The study was approved by the national research ethics committee (South West London committee, REC3) and the Research Office of the Royal Brompton and Harefield NHS Foundation Trust. Written informed consent was obtained from all patients before collecting nasal and blood samples. Nasal fluid and blood samples from all participants were anonymized and blinded before laboratory processing. All research laboratory staff were blinded, and all experiments were performed blind. Data were locked before unblinding the results. All analysis was performed on the availability of nasal fluid samples from participants.

Rhinoconjunctivitis Total Symptom Scores, Global Evaluation scores, and Mini-Rhinitis Quality of Life Questionnaire scores

Rhinoconjunctivitis Total Symptom Score (RTSS) were taken from patients during the pollen season. Individual nasal symptoms, itching, sneezing, runny nose, blocked nose, and eye redness and tears were scored on a 4-point scale. Changes in symptom severity compared to that of before treatment were quantified by using a retrospective global evaluation score.

TABLE I. Subjects' characteristics

	Patients with SAR (n = 13)	SCIT-treated patients (n = 10)	NA subjects (n = 12)
Sex (male/female)	6/7	6/4	6/6
Age (y), mean $\pm$ SD	33.0 $\pm$ 7.4	39.1 $\pm$ 12.2	38.8 $\pm$ 13.4
Timothy grass sIgE (IU/mL), mean $\pm$ SD	39.9 $\pm$ 29.4	33.7 $\pm$ 35.3	0.03 $\pm$ 0.04
Total IgE (IU/mL), mean $\pm$ SD	165.7 $\pm$ 198.2	583.2 $\pm$ 838.2	28.5 $\pm$ 27.9

Patients undergoing SCIT were asked to compare their hay fever symptoms score ranging from -3 (much worse) to $+3$ (much better) compared with before starting immunotherapy, whereas patients with SAR and NA subjects were asked to compare their symptoms in the current year with those in previous years. A Mini-Rhinitis Quality of Life Questionnaire²⁸ was completed by all participants.

Nasal fluid and serum collection in and out of the grass pollen season

Nasal fluid was collected from a single foam sponge (Zuschnitt Schaumstoff RG27 grau, ISO 5999, 1982 sponge; Gummi-Welz GmbH & Co, Neu-Ulm, Germany) inserted into each nostril under direct vision by using sterile forceps and left in place for 5 minutes. Sponges were then placed above a microfilter within a Spin-X 0.22- μ m centrifuge tube (Costar; Corning, Corning, NY) and kept briefly on ice (see the [Methods](#) section in this article's Online Repository for further description).

Immunoglobulin measurements in nasal fluid and serum

Total IgE levels were measured in serum by using the ImmunoCAP100 system (Phadia, Uppsala, Sweden), according to the manufacturer's instructions. Levels of sIgE and sIgG₄ to grass pollen components in nasal fluid and serum were measured by using the Immuno Solid Allergen Chip (ISAC) array (Thermo scientific, Winsford, United Kingdom), according to the manufacturer's protocol, and analyzed with Phadia MIA software.

CD23-mediated IgE-FAB

Using an *in vitro* IgE-Facilitated Allergen Binding (IgE-FAB) assay, we assessed the ability of IgE in nasal mucosa and serum to facilitate the cooperative binding of allergen-IgE complexes to CD23 on the surfaces of B cells carried out as previously described (see the [Methods](#) section in this article's Online Repository for further description).²⁹

Inhibition of CD23-mediated IgE-FAB

IgE-FAB to B cells was performed, as previously described.^{15,29-32} Briefly, serum from a grass pollen-sensitized donor (Phl p sIgE >100 kU/L) was incubated with 1 μ g/mL Phl p in the presence or absence of an equal volume of nasal fluid or serum 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. Binding of allergen-IgE complexes to the CD23 receptor on the surfaces of B cells was detected by using phycoerythrin-labeled anti-human IgE antibodies (Miltenyi Biotech, Auburn, Calif) and acquired with the BD FACSCanto II (BD Biosciences, Rockville, Md). Data were analyzed by using FACSDiva software (BD Biosciences). Binding of allergen-IgE complexes to B cells and inhibition relative to indicator serum from a patient with grass pollen allergy was calculated by using the following formula:

% Relative allergen IgE complex binding to B cells =

$$\left(\frac{\% \text{ IgE FAB using indicator and immunotherapy serum}}{\% \text{ IgE FAB indicator serum only}} \right) \times 100$$

IgG depletion from nasal fluid and serum from patients undergoing AIT

IgG depletion from nasal fluid samples and sera from SCIT-treated patients was carried out by using Proteus Protein G Antibody Purification Mini-Kits (Bio-Rad, Oxford, United Kingdom), according to the manufacturer's protocol (see the [Methods](#) section in this article's Online Repository for further description).

Quantification of Breg cells

Heparinized blood diluted 1:1 with RPMI 1640 media (Life Technologies, Paisley, United Kingdom) was layered on 30% Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) density gradient and centrifuged for 25 minutes at 2200 rpm at room temperature. The PBMC layer was collected, washed, and resuspended in RPMI 1640 medium. Cell viability of greater than 97% was assessed by using trypan blue exclusion. B cells were isolated from PBMCs obtained from NA subjects, patients with SAR, and patients undergoing SCIT using the CD19⁺ B-cell enrichment kit (STEMCELL Technologies, Vancouver, British Columbia, Canada), according to the manufacturer's protocol (see the [Methods](#) section in this article's Online Repository for further description). Moreover, CD4⁺CD45RO⁺ T cells enriched from patients with SAR (n = 12) were cocultured with antigen-presenting cells and 5 μ g/mL Phl p in the presence of B cells or CpG-primed B cells (IL-10⁺ Breg cells). Phl p-induced T-cell proliferation was measured by using tritiated thymidine. The effect of neutralizing anti-IL-10 mAb or isotype control was evaluated (see the [Methods](#) section in this article's Online Repository for further description).

Statistical analysis

The 2-tailed Mann-Whitney test determined statistical differences between individual groups. The Wilcoxon signed-rank test was used for within-group comparisons. One-way ANOVA with Friedman correction for repeated nonparametric measurements was performed where comparison against baseline measurement was required. Correlations between symptom scores and inhibitory activity of serum and nasal fluid was assessed by using the Spearman rank test. Analyses were performed by using Prism software (GraphPad Software, La Jolla, Calif), with *P* values of less than .05 considered as significant.

RESULTS

Symptom scores

RTSSs were significantly lower in the SCIT group compared to untreated SAR (*P* = .009; [Fig 1, A](#)). NA subjects did not exhibit any seasonal symptoms ([Fig 1](#); patients' demographics are presented in [Table I](#)). Retrospective global evaluation scores revealed a decrease in overall severity, which was greater in the SCIT group compared with that in the untreated SAR group (*P* = .006; [Fig 1, B](#)). The overall mean Mini-Rhinitis Quality of Life Questionnaire scores and some individual parameters observed were lower in the SCIT group compared with those in the SAR group (*P* = .024; [Fig 1, C](#), and see [Tables E1 and E2](#) in this article's Online Repository at www.jacionline.org).

