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Differential induction of allergen-specific IgA responses following timothy grass subcutaneous and sublingual immunotherapy

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Abstract

Introduction: There is no detailed comparison of allergen-specific immunoglobulin responses following sublingual immunotherapy (SLIT) and subcutaneous immunotherapy (SCIT).

Objective: We sought to compare nasal and systemic timothy grass pollen (TGP)-specific antibody responses during 2 years of SCIT and SLIT and 1 year after treatment discontinuation in a double-blind, double-dummy, placebo-controlled trial.

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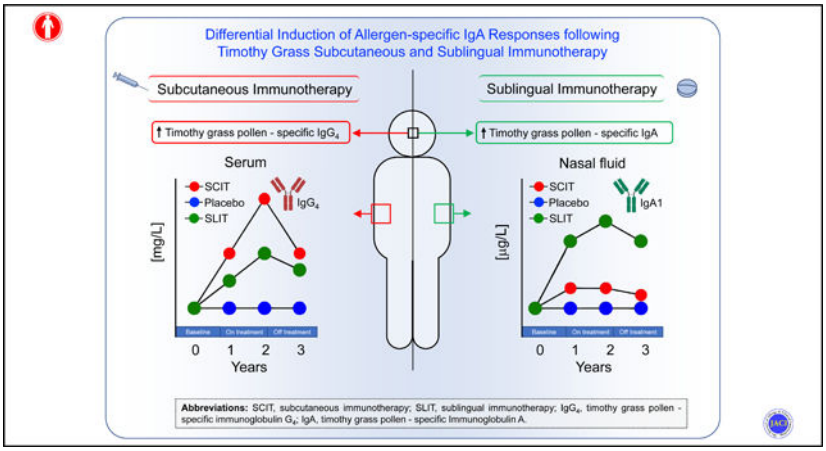
Methods: Nasal fluid and serum were obtained yearly (per-protocol population, n = 84). TGP-specific IgA_1, IgA_2, IgG_4, IgG, and IgE were measured in nasal fluids by ELISA. TGP-specific IgA_1, IgA_2, and *Phleum pratense* (Phl p)1, 2, 4, 5b, 6, 7, 11, and 12 IgE and IgG_4 were measured in sera by ELISA and ImmunoCAP, respectively.

Results: At years 2 and 3, TGP-$IgA_{1/2}$ levels in nasal fluid were elevated in SLIT compared with SCIT (4.2- and 3.0-fold for IgA_1, 2.0- and 1.8-fold for IgA_2, respectively; all $P < .01$). TGP-IgA_1 level in serum was elevated in SLIT compared with SCIT at years 1, 2, and 3 (4.6-, 5.1-, and 4.7-fold, respectively; all $P < .001$). Serum TGP-IgG level was higher in SCIT compared with SLIT (2.8-fold) at year 2. Serum TGP-IgG_4 level was higher in SCIT compared with SLIT at years 1, 2, and 3 (10.4-, 27.4-, and 5.1-fold, respectively; all $P < .01$). Serum IgG_4 levels to Phl p1, 2, 5b, and 6 were increased at years 1, 2, and 3 in SCIT and SLIT compared with placebo (Phl p1: 11.8- and 3.9-fold; Phl p2: 31.6- and 4.4-fold; Phl p5b: 135.5- and 5.3-fold; Phl p6: 145.4- and 14.7-fold, respectively, all at year 2 when levels peaked; $P < .05$). IgE to TGP in nasal fluid increased in the SLIT group at year 2 but not at year 3 compared with SCIT (2.8-fold; $P = .04$) and placebo (3.1-fold; $P = .02$). IgA to TGP and IgE and IgG_4 to TGP components stratified participants according to treatment group and clinical response.

Conclusions: The observed induction of $IgA_{1/2}$ in SLIT and IgG_4 in SCIT suggest key differences in the mechanisms of action.

Graphical Abstract



Keywords

Sublingual immunotherapy; subcutaneous immunotherapy; IgE-FAB; blocking antibodies; IgG_4; IgA_1; IgA_2; allergic rhinitis

Allergen immunotherapy (AIT) is currently the only disease-modifying treatment for allergic rhinitis, an IgE-mediated disease affecting up to 20% to 30% of adults and up to 40% of children.[1] Two routes of administration of AIT used clinically are subcutaneous (subcutaneous immunotherapy [SCIT]) and sublingual (sublingual immunotherapy [SLIT]). SCIT involves weekly updosing injections, which are then followed by monthly maintenance injections for at least 3 years,[2] whereas SLIT is self-administered and involves daily drops or tablets placed under the tongue for the same duration of 3 years.[3] SCIT[4,5] and

SLIT[6,7] are both disease-modifying and effective treatments in reducing clinical symptoms during the timothy grass pollen (TGP) season and the overall need of rescue medications.

After a 3-year course of AIT ends, long-term clinical benefit persists for at least 2 to 3 years.[5,6] However, we recently reported that, in a 2-year AIT study of both SCIT and SLIT, the Gauging Response in Allergic Rhinitis to Sublingual and Subcutaneous Immunotherapy (GRASS) trial, no difference in the nasal response to allergen challenge was observed in either SCIT- or SLIT-treated group compared with the placebo group, 1 year after cessation of treatment, indicating that 2 years of immunotherapy was not sufficient to induce sustained clinical tolerance.[8]

AIT confers its clinical effect through several mechanisms including immune deviation of T_H2-cell response toward a T_H1-cell response, suppression or deletion of T_H2 cells,[9–13] induction of regulatory T and B cells,[14,15] and induction of IgG-blocking antibodies, in particular IgG_4.[16–18] However, a detailed examination of the effect of SCIT and SLIT on a wider spectrum of humoral responses, such as nasal and systemic IgA_1, IgA_2, IgG, and IgG_4 to AIT allergens and their molecular components has not been conducted. Assessing the effect of AIT on IgA is of interest because IgA is a mucosal antibody likely to play a central role in binding allergens on environmental exposure; as such, it could reduce allergen contact with mucosal and submucosal mast cell–bound IgE.[19]

Using samples from the GRASS trial, we herein report for the first time differential induction of allergen-specific IgA responses following SLIT, compared with SCIT, in serum and nasal fluids. Moreover, we conducted an unsupervised cluster analysis of biomarkers to identify whether immunoglobulins measured after 1 year of treatment, in conjunction with other immunologic parameters, could stratify subjects according to treatment group and treatment response at year 2.