Grass pollen sIgE levels in serum and nasal fluid in the SAR, NA, and SCIT groups

Phl p 1 and Phl p 5 (major grass pollen allergens) sIgE levels were measured in nasal fluid and serum from NA subjects,

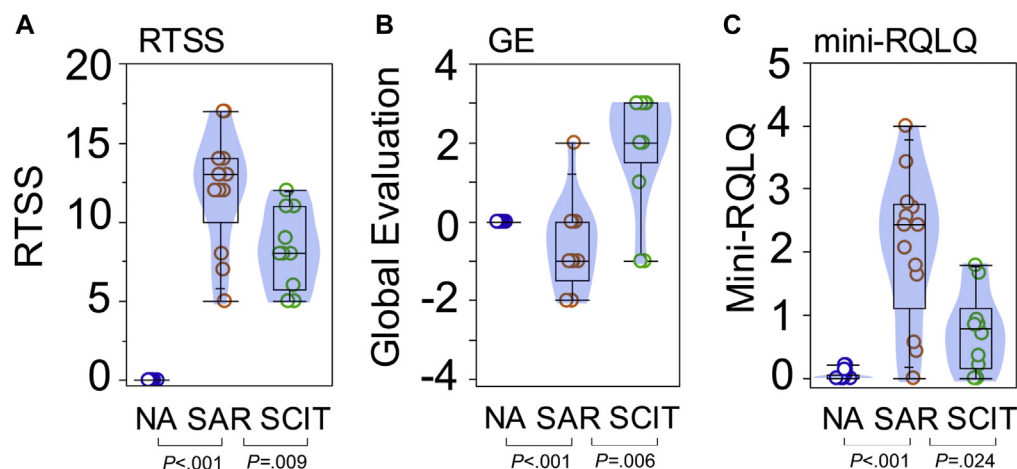


FIG 1. Clinical Symptom Scores. **A**, Rhinoconjunctivitis Symptom Scores (RTSS); **B**, Retrospective Global Evaluation Scores (−3, worse disease burden to +3, better clinical improvement); and **C**, Mini Rhinoconjunctivitis Quality of Life Questionnaire (mini-RQLQ) scores were evaluated in NA (n = 12), SAR (n = 13), and SCIT-treated (n = 10) groups. Data are shown as violin plots with individual data (open blue circles, NA subjects; orange circles, patients with SAR; and green circles, patients undergoing SCIT), quintile box plots, and data distribution. P values were obtained by using the 2-tailed Mann-Whitney test.

patients with SAR, and subjects undergoing SCIT. Nasal and serum Phl p 1 sIgE levels were greater in patients with SAR compared with NA subjects (all, $P < .001$; Fig 2). However, there was no difference in the levels of nasal and serum Phl p 1 sIgE in patients undergoing SCIT compared with the SAR group. Furthermore, nasal and serum Phl p 5 sIgE levels were higher in patients with SAR compared with NA subjects (all, $P < .001$; Fig 2, A-B). In contrast, Phl p 5 sIgE responses were lower in the SCIT-treated patients in nasal fluid compared with patients with SAR ($P < .001$; Fig 2, A) and in serum ($P = .073$; Fig 2, B). IgG depletion resulted in an increase in nasal and serum levels of Phl p 1 and Phl p 5 sIgE in each SCIT-treated patient (Fig 2, C and D). However, this did not influence the difference in the levels of sIgE to Phl p 1 and Phl p 5 between the SCIT and SAR groups.

sIgE in nasal fluid is functional and forms cooperative allergen-IgE binding to CD23 on the surfaces of B cells

After identifying the induction of sIgE in the local mucosa in patients with SAR, we determined the functional role of sIgE antibodies by assessing their ability to mediate CD23-dependent IgE-allergen complexes binding to B cells (Fig 3, A and B). A dose-dependent response of allergen-IgE complexes binding to CD23 on the surfaces of B cells was observed in the presence of Phl p but not an irrelevant birch pollen allergen (Fig 3, C and D). Optimal binding was observed at 1 $\mu\text{g/mL}$ Phl p (Fig 3, C). IgE dependency was assessed by diluting nasal fluid samples from patients with grass pollen allergy. A dilution-dependent linearity of allergen-IgE complexes binding to B cells was observed (Fig 3, E). The requirement of the low-affinity IgE receptor CD23 for allergen-IgE complexes binding to B cells was also investigated. Preincubation of B cells with increasing concentrations of anti-CD23 antibody resulted in a concentration-dependent decrease of allergen-IgE binding (Fig 3, F).

SCIT induces nasal immunoreactive and functional IgG₄ antibodies with inhibitory activity for IgE-mediated responses

Levels of immunoreactive and functional sIgG₄ to grass pollen components were measured in nasal fluid samples and sera in and out of the pollen season from NA subjects, patients with SAR, and SCIT-treated patients. Nasal Phl p 1 and 5 sIgG₄ levels were increased in patients with SAR during the pollen season compared with those out of season (all, $P < .01$; Fig 4, A). SCIT-treated group also had increased levels of nasal Phl p 1 and 5 sIgG₄ in season compared with those out of season (all, $P < .05$; Fig 4, A). Increases in Phl p 1 and 5 sIgG₄ levels were greater in patients undergoing SCIT compared with patients with SAR (all, $P < .01$). NA subjects did not exhibit any increase in Phl p 1 and 5 sIgG₄ levels in season compared with those out of the pollen season (data not shown). Serum Phl p 1 and 5 sIgG₄ levels did not increase in the SAR and SCIT group. However, levels were higher (all, $P < .01$; Fig 4, B) in the SCIT group compared with the SAR group. Seasonal allergen exposure did not affect serum IgG₄ levels (Fig 4, B). Interestingly, during grass pollen season, the Phl p 5 sIgG₄/sIgE ratio was significantly greater in nasal fluid samples compared with levels in serum in SCIT-treated patients ($P < .05$; see Fig E2 in this article's Online Repository at www.jacionline.org). A reduction in allergen-IgE complexes binding to B cells was also observed in nasal fluid and serum of SCIT-treated patients and was associated with the treatment period over 12 to 24 months when compared with those in patients with SAR (see Fig E3 in this article's Online Repository at www.jacionline.org). This was associated with an increase in IgG₄ levels over a treatment period of 12 to 24 months (see Fig E4 in this article's Online Repository at www.jacionline.org).

Cooperative allergen-IgE binding to B cells was decreased in SCIT-treated patients in and out of season, compared to SAR. Out of season, the nasal inhibitory activity of SCIT-treated patients was reduced to 74% ($P < .001$), and a further reduction was observed during the season, reaching 93% ($P < .001$; Fig 4, C). Similarly, the functional IgG₄ response from peripheral blood