METHODS

Subjects

The GRASS study was a randomized, double-blind, placebo-controlled, double-dummy, single-center trial conducted over 4 years. The details of the immunotherapy protocol and clinical outcome measures, including symptoms and rescue medication scores, have been previously reported.[8,11] Briefly, subcutaneous alum-adsorbed TGP immunotherapy (Alutard SQ Grass Pollen; ALK-Abelló, Hørsholm, Denmark) or matched placebo subcutaneous injections were given weekly for 15 weeks (updosing), followed by monthly maintenance injections for 2 years. TGP (*Phleum pretense* [Phl p], 15 μg) sublingual tablets (Grazax; ALK-Abelló) or matched placebo sublingual tablets were self-administered daily for 2 years. A 1:1:1 randomization (SCIT:-SLIT:placebo) strategy was used (see Fig E1 in this article's Online Repository at www.jacionline.org). All study participants were allergic to grass pollen, and individuals allergic to other seasonal allergens, such as birch pollen and alternaria, were excluded from the study.

Nasal allergen challenge and collection of nasal fluid and serum

Nasal fluid was collected from a single foam sponge (Zuschnitt Schaumst-off RG27 grau, Gummi-Welz GmbH & Co, Neu-Ulm, Germany) inserted into each nostril under direct vision. Sponges were then placed above a microfilter within a Spin-X 0.22-μm centrifuge tube (Corning Costar, Dorset, UK) and kept briefly on ice. Nasal fluid from each sponge was extracted by adding 75 μL of assay buffer (Millipore Corporation) and centrifuged at 4500 RCF at 4°C for 10 minutes. Supernatants were stored at −80°C until assayed. Serum was isolated from whole blood by centrifugation at 2200*g* for 10 minutes and stored at −80°C. Serum and nasal fluid, obtained for immunologic assessments, were collected outside the grass pollen season (between October and March each year), on the day of the nasal allergen challenge (NAC). Serum was collected before NAC, whereas nasal fluid was collected during the NAC.

Immunoglobulin measurements and inhibition of CD23-mediated IgE-facilitated allergen binding

TGP-specific IgA_1, IgA_2, IgG, IgG_4, and IgE were measured in nasal fluid collected at out-of-season NAC visits (before the challenge) by an in-house optimized and validated ELISA (see this article's Methods section in the Online Repository at www.jacionline.org). Specific IgE and IgG_4 to TGP and TGP components (Phl p1, 2, 4, 5b, 6, 7, 11, 12) as well as IgA_1 and IgA_2 to TGP extract were measured in prechallenge out-of-season sera by the ImmunoCAP system (Phadia US Inc, Portage, Mich) according to manufacturer's instructions and ELISA, respectively. Inhibition of IgE-facilitated allergen binding to B cells was measured as previously described.[16,17] Further details can be found in this article's Methods section in the Online Repository.

Statistical analysis

All immunologic data were assessed in the per-protocol (PP) population using a linear mixed model adjusted for baseline values. The PP population included participants who remained in the study at least 3 years and were compliant with study medications (defined as taking 50% or more of their study medication for the duration of the study), and who had an evaluable primary end point. Missing baseline serum (8 of 84) IgE and IgG_4 antibodies to TGP were imputed on the basis of corresponding antibody levels to TGP components. Serum antibodies to TGP and TGP components (*Phl p1, Phl p2, Phl p4, Phl p5b, Phl p6, Phl p7, Phl p11, Phl p12*) have a joint multivariate normal distribution. Therefore, a Markov Chain Monte-Carlo method was used to impute missing baseline serum TGP-specific IgE and IgG_4 antibody data by drawing on the joint multivariate normal distribution. Nasal challenge–induced total nasal symptom score (TNSS) area under curve (AUC) data were reanalyzed in the PP population using an Analysis of covariance (ANCOVA) model adjusted for baseline AUC. To determine how many clusters were present in the study cohort, a complete lineage hierarchical clustering method was used with an agglomerative approach (bottom-up). Participants were categorized as "Nonresponders," "Partial Responders," or "Responders" on the basis of changes in TNSS from baseline to year 2. Non-responders had less than 10% reduction in TNSS from baseline to year 2, Partial Responders had 10% to 40% reduction in TNSS from baseline to year 2, and Responders had more than

40% reduction in TNSS from baseline to year 2. The threshold for significance was *P* less than .05 (2-sided). Because all analyses were considered exploratory, *P* values were not adjusted for multiple comparisons. All analyses were performed with SAS, version 9.4 (SAS Institute, Inc, Cary, NC) and R version 3.2.4 (R Foundation for Statistical Computing). Data are accessible through TrialShare (the Immune Tolerance Network repository) at https://www.itntrialshare.org/GRASS_antibody.url.

RESULTS

Participant characteristics

Table I summarizes demographic and clinical characteristics of the 84 PP GRASS study participants. Age, sex, and ethnicity were not significantly different between the 3 treatment groups. On the basis of grass component IgE, and with sensitization defined as IgE more than 0.7 kU/L, most participants were sensitized at baseline to Phl p1 (97.6%), Phl p2 (78%), Phl p4 (82.9%), Phl p5b (91.5%), and Phl p6 (90.2%). No significant baseline differences in skin prick test wheal sizes, total IgE, or IgE to timothy grass components were observed between treatment groups.

Total nasal symptom scores

As previously reported for the intent-to-treat population,[8] baseline TNSSs were obtained from an NAC performed between October and March, before the start of the treatment. There were no significant differences in the PP population between treatment groups in the baseline NAC-induced TNSS. At year 1, TNSS was lower in the SCIT-treated group compared with placebo, whereas a trend for lower TNSS was observed in the SLIT-treated group. After the 2 years of active treatment, the NAC-induced TNSS was lower in both SCIT and SLIT groups compared with placebo (SCIT: $P < .001$; SLIT: $P = .001$). However, neither SCIT nor SLIT treatment led to persistent clinical improvement as the TNSS increased at year 3, 1 year off treatment, with no difference from placebo (Fig 1, https://www.itntrialshare.org/GRASS_antibody_fig1.url).

Induction of local and systemic IgA_1 and IgA_2 to timothy grass following SLIT, SCIT, or placebo

Within the 3 treatment groups, only SLIT participants had increased levels of TGP-specific IgA_1 and IgA_2 in nasal fluid at years 2 (3.35- and 2.72-fold over baseline, respectively, $P < .001$) and 3 (3.37- and 2.77-fold over baseline, respectively; $P < .001$). As a result, levels of TGP-specific IgA_1 and IgA_2 were significantly higher in nasal fluids from the SLIT group compared with the placebo group or the SCIT group at years 2 (all, $P < .01$) and 3 (all, $P < .01$) (Fig 2, A and B; see Table E1 in this article's Online Repository at www.jacionline.org; https://www.itntrialshare.org/GRASS_antibody_fig2.url). Year 1 specimens were not assayed because of low sample volumes.