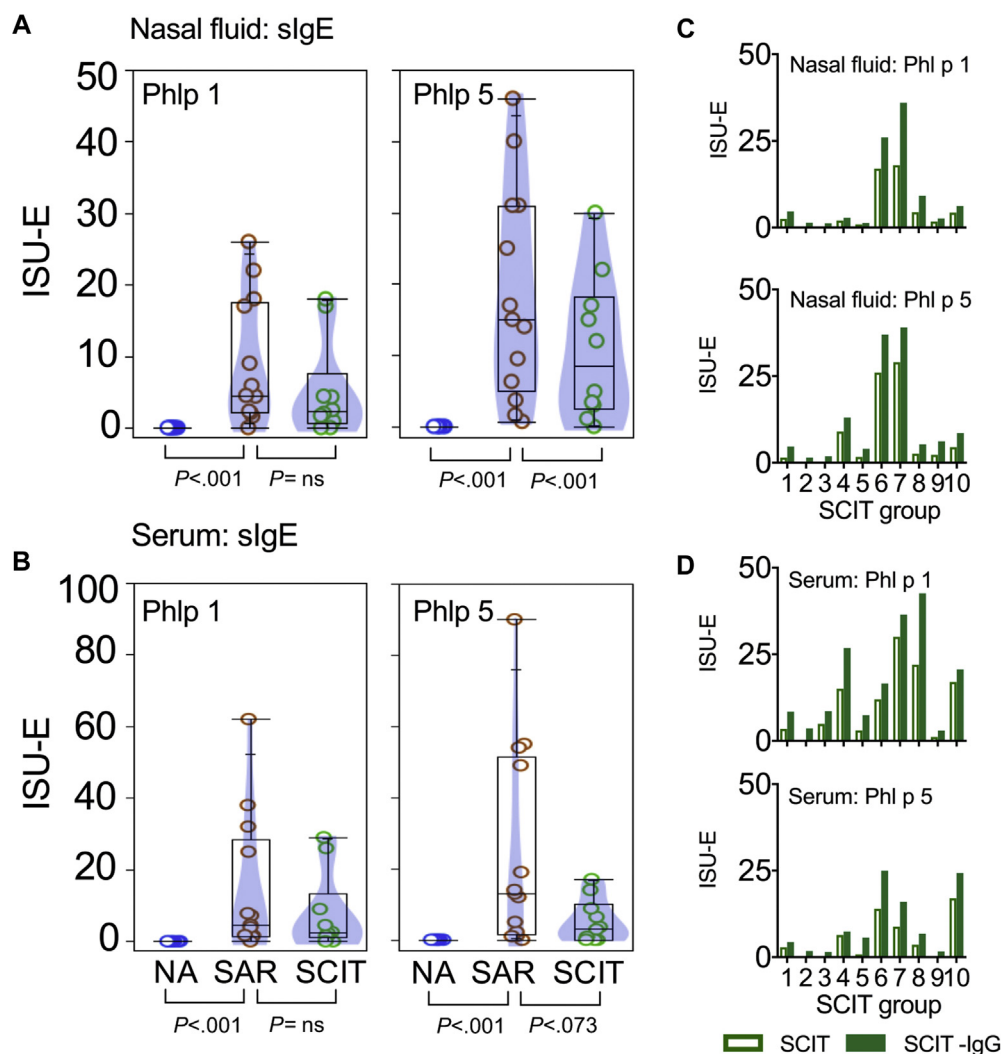


FIG 2. IgE responses in nasal mucosa and peripheral blood in nonatopic subjects, patients with grass pollen allergy, and SCIT-treated patients. **A** and **B**, Timothy grass pollen component (Phl p 1 and 5) sIgE levels were measured in nasal fluid (**A**) and serum (**B**) from NA subjects, patients with SAR, and SCIT-treated patients by using the ISAC assay. Data are shown as violin plots with individual data (open blue circles, NA subjects; orange circles, patients with SAR; and green circles, patients undergoing SCIT), quintile box plots, and data distribution. **C** and **D**, Depletion of IgG from nasal fluid and serum from SCIT-treated patients. Data are shown as bar charts for individual data (white bar, SCIT; green bar, IgG-depleted SCIT samples). *P* values were obtained by using the 2-tailed Mann-Whitney test.

was also reduced to 66% ($P < .001$) in season and 60% ($P < .001$) out of season (**Fig 4, D**). This reduction in allergen-IgE binding to B cells correlated with the increased levels of IgG₄ in SCIT-treated patients.

IgG depletion from SCIT samples was performed to further elucidate the protective role of IgG for having a suppressive effect on allergen-IgE complexes binding to B cells. Depletion of total IgG from SCIT samples resulted in higher binding of allergen-IgE complexes to B cells ($P < .001$) compared with non-depleted samples from the SCIT group and restored the level of IgE-FAB binding close to that observed for the untreated SAR samples (**Fig 4, E**). When the IgG eluate from the column was added back to the IgG-depleted complex mixture, inhibition of allergen-IgE complex binding to B cells was again seen and comparable with that observed for the non-depleted SCIT serum. In contrast, this

allergen-IgE complex binding inhibitory activity was not restored by addition of control purified IgG₄ from a nonatopic donor (ie, not specific to grass pollen). Levels of nasal Phl p 5 sIgG₄ inversely correlated against nasal IgG-associated inhibitory activity ($r = -0.85$, $P < .0001$). However, the magnitude of this association was less in serum ($r = -0.55$, $P = .0095$; see **Table II**).

Influence of natural grass pollen allergen exposure on IL-10⁺ Breg cell subsets in patients undergoing SCIT, patients with SAR, and NA subjects

IL-10⁺ Breg cell subsets were quantified in and out of the grass pollen season in NA subjects, patients with SAR, and SCIT-treated patients. Numbers of IL-10⁺CD19⁺CD5^{hi}CD1d⁺ B cells and IL-10⁺CD19⁺CD24^{hi}CD38^{hi} Breg cells were increased in season

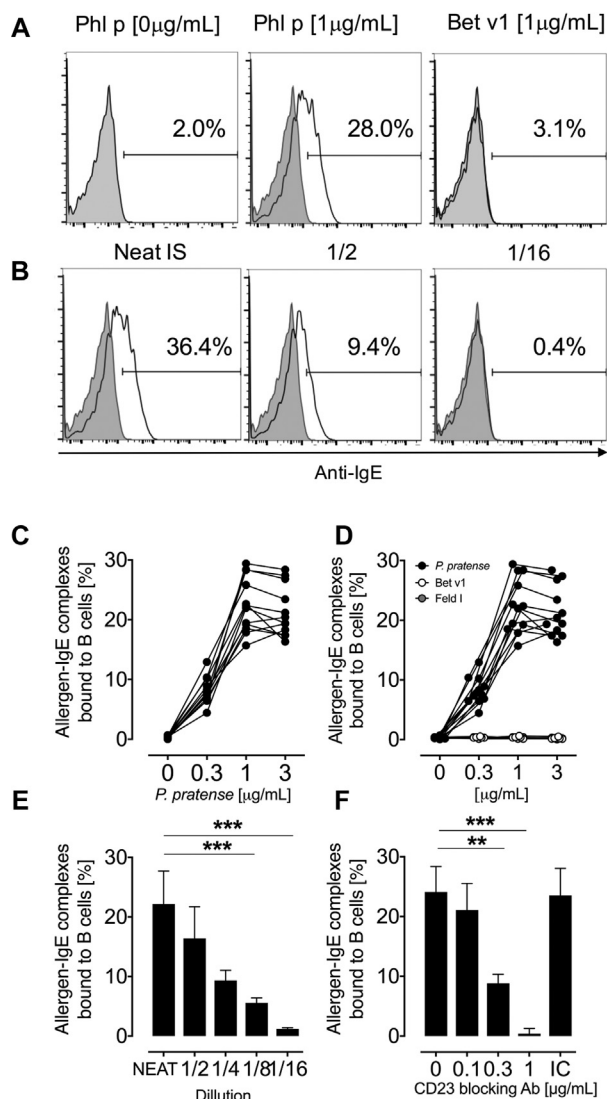


FIG 3. sIgE antibodies in nasal fluid are functional and promote CD23-mediated IgE-facilitated binding of allergen-IgE complexes to B cells. **A**, Representative flow cytometric data showing cooperative binding of allergen-IgE complexes to B cells optimal at 1 μ g/mL Phl p allergen and specific to the sensitizing allergen. **B**, Reduced cooperative allergen-IgE binding to B cells on dilution of indicator serum. **C**, The IgE-FAB assay was performed to assess the ability of sIgE in nasal fluid obtained from patients with grass allergy ($n = 12$) to elicit allergen-IgE complex binding to B cells across varying concentrations of Phl p allergen. **D**, Specificity of sIgE in nasal fluid by means of incubation with Phl p, Bet v 1, or Fel d 1 allergen at various concentrations. Nasal fluid from 12 patients with grass pollen allergy were incubated with Phl p, Bet v 1, or Fel d 1 allergen at various concentrations. Cooperative binding of allergen-IgE to B cells was measured using the FAB assay. **E**, IgE dependency and linearity were assessed by diluting nasal fluid obtained from patients with grass allergy. **F**, CD23 dependency was tested by pretreating EBV-transformed B cells with anti-CD23 blocking antibody at varying concentrations. Allergen-IgE complex binding to B cells was detected by using nasal fluid obtained from only 6 of 13 patients with grass allergy caused by insufficient volumes of nasal fluid from the rest of the patients in the presence of 1 μ g/mL allergen. Comparisons with baseline measurements were determined by using 1-way ANOVA with Friedman correction for repeated nonparametric measurements. * $P < .05$, ** $P < .01$, and *** $P < .001$, respectively.

compared with out of season in SCIT-treated patients (all $P < .01$, Fig 5). No change was observed in the SAR group. Most importantly, the SCIT-treated group had increased IL-10⁺CD19⁺CD5^{hi}CD1d⁺ and IL-10⁺CD19⁺CD24^{hi}CD38^{hi} Breg cells compared with the SAR group ($P < .01$ and $P < .001$, respectively; Fig 5). The SAR group had lower proportions of IL-10⁺CD19⁺CD5^{hi}CD1d⁺ B cells and IL-10⁺CD19⁺CD24^{hi}CD38^{hi} Breg cells compared with the NA group ($P < .05$ and $P < .001$, respectively; Fig 5). Moreover, numbers of IL-10⁺CD19⁺CD5^{hi}CD1d⁺ Breg cells correlated more closely with peripheral sIgG₄ levels ($r = 0.81$, $P = .01$) than with nasal sIgG₄ levels ($r = 0.67$, $P = .05$). Moreover, to address the functional role of Breg cells, CpG-primed B cells (Bregs) or non-primed B cells were cocultured with equal numbers of memory CD4⁺ T cells and irradiated non-CD4⁺ PBMCs in the presence of 5 μ g/mL Phl p extract. CpG-primed B cells (Bregs) inhibited T-cell proliferation ($P < .01$, see Fig E5 in this article's Online Repository at www.jacionline.org) and IL-5 ($P < .001$) and IL-13 ($P < .001$) production (see Fig E6 in this article's Online Repository at www.jacionline.org). This effect was lost in the presence of IL-10-neutralizing antibody ($P < .001$, see Fig E5). We also show that IL-10-producing CD19⁺CD5^{hi} and CD24^{hi}CD38^{hi} Breg cells were also induced by SCIT compared with the SAR group (see Fig E7 in this article's Online Repository at www.jacionline.org).

Functional nasal IgG levels correlate with clinical response to treatment

The relationship between the clinical response to grass pollen SCIT was measured based on global improvement, RTSSs, and visual analog scale (VAS), along with IgG-associated inhibitory activity assessed through IgE-FAB, Phl p 5 sIgG₄, and the Phl p 5 sIgG₄/sIgE ratio in nasal fluid and serum.

Levels of Phl p 5 sIgG₄ in nasal fluid were correlated with global improvement (Spearman $r = 0.49$, $P = .024$) but inversely correlated with RTSSs ($r = -0.59$, $P = .005$) and VASs ($r = -0.58$, $P = .0091$). Serum Phl p 5 sIgG₄ levels did not correlate with global improvement ($r = -0.37$, $P = .0955$), RTSSs ($r = -0.37$, $P = .09$), and VASs ($r = -0.06$, $P = .766$) (Table II). The Phl p 5 sIgE/sIgG₄ ratio in nasal fluid correlated with global improvement ($r = 0.56$, $P = .012$) and inversely correlated with RTSSs ($r = -0.49$, $P = .035$) but did not correlate with VASs ($r = 0.26$, $P = .290$). The Phl p 5 sIgE/sIgG₄ ratio in serum also correlated with global improvement ($r = 0.47$, $P = .032$) and RTSSs ($r = -0.49$, $P = .028$) but not VASs ($r = -0.13$, $P = .56$) (Table II). Interestingly, IgA₁ and IgA₂ levels in nasal fluid and serum of the SCIT-treated group were significantly greater than those in the SAR group (see Fig E1 in this article's Online Repository at www.jacionline.org). Levels of grass pollen-specific IgA₂ in nasal fluid and serum correlated with global improvement ($r = -0.064$, $P = .78$, and $r = 0.49$, $P = .025$, respectively) (Table II). IgG-associated inhibitory activity in nasal fluid inversely correlated with global improvement ($r = -0.69$, $P = .0005$), RTSSs ($r = 0.62$, $P = .0028$), and VASs ($r = 0.57$, $P = .007$). However, IgG-associated inhibitory activity in serum had a modest inverse correlation against global evaluation ($r = -0.55$, $P = .0095$), RTSSs ($r = 0.50$, $P = .02$), and VASs ($r = 0.31$, $P = .169$) (Table II).

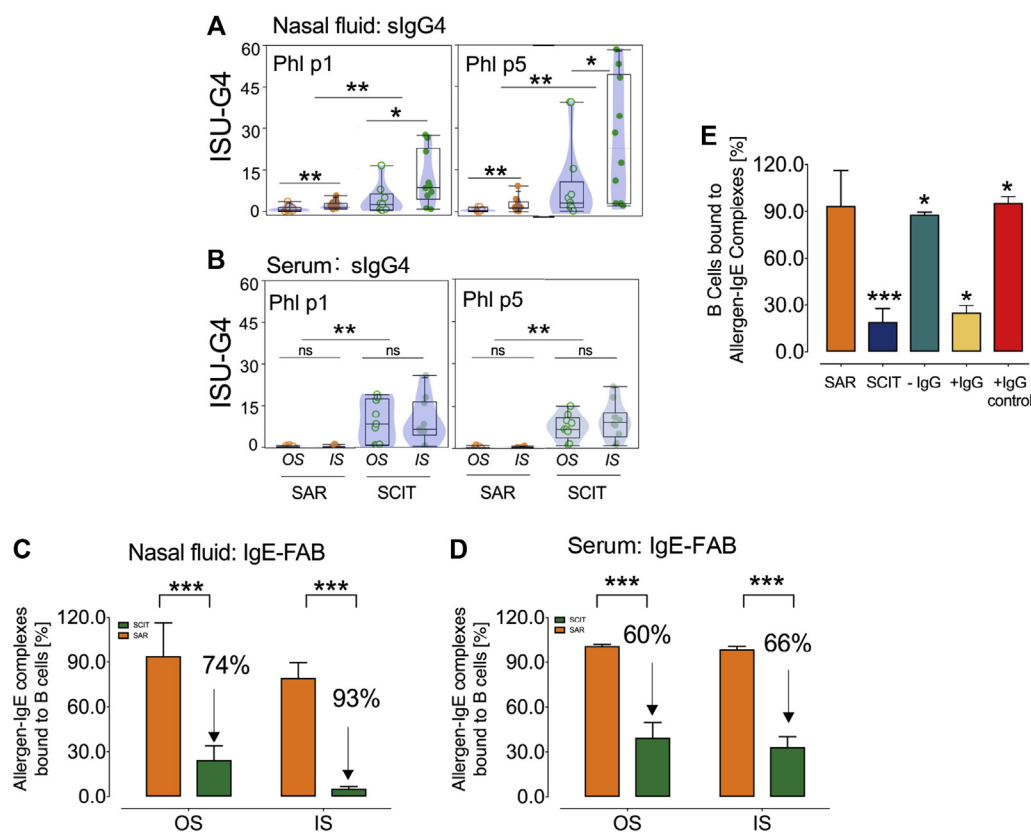


FIG 4. Immunoreactive and functional IgG₄ responses in nasal fluid and peripheral blood in NA subjects, untreated patients with grass pollen allergy, and SCIT-treated patients. **A** and **B**, Timothy grass pollen components (Phl p 1 and Phl p 5) sIgG₄ levels were measured in nasal fluid (Fig 4, **A**) and serum (Fig 4, **B**) from patients with SAR and SCIT-treated patients. Data are shown as violin plots with individual data (open blue circles, NA subjects; orange circles, patients with SAR; and green circles, SCIT-treated patients), quintile box plots and data distribution, and means (SEMs). **C** and **D**, Cooperative allergen-IgE binding to B cells and inhibitory activity for IgE-FAB was measured in nasal fluid (Fig 4, **C**) and serum (Fig 4, **D**) from patients with SAR and SCIT-treated patients in season (IS) and out of season (OS). **E**, Binding of allergen-IgE complexes to B cells following IgG depletion was determined using IgE-FAB Assay. Data are represented as bar charts (orange bar, untreated SAR group; blue bar, SCIT-treated group). *P* values were obtained by using the 2-tailed Mann-Whitney test (between groups) and the Wilcoxon signed rank test (within groups). **P* < .05, ***P* < .01, and ****P* < .001, respectively.

DISCUSSION

For the first time, we have shown that grass pollen-specific immunotherapy, in particular SCIT, is associated with induction of local (nasal) and functional antibodies that inhibit IgE-FAB in the nasal mucosa. Furthermore, we demonstrated that inhibitory activity against IgE in nasal fluid from SCIT-treated patients correlates better with clinical outcome than the inhibitory activity of serum samples, highlighting its potential as a novel biomarker for monitoring treatment effect. Moreover, we have demonstrated that grass pollen SCIT induces an increase in numbers of IL-10⁺ Breg cells that correlates with the increase in IgG₄ levels. Our findings suggest that the functional inhibitory activity in both nasal fluid and serum is dependent on the functionality of grass pollen sIgG₄ blocking antibodies, given that depletion of IgG in SCIT nasal fluid and serum samples led to recovery of allergen-IgE complexes binding to CD23 on B cells.

Allergy is characterized by an increase in serum sIgE levels in the context of symptoms on exposure to the relevant allergen. Our findings show a seasonal increase in levels of local sIgE in the

nasal fluid of allergic patients compared to healthy control subjects. Furthermore, sIgE in the nasal mucosa is functional and has the ability to mediate CD23-dependent IgE-facilitated allergen binding and presentation by B to T cells. AIT did not diminish levels of sIgE in the nasal mucosa. However, depletion of IgG resulted in an increase in levels of IgE, suggesting the potential of IgG to compete with IgE.³³

AIT is known to be associated with the induction of sIgG₄ antibodies.^{2,22,34} This increase is likely to be under the influence of newly induced IL-10-producing regulatory T cells, as well as Breg cells.^{22,34} In this study we illustrated that both nasal fluid and serum from SCIT-treated patients inhibited allergen-IgE complex binding to B cells, as measured through the IgE-FAB assay. In addition, we also illustrated that inhibition of IgE-FAB to B cells in nasal fluid and serum was dependent on the duration of the SCIT treatment. A greater inhibition of allergen-IgE complex binding to B cells was observed in nasal fluid compared to serum, and this is likely due to the higher amount of sIgG₄ antibodies and a high IgG₄/IgE ratio.