Serum TGP-specific IgA_1 levels were increased in the SLIT-treated group at years 1, 2, and 3 compared with baseline (4.96-, 7.02-, and 2.89-fold, respectively, all $P < .001$) (Fig 2, *C*; Table E1). However, serum IgA_1 levels declined in the SLIT-treated group at year 3 compared with year 2 ($P < .001$). Serum TGP-specific IgA_1 levels were not increased in

the SCIT-treated group at years 1, 2, or 3, compared with baseline. Similarly, no changes in serum IgA_1 levels were observed in the placebo group. The SLIT group serum TGP-specific IgA_1 levels were higher than the placebo- and SCIT-treated groups levels at year 1 (all, $P <$ .001), year 2 (all, $P < .001$), and year 3 (all, $P < .001$) (Fig 2, *C*).

Serum TGP-specific IgA_2 levels were increased in the SCIT- and SLIT-treated groups at year 1 (all, $P <.01$) and year 2 (all, $P <.05$) compared with baseline. No significant change in serum IgA_2 levels from baseline were observed in placebo-treated participants. The SLIT group was not statistically different from placebo or from SCIT at any of the 3 study years. SCIT resulted in significantly higher serum IgA_2 compared with placebo, but only in year 1 (Fig 2, *D*).

We examined whether the clinical effect of AIT on nasal allergen-induced symptoms was associated with changes in nasal fluid or serum IgA. Fig 3 presents the relationships between AIT-induced log_{10}-fold change in nasal TGP-specific IgA_1 and log_{10}-fold changes in nasal allergen challenge–induced TNSS from baseline to year 2. We found a significant, albeit modest, correlation between nasal fluid IgA_1 and TNSS after SLIT, but not SCIT or placebo (Pearson $r = -0.52$; $P = .006$). No significant relationships between changes in TNSS and serum IgA_1 (Fig 3, *A* and *B*; https://www.itntrialshare.org/GRASS_antibody_fig3.url), serum IgA_2 or nasal fluid IgA_2 were observed (see Fig E2 in this article's Online Repository at www.jacionline.org). We further confirmed this observation by applying a linear mixed-effect model, with TNSS AUC as the dependent variable. When only 1 biomarker was included at a time within the model along with visit, treatment, and visit treatment interaction, nasal IgA_1 and IgA_2 level was found significant, indicating that the level of nasal IgA_1 and IgA_2 had an effect on TNSS AUC in the expected direction, confirming our earlier observations (see Table E2 in this article's Online Repository at www.jacionline.org).

Nasal fluid and serum IgG, IgG_4, and IgE to grass pollen following SLIT, SCIT, or placebo

TGP-specific IgG in nasal fluid and serum was elicited by both SCIT and SLIT. The levels in nasal fluid were increased from baseline at year 2 in both SCIT and SLIT groups ($P <$ .05) and at year 3 in the SLIT group ($P < .05$) (Fig 4, *A*). Similarly, serum TGP-specific IgG levels were increased from baseline at years 2 ($P < .001$) and 3 ($P < .001$) in both SCIT and SLIT groups (Fig 4, *B*). Levels of GP-specific IgG_4 in nasal fluids increased from baseline for the SLIT and SCIT groups at years 2 and 3 ($P < .001$), (Fig 4, *C*; see Table E3 in this article's Online Repository at www.jacionline.org). Similarly, serum IgG_4 to TGP increased from baseline in both SLIT and SCIT groups (Fig 4, *D*; Table E3). A decline in IgG (87%, SCIT, $P < .001$; 16%, SLIT, $P = .7$) and IgG_4 (84%, SCIT, $P = .001$; 49%, SLIT, $P = .16$) was observed in nasal fluids at year 3 compared with year 2. The observations in nasal fluids were paralleled by serum IgG (68%, SCIT, $P < .001$; 45%, SLIT, $P = .01$) and IgG_4 (92%, SCIT, $P < .001$; 57%, SLIT, $P < .001$) at year 3 compared with year 2.

In nasal fluids, levels of IgG_4 to timothy grass were significantly higher in the SCIT group at year 2 compared with the SLIT group ($P < .05$). In serum, levels of both IgG and IgG_4 were significantly higher in the SCIT group compared with the SLIT group at year 2 ($P < .01$). This differential treatment effect persisted for IgG_4 in serum at year 3.

TGP-specific nasal fluid IgE level was higher in SLIT compared with SCIT (2.76-fold, $P = .04$) and placebo groups (3.07-fold, $P = .02$) at year 2, but not at year 3 (Fig 4, *E*; see Table E3). This differential treatment effect on IgE levels in nasal fluids was paralleled in serum (Fig 4, *F*; Table E3; https://www.itntrialshare.org/GRASS_antibody_fig4.url). Moreover, longitudinal changes in serum IgG_4 and IgE to Phl p1, 2, 4, 5b, 6, 7, 11, and 12 following SLIT, SCIT, or placebo were observed (see Figs E3 and E4 and Tables E4 and E5 in this article's Online Repository at www.jacionline.org). Further details on this observation can be found in this article's Results section in the Online Repository at www.jacionline.org.

Inhibitory activity against *in vitro* allergen-IgE complex binding to B cells

The allergen-IgE binding to B cells as measured by the IgE-facilitated allergen binding assay using nasal fluid was similar at baseline in SLIT-, SCIT-, and placebo-treated patients. At year 2, allergen-IgE complex binding to B cells was lower in SLIT (62.9%, $P < .01$) and SCIT (56.5%, $P < .05$) groups compared with the placebo group (Fig 5, *A*), indicating treatment-induced, blocking antibody activity in nasal fluids. At year 3, the reduction in allergen-IgE complexes binding to B cells persisted in SLIT (61.2%, $P < .01$) compared with placebo, but not in SCIT ($P = .21$). Serum from both SCIT- and SLIT-treated groups blocked allergen-IgE complexes from binding to B cells at years 2 and 3, when compared with placebo, as previously reported[10,16] (Fig 5, *B*; see Table E6 in this article's Online Repository at www.jacionline.org; https://www.itntrialshare.org/GRASS_antibody_fig5.url).