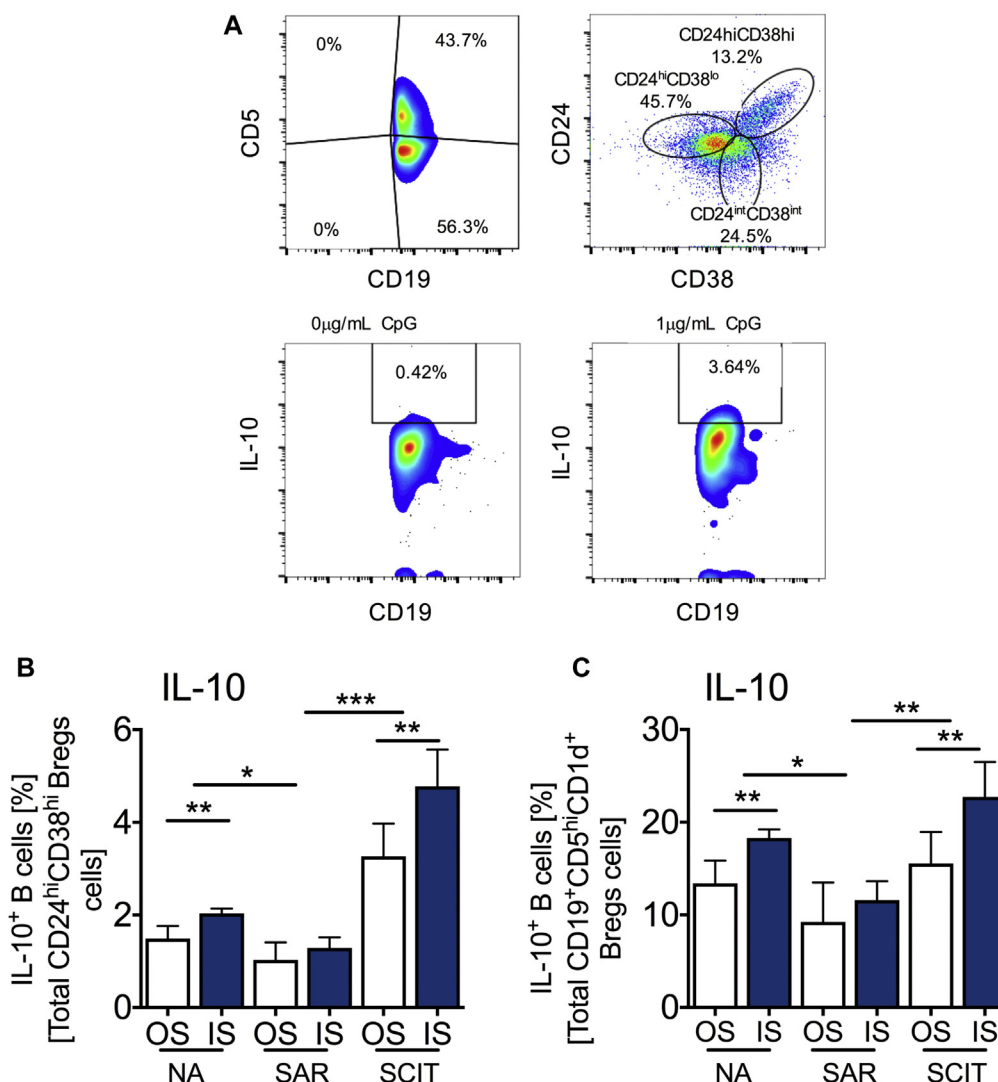


FIG 5. Breg cell (IL-10⁺CD24^{hi}CD38^{hi}CD19⁺ B cells and IL-10⁺CD5^{hi}CD19⁺ B cells) responses in NA subjects, patients with SAR, and SCIT-treated patients outside and during the grass pollen season. **A**, Representative flow cytometric plot for enumerating Breg cell subsets. **B** and **C**, Proportion of IL-10⁺CD19⁺CD5^{hi} (Fig 5, **B**) and IL-10⁺CD19⁺CD24^{hi}CD38^{hi} (Fig 5, **C**) Breg cells in NA subjects (n = 12), patients with SAR (n = 13), and SCIT-treated patients (n = 10) in and out of the allergen season. *P* values were obtained by using the Wilcoxon signed rank test (within groups) and 2-tailed Mann-Whitney test (between groups). **P* < .05, ***P* < .01, and ****P* < .001, respectively.

Our study confirms the induction of sIgG₄ not only in serum but also in nasal fluid of grass pollen SCIT-treated participants, specific to several components of grass pollen, when measured semiquantitatively with the solid-phase ISAC assay. However, a limitation of this assay is that it provides a semiquantitative measurement of IgG₄ in ISAC units, as opposed to total quantified levels that can be measured by using ImmunoCAP. However, ISAC was preferred because of its use in smaller sample volumes. Furthermore, allergen-sIgG₄ can compete with sIgE with allergen for allergen binding within the assay, which might lead to detection of lower levels of sIgE than actual levels.

In spite of this, SCIT-treated patients demonstrated significantly increased Phl p 1 and Phl p 5 sIgG₄ levels in both nasal fluid and serum despite similar levels of Phl p 1 and Phl p 5 sIgE in both groups. The Phl p 5 sIgG₄/sIgE ratio was found to be significantly greater in nasal fluid when compared

with that in serum. A previous study identified local nasal increases in sIgG₁ but not sIgG₄ levels by using ELISA in patients with birch allergy who received SCIT with a recombinant birch vaccine.¹⁸ In addition to this, a previous study reported that ragweed-specific IgA and sIgG antibodies could inhibit basophil histamine release.³⁵ Despite these previous findings, the inhibitory effect of serum from SCIT-treated patients that arises from the functional CD23-mediated blocking activity of sIgG₄ has not been assessed in nasal fluid. Nasal fluid and serum samples from SCIT-treated patients were depleted of IgG to test the role of sIgG antibodies in eliciting these blocking effects. In both serum and nasal fluid, depletion resulted in significant recovery of allergen-IgE complex binding to B cells. We conclude that functional inhibitory activity in nasal fluid and serum is IgG dependent. We confirmed that the previously described mechanisms of IgG₄ blocking activity are present not only in serum

TABLE II. Correlation statistics of clinical response, levels of immunoglobulins, and IgE-FAB in SCIT-treated patients

	Global evaluation		RTSS		VAS	
	Spearman <i>r</i>	<i>P</i> value	Spearman <i>r</i>	<i>P</i> value	Spearman <i>r</i>	<i>P</i> value
Nasal fluid measurements						
Phl p 5 sIgG ₄	0.4905	.024	−0.5907	.0048	−0.581	.0091
Grass pollen-specific IgA ₁	−0.08001	.7303	−0.0275	.9058	−0.3859	.0841
Grass pollen-specific IgA ₂	−0.06412	.7824	−0.004581	.9843	−0.08268	.7216
IgG ₄ /IgE (Phl p 5)	0.5648	.0118	−0.487	.0345	−0.2562	.2898
IgE-FAB	−0.6897	.0005	0.6186	.0028	0.5737	.0065
Serum measurements						
Phl p 5 sIgG ₄	0.3734	.0955	−0.3772	.0918	−0.06909	.766
Grass pollen-specific IgA ₁	0.3451	.1255	−0.07134	.7586	−0.3229	.1534
Grass pollen-specific IgA ₂	0.4993	.025	−0.2774	.2364	−0.5882	.0064
IgG ₄ /IgE (Phl p 5)	0.4797	.0323	−0.4905	.0281	−0.1374	.5635
IgE-FAB	−0.552	.0095	0.5039	.0198	0.3119	.1688

but extend to nasal fluid harvested directly from the nasal mucosa. The SAR group also showed a seasonal increase in sIgG₄ levels in nasal fluid, although this increase was very modest compared to the SCIT-treated group. Immunotherapy exerts its effect in the target organ, and therefore it is paramount to assess the immunomodulatory effect in the nasal mucosa. In our study IgG₄ blocking antibodies measured in nasal fluid correlated more closely with clinical efficacy (ie, RTSS or VAS) compared to serum. These findings clearly highlight the need to measure IgG₄ and IgG₄ blocking activity during AIT in the target organ rather than in serum. Although serum IgG₄ levels can reflect immunologic response and treatment compliance. It is also important to note that this was a pilot study, and a large study of SCIT is needed to confirm nasal IgG₄ blocking antibody as a biomarker of SCIT.

Some studies have suggested that IL-10 and Breg cells are responsible for the observed increase in sIgG₄ levels.³⁶ We have also confirmed an increase in the population of IL-10⁺ Breg cell subsets (CD19⁺CD24^{hi}CD38^{hi} and CD19⁺CD5^{hi}CD1d⁺) in the patients receiving SCIT compared with the SAR group. We have shown that the proportion of Breg cells were lower in patients with SAR compared with NA subjects and that grass pollen SCIT was associated with the restoration of Breg cell subsets to levels near those in NA subjects.

We have also demonstrated a correlation between IL-10 Breg cell counts and peripheral IgG₄ levels. It would be insightful to reproduce these findings by enumerating allergen-specific Breg cells. However, identifying these cells is technically challenging because the frequency of these cells is extremely low in peripheral blood, which makes it very challenging to acquire sufficient cells by using flow cytometry to have reliable and reproducible data. To further address the functionality of Breg cells, we showed that IL-10⁺ Breg cells were able to inhibit allergen-driven T-cell proliferation and cytokine (IL-5 and IL-13) production and that this inhibition was dependent on IL-10. We further highlight that IL-10-producing Breg cells were induced by SCIT when compared with those in patients with SAR over a period of 12 to 24 months of treatment.

We have identified a correlation between functional activity in nasal fluid and clinical response treatment. We confirmed our previously reported correlation of the functional inhibitory activity of serum IgG₄ and symptom scores and clinical outcome.²

The correlation between functional inhibition by nasal fluid for RTSS and global evaluation was stronger than the correlation between functional inhibition by serum for RTSS and global

evaluation, suggesting that functional activity in nasal fluid represents a more predictive and accessible biomarker for monitoring clinical responses to treatment. Further studies with larger cohorts are needed to support these findings.

This cross-sectional study, performed in and out of the pollen season, provides us with a snapshot regarding the change in biomarkers after treatment. A prospective study is needed to investigate changes in levels of baseline biomarkers before and after treatment with AIT. This will provide us with a clearer indication as to whether the biomarkers identified in this study are a true marker of treatment response.

In conclusion, for the first time, we report that after grass pollen SCIT, both local IgE inhibitory activity and allergen-sIgG₄ antibody levels were increased in nasal fluid. The increase in IgG₄ and IgE inhibitory activity in nasal fluid correlated more closely with clinical outcomes when compared with serum samples. Larger clinical studies with samples before/after treatment will determine whether changes in local IgG-associated IgE-inhibitory activity will be applicable as a potential biomarker to predict clinical response to treatment in individual patients.

Key messages

- Grass pollen-specific immunotherapy, in particular SCIT, is associated with induction of local (nasal) and functional antibodies that inhibit IgE-FAB in the nasal mucosa.
- Inhibitory activity against IgE in nasal fluid from SCIT-treated patients correlates more closely with clinical outcome than the inhibitory activity of serum samples, highlighting its potential as a novel biomarker for monitoring treatment effect.
- Grass pollen SCIT induces an increase in IL-10⁺ Breg cells that correlates with the increase in IgG₄ levels.

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ONLINE REPOSITORY METHODS

TITLE: Nasal Allergen Neutralising IgG4 Antibodies Block IgE-mediated Responses: Novel biomarker of Subcutaneous Grass Pollen Immunotherapy

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Supplementary methods

28

29 ***Subjects: inclusion and exclusion criteria***

30 All allergic participants were skin prick test positive (wheal of >3mm) to *Phleum*
31 *pratense* (Phl p) and suffered from moderate-severe grass-pollen induced symptoms
32 for at least two years. Anti-histamine tablets and nasal sprays were excluded at least
33 3 days before sample collection, while topical corticosteroids were excluded for 14
34 days. AIT-treated subjects included those currently on Alutard SQ and having
35 received the treatment for a minimum of 12 months. NA had no history of allergic
36 symptoms and had a negative skin prick test to Phl p and to a panel of eleven
37 common aeroallergens. Participants were excluded if they had a clinical history of
38 other allergic diseases, asthma, chronic-obstructive pulmonary disease (COPD),
39 recurrent acute sinusitis, chronic sinusitis, or other significant illness.

40

41 ***Nasal fluid and serum collection in and out of grass pollen season***

42 Nasal fluid from each sponge was extracted with 75 µL of assay buffer (Millipore
43 Corporation, USA) and centrifuged at 4500 RCF at 4°C for 10 minutes. After
44 weighing tubes to determine fluid volume, nasal fluid samples were stored at -80°C
45 until further use. Serum was isolated from blood by centrifugation at 2200 g for 10
46 minutes.

47

48 ***Inhibition of CD23-Mediated IgE-facilitated allergen binding***

49 IgE-facilitated allergen binding to B cells was performed as previously described.^{16, 17}
50 Briefly, serum from a grass pollen-sensitised donor (Phl p-sIgE >100 kU/L) was
51 incubated with 1 µg/mL of Phl p in the presence or absence of an equal volume of
52 nasal fluid or sera from study patients for 1 hour at 37°C. EBV-transformed B cells

(100,000 cells/test) were added for 1 hour at 4°C and washed. The binding of allergen-IgE complexes to the CD23 receptor on surface of B cells was detected using PE-labelled anti-human IgE antibodies (Miltenyi Biotech, Auburn, CA) and acquired by BD FACS Canto II (BD Biosciences, Rockville, M.D.). The data was analysed using FACS DIVA software (BD Biosciences, Rockville, M.D.). The binding of allergen-IgE complexes to B cells and the inhibition relative to indicator serum from a grass pollen allergic subject was calculated using the formula below:

$$\% \text{ relative allergen IgE complex binding to B cells} = \left(\frac{\% \text{ IgE FAB using indicator and immunotherapy serum}}{\% \text{ IgE FAB indicator serum only}} \right) \times 100$$

IgG depletion from nasal fluids and sera from AIT patients

The protein G spin column was equilibrated with 650 µL of binding buffer A (pH 7.4), centrifuged at 4400 rpm for 1 minute. Sera and nasal fluid samples were diluted 1:1 with binding buffer before applying to the protein G spin column and centrifuged at 2600 rpm for 10 minutes. The column was washed three times with 650 µL of binding buffer to remove any contaminants. The mini plugs were eluted twice, in separate Proteus spin columns, by adding 65 µL of neutralising buffer (pH 9.0) and 500 µL of elution buffer (pH 2.0) on top of the protein G mini plug, centrifuged at 4400 rpm for 1 minute. The IgG depleted samples and elutions were measured for levels of IgG₄ using ImmunoCAP100 system (Phadia, Uppsala, Sweden) according to manufacturer's instructions (Pharmacia, Uppsala, Sweden). Non-allergen-specific IgG₄ standard (ThermoFisher, Paisley, UK) was used as a control.

Measurement of IgA₁/IgA₂ in Nasal Fluid and Serum using ELISA

Plates were coated with 50 µl of desired concentration of Phl p for 1 hour at 37°C. Each well was washed three times using the 0.05% Tween 20 in PBS and

completely dried. Plate was blocked with 1% Bovine Serum Albumin (BSA) in PBS at 37°C for 1 hour, washed five times with wash buffer and dried completely. Standards and serum samples were added into corresponding wells and incubated overnight at 4°C. Following overnight incubation, plates were washed and mouse monoclonal antibodies (mAbs) to IgA₁ (clone B3506) or IgA₂ (clone A9603) were added and incubated for 2 hours at 650 rpm. 100 µl of HRP-conjugated Streptavidin (Biolegend) was added to each well and incubated on a shaker for 30 minutes. Plate was washed and 100 µl of TMB substrate was added and incubated in the dark for 8 minutes, after which 50 µl of stop solution (H₂SO₄) was added. The plates were read at OD₄₅₀ using an ELISA microplate reader (Molecular Probes, Eugene, OR, USA).