Relationship between local and systemic grass pollen–specific antibody responses to clinical response

We next explored whether relationships between clinical outcomes and biomarkers were present. We found that biomarkers after 1 year of AIT treatment were related to the 2-year treatment outcomes. We performed hierarchical clustering using the $\log_{10}$-fold changes in variables from baseline to year 1 and an agglomerative approach (bottom-up). We first used a set of already published biomarkers from the same study that included early- and late-phase skin reactions,[8] basophil activation test,[11] serum IgE-facilitated allergen binding data, tetramer data,[11] and serum IgE and IgG_4 data. Hierarchical clustering of these biomarkers allowed for relatively good distinction between placebo and active treatment, but less distinction between SCIT and SLIT (Fig 6, *A*). When serum IgA data and serum component IgE and IgG_4 data were added to the analysis, the ability to cluster study participants by treatment and response to treatment was greatly improved (Fig 6, *B*). The dendogram in Fig 6, *B* shows that study participants could be separated into 3 clusters. One cluster contained all 7 participants who had less than 10% TNSS reduction from baseline to year 2. In general, these participants produced less serum IgA to grass extract, and less IgG_4 and IgE to grass components from baseline to year 1. Fourteen of the 18 members of this cluster belonged to the placebo arm. In contrast, the other 2 clusters contained the majority of participants who had more than 40% TNSS reduction from baseline to year 2. These 2 clusters had increased serum IgA to grass extract, and more IgG_4 and IgE to grass components from baseline to year 1 (Fig 6, *A* and *B*; https://www.itntrialshare.org/GRASS_antibody_fig6.url). Of all the participants in the second cluster, 0 were SCIT recipients, whereas 9 of the 11 participants in the third cluster received SCIT. All the participants in the second cluster (9 of 9), but only 2 of the 11 participants in the third cluster, received SLIT.

DISCUSSION

Here, we report a detailed longitudinal assessment of nasal and systemic antibody responses during sublingual and subcutaneous grass pollen immunotherapy. IgA_1 and IgA_2 antibody induction was higher in SLIT, compared with SCIT and placebo. This effect was most prominent in nasal fluids. In contrast, IgG and IgG_4 antibodies both in nasal fluids and in the circulation were greater in SCIT, compared with SLIT and with placebo. Moreover, adding serum IgA, IgG_4, and IgE measurements, taken 1 year after initiation of AIT, to an unbiased biomarker cluster analysis enabled participants to be relatively well stratified on the basis of treatment they received and also identified subjects who did not respond to treatment.

AIT treatment has been previously associated with the induction of a serum allergen-specific IgA_2 antibody response.[20] Increases in salivary concentrations of grass pollen–specific total IgA have also been observed in children following 3-year grass pollen SLIT that persisted for 2 years after treatment cessation.[21] Our study extends these observations by directly comparing local nasal and systemic IgA_1 and IgA_2 concentrations following grass pollen SCIT and SLIT. Elevated IgA in SLIT was observed during immunotherapy treatment and persisted even after discontinuation of treatment. Changes in allergen-induced symptoms and changes in the level of nasal fluid IgA_1 showed a significant inverse correlation in the SLIT arm, but not in SCIT or placebo, suggesting that IgA induced by SLIT may be related to clinical improvements observed during therapy. However, this correlation was relatively modest and, in year 3, after discontinuation of treatment, nasal fluid IgA_1 and IgA_2 remained elevated, whereas allergen-induced nasal symptoms did not differ between SLIT and placebo.

IgA produced during grass pollen immunotherapy may participate in blocking allergen from binding IgE receptors. The blocking capacity of IgA antibody has been previously reported in a study in which ragweed-specific IgA from nasal secretions was shown to inhibit basophil histamine release.[22] Here, we show that, compared with baseline, nasal fluids collected after 2 years of treatment had increased blocking activity against IgE-allergen complex binding to B cells. It is notable that the magnitude of this effect was equal between SLIT and SCIT. However, SLIT, compared with SCIT, had lower levels of nasal fluid IgG_4, the conventional “blocking antibody” subclass, and higher levels of IgA_1 and IgA_2. This is compatible with a hypothesis that IgA may also play the role of a “blocking antibody” in nasal fluids. This notion is further supported by the experiments with serum in which the differences between SLIT- and SCIT-induced IgA_1 versus IgG_4 were even more striking, but the blocking activity of serum against IgE-allergen complex binding to B cells was no different. Our data underscore that SCIT and SLIT likely involve differences in the mechanism of action, with IgG_4 playing a predominant role in SCIT and IgA in SLIT.

The addition of serum IgA, as well as serum grass component IgE and IgG_4, to various other parameters, as part of an exploratory cluster analysis of the changes from baseline to year 1, yielded 3 clusters that largely separated participants according to the treatment they received (placebo, SLIT, SCIT). This suggests that differences in the production of IgA, IgE, and IgG_4 antibodies are defining characteristics of SCIT and SLIT treatment. Including the baseline to year 1 changes in these data sets (https://www.itntrialshare.org/

GRASS_antibody.url) also allowed for the identification of participants who responded to therapy at year 2 and clustered all participants who did not respond to therapy into a separate group, suggesting that treatment efficacy may involve various aspects of the immune response and that it may be possible to identify patients early in treatment who might benefit from adjunct therapy. Furthermore, because SLIT and SCIT may act by distinct mechanisms, this raises the question whether it may be helpful to combine the 2 therapies in treatment-resistant cases. This would require testing in an adequately powered controlled comparative clinical trial.

Local antibody responses are reflective of immunologic and clinical responses to individual allergen components. However, because of limitation in the amount of nasal fluid that was obtained in this study, only IgE, IgG, IgG_4, IgA_1, and IgA_2 measurements to the allergen extract, and not to individual grass pollen components, were assessed in this study. Although it is possible to use an assay such as ImmunoCAP solid-phase allergen chip technology to measure reactivity against individual allergen components in nasal fluids, studies have shown that allergen-specific IgG_4 may compete with specific IgE for allergen binding within the assay. This may therefore underestimate the levels of specific IgE obtained by Immuno Solid Allergen Chip.[23] Further to this, the baseline nasal fluid and serum samples measured in this study were collected at the end of the grass pollen season. For this reason, it would be challenging to derive any observations on the blunting of seasonal IgE increases in the local target organ or within the circulation.

During the GRASS trial, peak pollen count was found within study year 2. Despite this peak, each treatment group was exposed to similar levels of pollen because study participants were randomized into 1 of 3 treatment arms at the start of the study, indicating that differences in antibody levels between treatment groups are not due to differences to pollen exposure. In addition, when looking at antibody trends within the placebo group, there were no consistent increases between year 1 and 2, and no consistent decrease between years 2 and 3, highlighting that exposure to pollen has minimal priming effect on the out-of-season time points used for antibody measurements.

Despite our speculation that IgA may act as a blocking antibody, we have no direct evidence of this. Our data suggest that there is an interaction between IgA_1, IgA_2, IgG_4, and IgE, but the nature of this interaction is not clear. Shared repertoires between IgG and IgE have been illustrated previously in a murine study[24] and more recently, in a human study involving subjects with and without allergies.[25,26] Similar analyses for IgA may be worth pursuing to help determine whether IgA induced by SLIT is capable of inhibiting IgE-mediated immune responses. However, this was beyond the scope of this study.