Quantification of B regulatory cells

Isolated B cells (1×10^6) were cultured with CpG (1 µg/mL) and CD40L for 48 hours at 37°C, 5% CO₂, and were stimulated with 20 ng/mL PMA (Invitrogen, CA, USA) and 1 µg/mL of Ionomycin (Sigma-Aldrich, MO, USA) followed by Brefeldin A (10 µg/mL) (Biolegend, London, UK). Cells were blocked with FcR blocker (Miltenyi Biotech, Auburn, CA) for 15 minutes in the dark and then surface stained with fluorescent-labelled antibodies: CD5 FITC (Biolegend, London, UK), CD19 Amcyan (BD Biosciences, Devon, UK), CD24 PE (Biolegend, London, UK), and CD38 PE/Cy7 (Biolegend, London, UK). Cells were then incubated for 20 minutes with Cytofix/Perm buffer (BD Biosciences, Devon, UK) and washed with Perm/wash buffer (BD Biosciences, Devon, UK). Cells were immunostained with fluorescence-conjugated antibody IL-10 APC (Biolegend, London, UK) for 30 minutes at 4°C and then washed twice in Perm/wash buffer before being resuspended in cell stain buffer and analysed using BD FACS Canto II instrument.

102

103 ***Memory T cell and Breg co-culture***

104

105 To further assess the effect of IL-10 producing Bregs, isolated CD19⁺ B cells (1 x
106 10⁶) were cultured with/without CpG (1 µg/mL) and CD40L for 48 hours at 37°C, 5%
107 CO₂. After 48hrs, enriched CD4⁺CD45RO⁺ T cells from PBMCs (1 x 10⁶) of SAR
108 subjects were co-cultured at with irradiated antigen-presenting cells (APC, non-CD4⁺
109 PBMCs) and 5µg/mL of Phl p (ALK-Abello, Horsholm, Denmark) in the presence or
110 absence of CpG-primed B cells. Cells were cultured at 37°C, 5% CO₂ for a further 7
111 days. T cell proliferation was determined by tritiated thymidine (³H-thymidine)
112 incorporation. Supernatants from cell cultures were collected to measure the Th2
113 cytokine response by multiplex assay (EMD Millipore).

114

115

Figure S1. Measured Levels of Phl p specific IgA₁ and IgA₂ in SAR and SCIT-treated groups. Levels of Phl p specific IgA₁ in nasal fluid (A) and serum (B) were measured and compared between each group. Also, the levels of Phl p specific IgA₂ was also assessed in Nasal Fluid (C) and Serum (D). All values are expressed as Arbitrary Units (AU), with the horizontal bar representing the Median Value. *P*-values were calculated using the Mann-Whitney *U* test, with *P* < .05 regarded as statistically significant. Phl p, *Phleum pratense*; SAR, Seasonal-allergic rhinitis; SCIT, Subcutaneous Immunotherapy.

Figure S2: Functional IgG₄ blocking antibodies compete with IgE in SCIT-treated subjects. Timothy grass pollen component Phl p 5 specific IgG₄ to IgE ratio was determined in the nasal and serum of SCIT- treated subjects during the grass pollen season. Data are shown as mean ± SEM. *P* values were obtained by two-tailed Mann-Whitney *U* test, where * represent *P* < .05.

Figure S3: Allergen-IgE complexes bound to B cells in SAR and SCIT treated subjects. The IgE-facilitated allergen-IgE complexes binding to CD23 on B cells were measured in (A) nasal fluid and (B) serum from SAR and SCIT- treated subjects over a period of 12 to 24 months. Data are shown as mean ± SEM.

Figure S4: Induction of IgG₄ antibodies. Functional IgG₄ levels in the (A) nasal fluid and (B) serum of SAR and SCIT-treated subjects collected at 12-13, 18 and 24 months as measured by ImmunoSolid Allergen Chip (ISAC) assay. Data are shown as mean ± SEM.

Figure S5: B regulatory cells suppress allergen-induced memory T cells proliferation. (A) CD4⁺CD45RO⁺ T cells enriched from SAR (n=12) were co-cultured with antigen presenting cells and 5 µg/mL of Phl p in the presence of B cells or CpG-primed B cells. Phl p-induced T cell proliferation was measured by ³H thymidine. (B) The effect of neutralizing anti-IL10 monoclonal Ab and isotype control (IC) was evaluated. Data is shown as mean ± SEM. *P* values were obtained by two-tailed Mann-Whitney *U* test, where *, ** and *** represent *P* < .01.

Figure S6: B regulatory cells suppress allergen-induced IL-5 and IL13. (A) CD4⁺CD45RO⁺ T cells enriched from SAR (n=12) were co-cultured with antigen presenting cells and 5 µg/mL of Phl p in the presence of B cells or CpG-primed B cells. IL-5 and IL-13 were measured in cell culture supernatants by magpix multiplex cytokine assay. Data is shown as mean ± SEM. *P* values were obtained by two-tailed Mann-Whitney *U* test, where *** represent *P* < .001.

Figure S7: IL-10⁺ B regulatory cells were induced following SCIT. Flow cytometry analysis to enumerate (A) IL-10⁺ B cells as a percentage total of CD19⁺CD5^{hi} Bregs (n=12) and (B) IL-10⁺ B cells as a percentage total of CD24^{hi}CD38^{hi} Bregs. Orange bar represents SAR while green bars represent SCIT-treated subjects at 12-13, 18 and 24 months. Data is shown as mean ± SEM. *P* values were obtained by two-tailed Mann-Whitney *U* test.

ONLINE REPOSITORY TABLES

TITLE: Nasal Allergen Specific IgG₄ antibodies with Neutralising Capacity to Block IgE-mediated Responses: A Novel biomarker of Subcutaneous Grass Pollen Immunotherapy Evaluated in a Cross-Sectional Pilot Study.

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Table E1: Activities of SAR and SCIT subjects

Activities	SAR			SCIT			SCIT vs SAR (P value)
	In season	Out season	I - O	In season	Out season	I - O	
Impaired Regular Activities at Home and at Work	1.85	0.27	1.62	0.78	0.3	0.4	0.0297
Increased Recreational Activities	1.92	0.091	1.85	0.67	0.4	0.2	0.0045
Sleep deprivation	1.54	0.091	1.46	1.00	0.4	0.5	0.2495
Need to rub nose	2.69	0.636	2.15	1.78	0.3	1.3	0.1711
Need to Blow Nose Repeatedly	2.31	0.182	2.15	1.56	0.7	0.7	2.154
Sneezing	1.92	0.545	1.46	1.33	0.3	0.9	0.225
Stuffy/Blocked Nose	2.38	0.545	1.92	2.00	1	0.8	0.1607
Runny Nose	2.00	0.182	1.85	1.11	0.7	0.3	0.0109
Itchy Eyes	1.69	1.364	0.54	1.78	0.2	1.4	0.6755
Sore Eyes	1.46	0.091	1.39	0.33	0	0.3	0.0221
Watering Eyes	1.62	0.364	1.31	0.67	0	0.6	0.1834
Tiredness and/or Fatigue	2.15	0.727	1.54	1.33	0.4	0.8	0.1632
Thirst	1.54	0.182	1.39	0.44	0.1	0.3	0.108
Feeling Irritable	2.08	0.182	1.92	1.11	0.3	0.7	0.1052

I-O: In Season – Out Season

Mann-Whitney Test, P<0.05 was considered statistically significant

Table E2. Mini Rhinoconjunctivitis Quality of Life Questionnaire

Mini-RQLQ © QOL Technologies Ltd.

Please complete all questions by circling the number that best describes how troubled you have been during the last week as a result of your nose/eye symptoms.

	Not troubled	Hardly troubled at all	Somewhat troubled	Moderately troubled	Quite a bit troubled	Very troubled	Extremely troubled
ACTIVITIES							
1. REGULAR ACTIVITIES AT HOME AND AT WORK (your occupation or tasks that you have to do regularly around your home and/or garden)	0	1	2	3	4	5	6
2. RECREATIONAL ACTIVITIES (indoor and outdoor activities with friends and family, sports, social activities, hobbies)	0	1	2	3	4	5	6
3. SLEEP (difficulties getting a good nights sleep and/or getting to sleep at night)	0	1	2	3	4	5	6

PRACTICAL PROBLEMS

4. NEED TO RUB NOSE/EYES	0	1	2	3	4	5	6
5. NEED TO BLOW NOSE REPEATEDLY	0	1	2	3	4	5	6

Not troubled	Hardly troubled at all	Somewhat troubled	Moderately troubled	Quite a bit troubled	Very troubled	Extremely troubled
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NOSE SYMPTOMS

6. SNEEZING	0	1	2	3	4	5	6
7. STUFFY/BLOCKED NOSE	0	1	2	3	4	5	6
8. RUNNY NOSE	0	1	2	3	4	5	6

EYE SYMPTOMS

9. ITCHY EYES	0	1	2	3	4	5	6
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10. SORE EYES	0	1	2	3	4	5	6
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11. WATERING EYES	0	1	2	3	4	5	6
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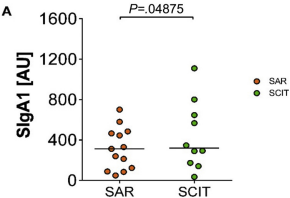
OTHER SYMPTOMS

12. TIREDNESS AND/OR FATIGUE	0	1	2	3	4	5	6
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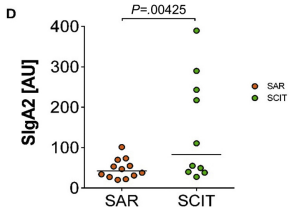
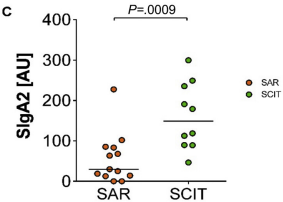
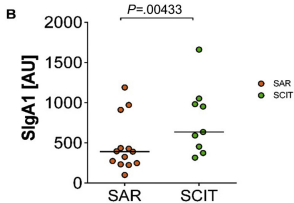
13. THIRST	0	1	2	3	4	5	6
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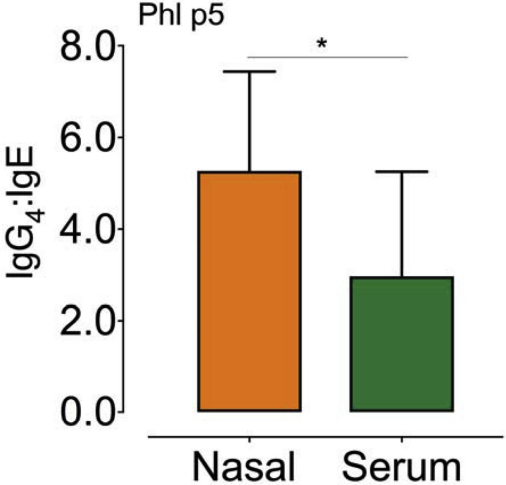
14. FEELING IRRITABLE	0	1	2	3	4	5	6
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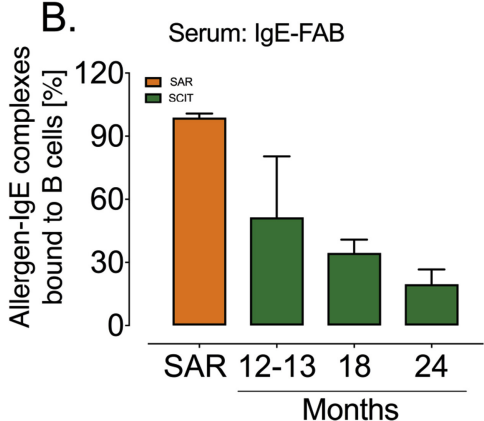
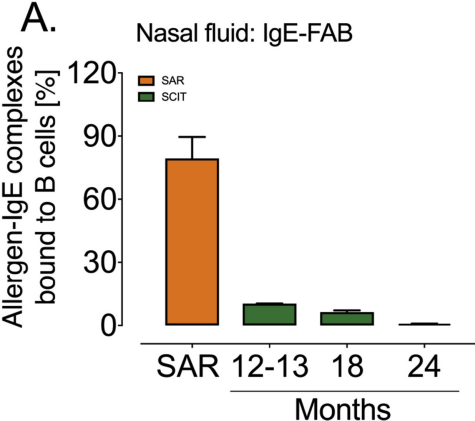
Nasal Fluid



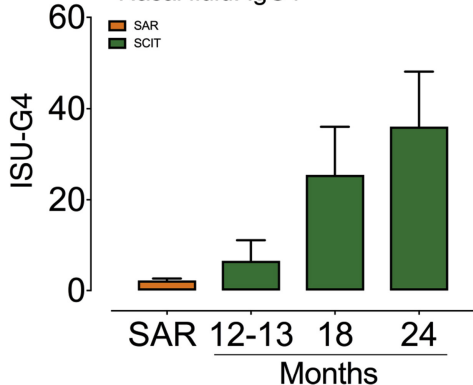
Serum



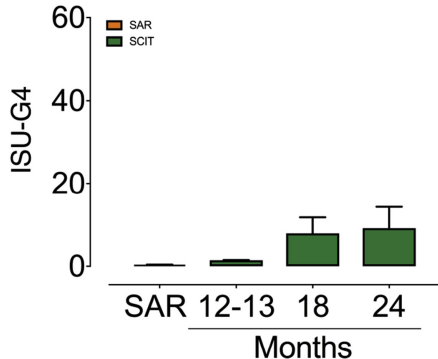




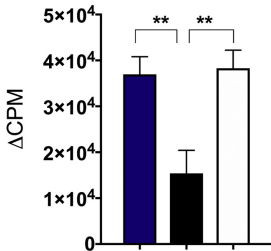
A. Nasal fluid: IgG4



B. Serum: IgG4

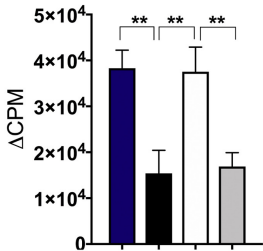


A.



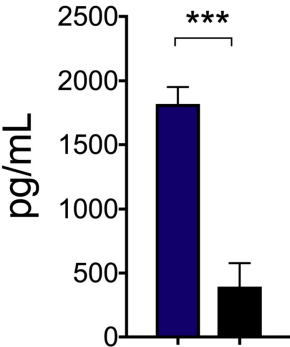
CD4 ⁺ CD45RO ⁺ T cells	+	+	+
APC	+	+	+
Phl p [5 $\mu\text{g/mL}$]	+	+	+
CpG-primed B cells	-	+	-
B cells	-	-	+

B.



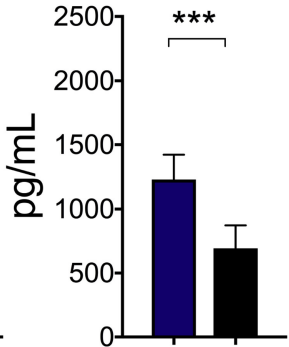
CD4 ⁺ CD45RO ⁺ T cells	+	+	+	+
APC	+	+	+	+
Phl p [5 $\mu\text{g/mL}$]	+	+	+	+
CpG-primed B cells	-	+	+	+
B cells	+	-	-	-
α -IL-10 [10 $\mu\text{g/mL}$]	-	-	+	-
IC [10 $\mu\text{g/mL}$]	-	-	-	+

IL-5



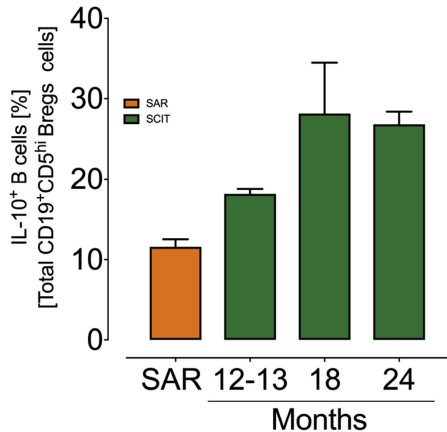
B cells

IL-13



CpG-primed B cells

A.



B.