Conclusions

We have identified that production of IgA is a major biological difference between SLIT and SCIT. Although, as expected, SCIT induced higher specific IgG_4 levels than SLIT, SLIT led to higher IgA levels, both in serum and in nasal fluid. Furthermore, the levels of IgA_1 in nasal fluids correlated with SLIT's suppression of nasal symptoms during NAC. Specific

IgA antibody production may therefore represent a distinct mechanism through which SLIT exerts its therapeutic effects, which needs to be further investigated.

METHODS

Measurement of IgA_1/IgA_2 in nasal fluid and serum using ELISA

Plates were coated with 50 μL of Phl p (5 μg/mL) for 1 hour at 37C. Wells were washed 3 times using 0.05% Tween 20 in PBS, dried, and blocked using 1% BSA in PBS at 37C for 1 hour. Plates were washed with wash buffer and dried completely. Standards and serum samples were added into corresponding wells and incubated overnight at 4°C. Following overnight incubation at 4°C, the plates were washed and incubated with mAbs to IgA_1 or IgA_2 (Abcam, Cambridge, UK) for 30 minutes. Plates were washed, dried, and 100 μL of horseradish peroxidase–conjugated streptavidin (Biolegend, London, UK) was added to each well and incubated on a shaker for 30 minutes. After washing, TMB substrate was added in the dark for 8 minutes and 50 μL stopping solution (H_2SO_4) was added. Plates were read at OD_{450} using an ELISA microplate reader (Molecular Probes, Eugene, Ore). The anti-IgA_1 antibody binds to the Fc portion of the heavy chain of human IgA_1. Meanwhile, anti-IgA_2 antibody binds to the Fc portion of the heavy chain of human IgA_2 and can detect both monomeric and dimeric IgA_2.

Measurement of nasal IgG, nasal IgG_4, and serum IgG by ELISA

Plates were coated with 50 μL Phl p (5 μg/mL) diluted in bicarbonate-coating buffer at 37C for 1 hour. Wells were washed 3 times with 0.05% Tween 20 in PBS, dried, and blocked using 1% BSA (Sigma, Gillingham, United Kingdom) in PBS at 37C for 1 hour. Plates were washed, dried, and SCIT serum added in serial dilutions (1:1) with PBS to standard wells. Samples were added 1:1000 (for IgG) or 1:3.3 (for IgG_4) dilution with PBS to corresponding wells. The concentration of the standard serum was determined using ImmunoCAP as per manufacturer's instructions. Following overnight incubation at 4C, the plates were washed and incubated with polyclonal antibody IgG (Abcam, Cambridge, United Kingdom, 1:100,000) or mAb IgG_4 (Sigma, 1:60,000) for 30 minutes. Plates were washed and dried, and 100 μL horseradish peroxidase–conjugated streptavidin (Biolegend, London, United Kingdom, 1:000) added for 30 minutes. After washing, TMB substrate was added in the dark for 8 minutes (for IgG) or 12 minutes (for IgG_4) and stopping solution (1 mol H_2SO_4) was added. Wells were read at 450 nm using an ELISA microplate reader (Molecular Probes).

Immunoglobulin measurements in serum by ImmunoCAP

Total IgE, specific IgE, and specific IgG_4 in patient serum and SCIT sera used for ELISA standards were measured by ImmunoCAP 100 system (Phadia, Uppsala, Sweden) according to manufacturer's instructions.

IgE-facilitated antigen binding

Highly expressing CD23 B cells were transformed by the EBV and maintained as previously described.[E1] Sera from patients with allergy with high specific IgE to grass pollen (Phl p–specific IgE >100 kU_A/L) were pooled and used as indicator serum. Patient nasal fluid or

sera was added in equal volumes to indictor serum to test inhibition of facilitated antigen binding. RPMI with L-glutamine (Life Technologies, Paisley, United Kingdom) was added to indicator serum and B cells as a control to measure percentage change from baseline. Allergen-IgE complexes were formed by adding 0.3 μg/mL of *P pratense* at 37°C, 5% CO_2 for 1 hour, and EBV-transformed B cells were added at 100,000 cells/5 μL for a further 30 minutes on the shaker. Cells were washed twice with FAB buffer (138.6 mM NaCl, 1.3 mM NaH_2PO_4, 6.9 mM Na_2HPO_4, 0.1% BSA), and polyclonal human anti-IgE PE-labeled antibody (Miltenyi Biotech, Auburn, Calif; 1:30) was added for 30 minutes on the shaker at room temperature. Monoclonal mouse IgG_1 PE-labeled antibody (Miltenyi Biotech; 1:20) was used as an isotype control for gating and was added to B cells incubated with RPMI alone. Cells were washed, fixed with BD CellFix (BD Biosciences, Rockville, Md; 1:10), and acquired using BD FACS Canto II flow cytometer (BD Biosciences). Analysis was done using FACS DIVA software (BD Biosciences) by gating for B cells using forward and side scatter, and a positive gate set using the isotype control.

The percentage of allergen-IgE complexes bound to B cells from indicator serum was compared with the addition of patient nasal fluid or sera, and the inhibition calculated using the following formula:

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

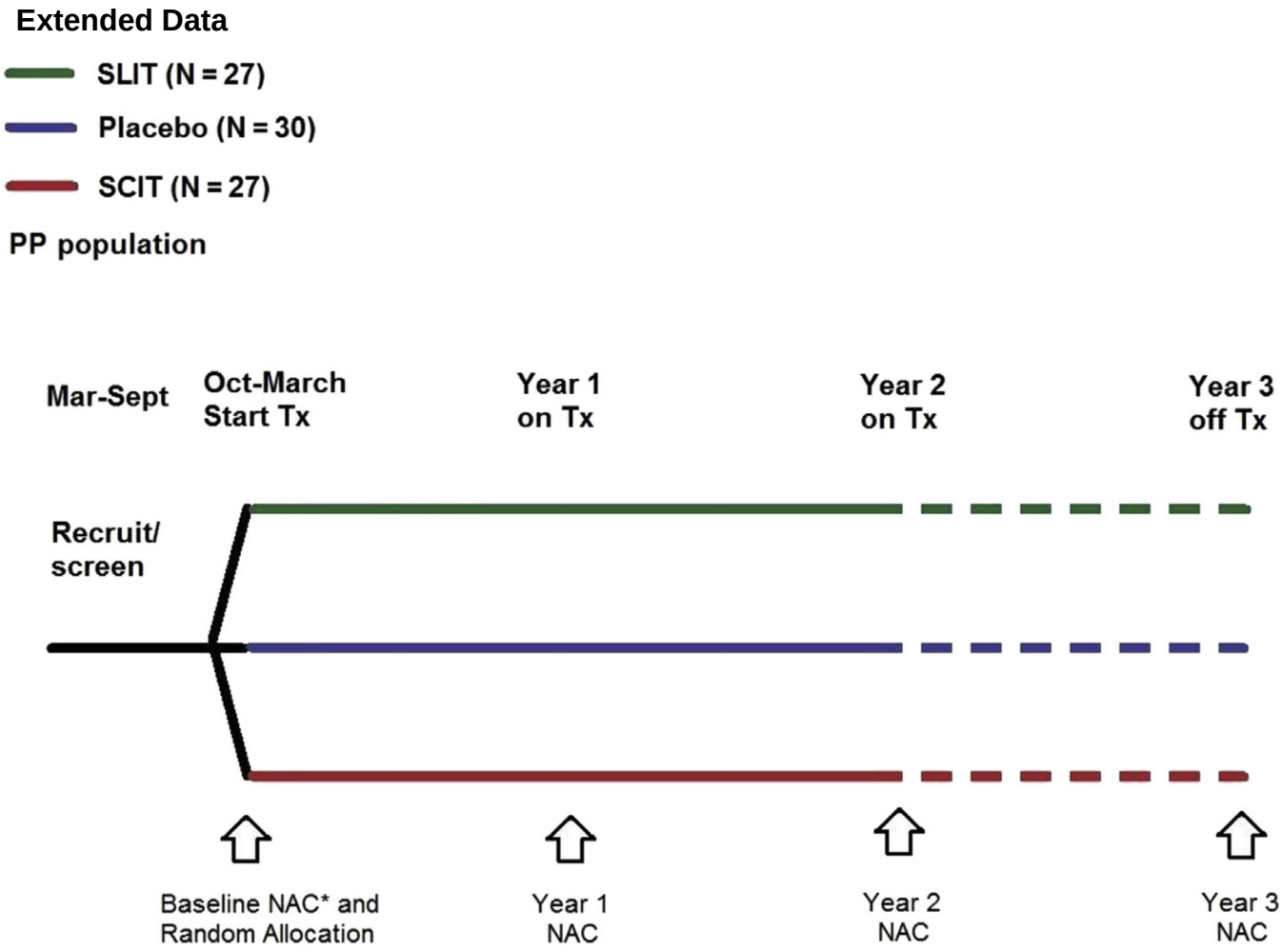


FIG E1.

Timing of clinical and mechanistic assessment for SLIT (green), placebo (blue), and SCIT (red) participants (https://www.itntrialshare.org/GRASS_antibody_figS1.url). *Tx*, Treatment. Sera and nasal fluids for antibody measurements were obtained at NAC visits, which occurred between October and March each year. *Baseline NAC was performed before treatment.

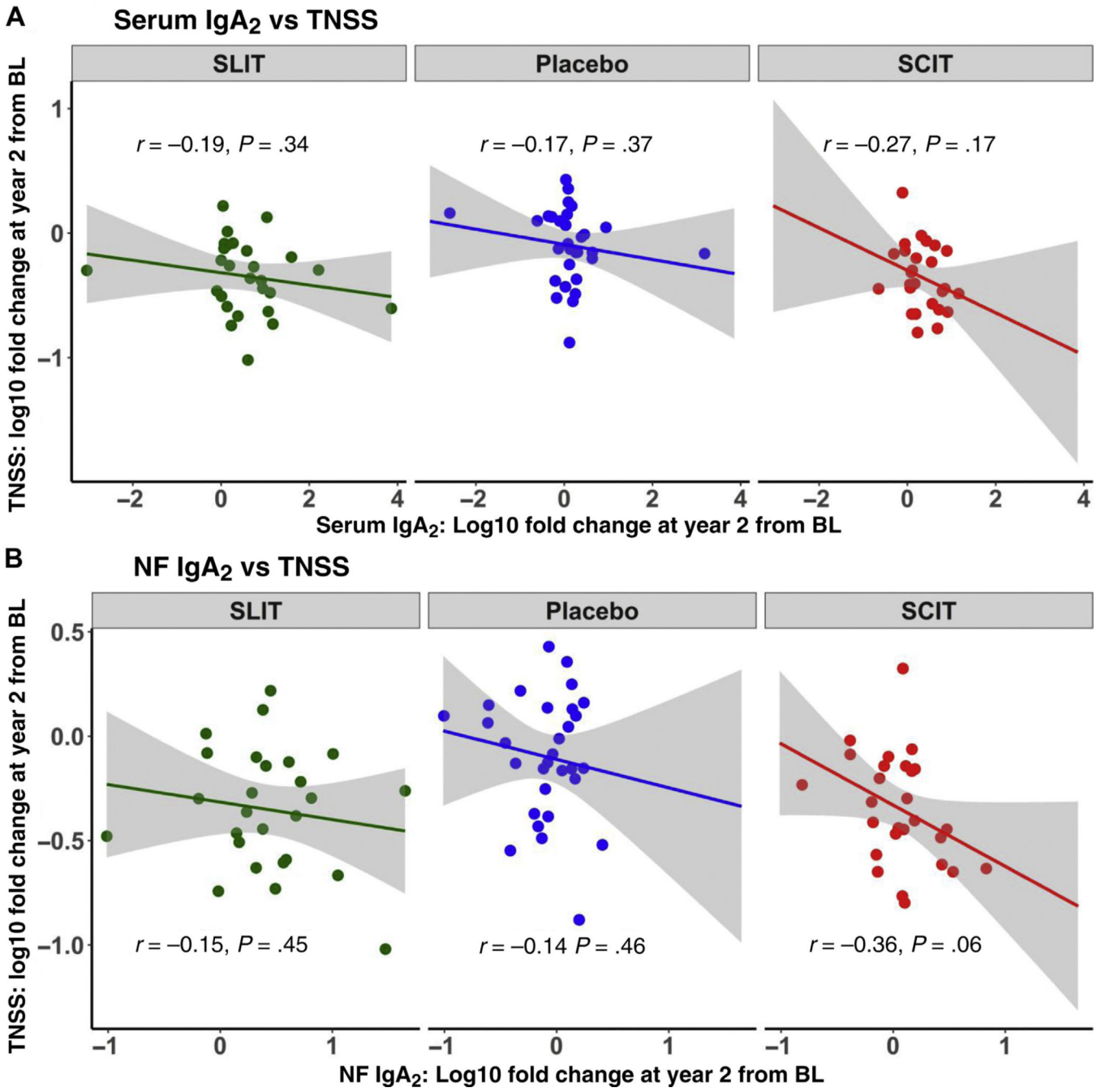


FIG E2.

Nasal and serum IgA_2 measured at year 2 did not correlate with TNSSs. Correlation of log_{10} change from baseline of TNSSs with log_{10} change from baseline of (**A**) serum or (**B**) nasal IgA_2 of SLIT-, placebo-, and SCIT-treated groups. *BL*, Baseline; *NF*, nasal fluid. Correlations were obtained using Pearson correlation method (https://www.itntrialshare.org/GRASS_antibody_figS2.url).

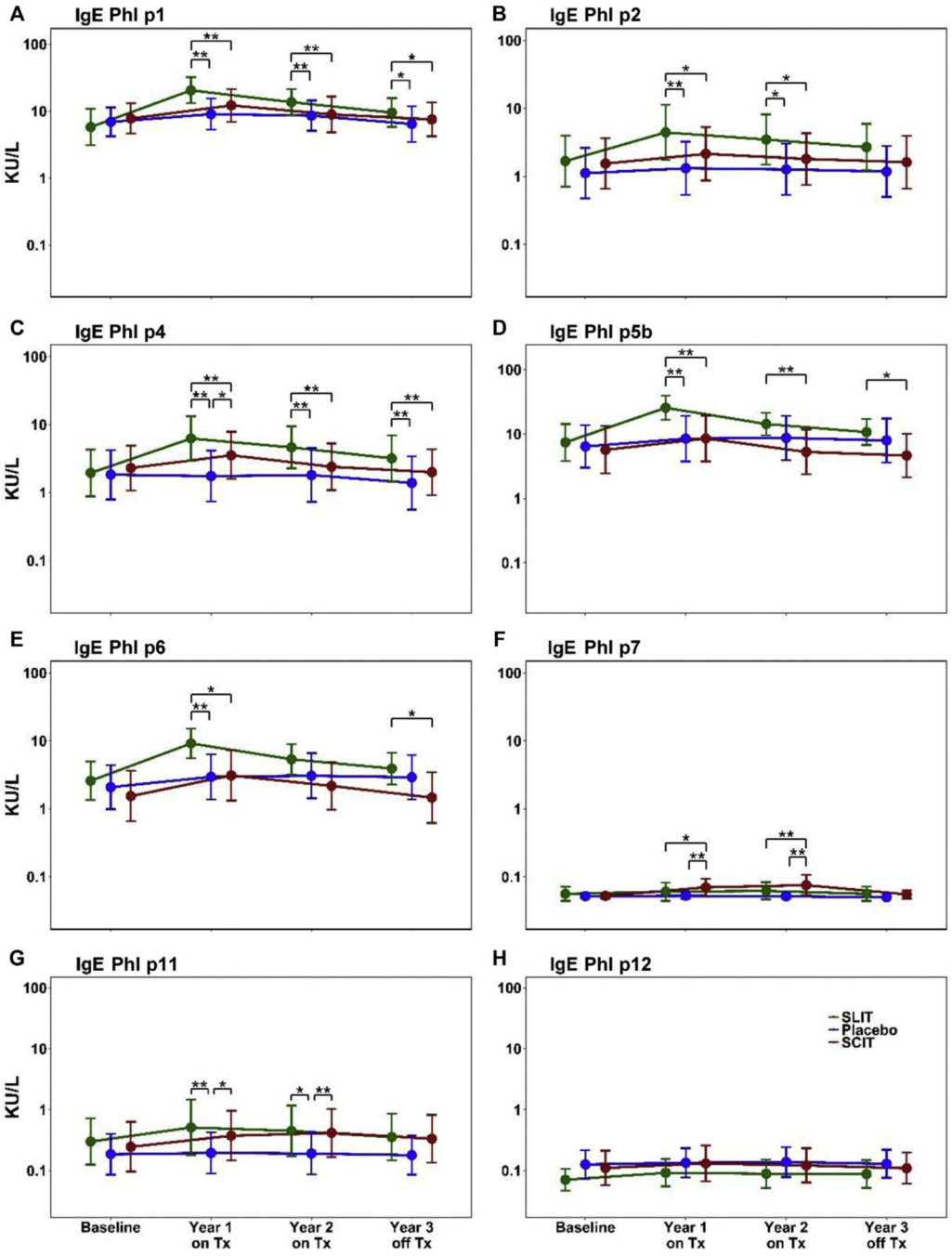


FIG E3.

Longitudinal changes in serum IgE to grass pollen components following SLIT, SCIT, or placebo. Serum specific IgE (kU/L) to grass pollen components (**A**) Phl p1, (**B**) Phl p2, (**C**) Phl p4, (**D**) Phl p5b, (**E**) Phl p6, (**F**) Phl p7, (**G**) Phl p11, and (**H**) Phl p12 were measured by ImmunoCAP system from SCIT-, SLIT-, and placebo-treated groups. *Tx*, Treatment. Data are presented as mean ± 95% CI. A linear mixed model was used with adjustment for baseline value. **P* < .05. ***P* < .01 (https://www.itntrialshare.org/GRASS_antibody_figS3.url).

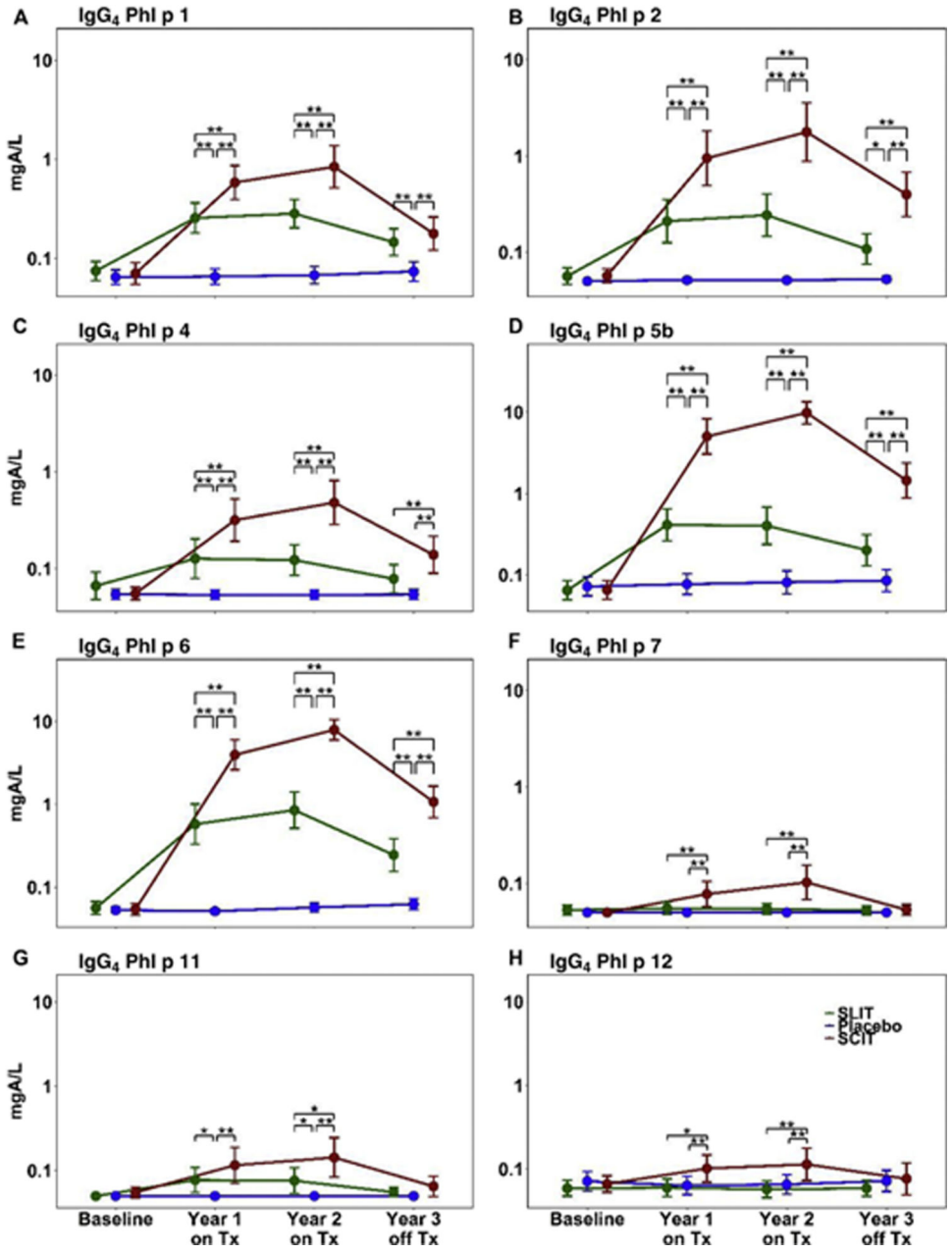


FIG E4.

Grass pollen–specific IgG_4 to grass pollen components following SLIT, SCIT, or placebo. Specific IgG_4 (mgA/L) to grass pollen components (**A**) Phl p1, (**B**) Phl p2, (**C**) Phl p4, (**D**) Phl p5b, (**E**) Phl p6, (**F**) Phl p7, (**G**) Phl p11, and (**H**) Phl p12 in serum collected from SCIT-, SLIT-, and placebo-treated groups was measured by ImmunoCAP. *Tx*, Treatment. Data are presented as mean ± 95% CI. A linear mixed model was used with adjustment for baseline value. $*P < .05$. $**P < .01$ (https://www.itntrialshare.org/GRASS_antibody_figS4.url).

TABLE E1.

Levels of specific IgA_1 and IgA_2 to grass pollen in nasal fluid and serum

Measurements (unit)	Year	Placebo				*P* value (year vs baseline)	SCIT				*P* value (year vs baseline)	*P* value (SCIT vs placebo)		
		n	Mean	LCLM	UCLM		n	Mean	LCLM	UCLM			n	Me
Nasal fluid grass pollen–specific IgA_1 (μg/mL)	Baseline	30	146.8	99.8	215.9		26	121.3	48.4	304.4			26	18
	Year 2	30	91.8	50.6	166.6	.0718	27	112.1	48.1	261.1	.7693	.3821	27	59
	Year 3	30	152.5	113.2	205.5	.8830	27	159.8	81.3	314.3	.3231	.6083	25	59
Nasal fluid grass pollen–specific IgA_2 (μg/mL)	Baseline	30	633.4	454.6	882.6		27	749.7	477.6	1176.9			27	52
	Year 2	30	513.3	389.0	677.4	.2098	27	860.4	610.8	1211.9	.4352	.0458	26	136
	Year 3	30	596.9	443.8	802.8	.7227	27	978.6	635.6	1506.6	.132	.058	26	147
Serum grass pollen–specific IgA_1 (μg/mL)	Baseline	30	88.0	54.9	141.1		27	127.5	66.0	246.0			27	11
	Year 1	30	77.1	48.6	122.5	.5308	27	134.3	52.3	345.0	.8128	.4044	27	57
	Year 2	30	66.5	41.2	107.4	.1841	27	171.7	67.0	440.2	.1797	.033	27	82
	Year 3	30	73.9	49.0	111.4	.4059	27	77.4	37.6	159.5	.0252	.39	27	33
Serum grass pollen–specific IgA_2 (μg/mL)	Baseline	30	52.0	30.5	88.7		26	78.7	54.3	114.1			27	38
	Year 1	30	87.9	68.7	112.5	.1117	27	226.4	143.7	356.7	.0017	.0474	27	97
	Year 2	30	71.1	42.6	118.5	.3440	27	165.2	112.9	241.8	.0243	.0765	27	13
	Year 3	30	74.1	61.2	89.8	.2826	27	63.7	29.5	137.5	.6492	.9705	27	10

LCLM, Lower confidence limit; *UCLM*, upper confidence limit.

TABLE E2.

Linear mixed-effect model with 1 bioassay covariate in the model

Effect	Estimate	SE	*P* value
Nasal fluid IgA_1	−0.446	0.23	.0495
Nasal fluid IgA_2	−0.776	0.36	.0365
Nasal fluid IgG_4	−0.121	0.14	.40
Nasal fluid IgE-facilitated allergen binding	0.09	0.19	.64
Serum IgG_4	−0.483	0.32	.13

Dependent variable: TNSS AUC; Independent variables: 1 time-varying covariate listed in the table and visit, treatment, and visit and treatment interaction.

TABLE E3.

Levels of specific IgE, IgG, and IgG_4 to grass pollen in nasal fluid and serum

Measurements (unit)	Year	Placebo				*P* value (year vs baseline)	SCIT				*P* value (year vs baseline)	*P* value (SCIT vs placebo)		
		n	Mean	LCLM	UCLM		n	Mean	LCLM	UCLM			n	Me
Nasal fluid grass pollen–specific IgE (kU/L)	Baseline	28	7.7	3.1	19.0		26	6.4	2.5	16.6			26	13
	Year 2	30	3.3	1.2	9.2	.0644	27	4.2	1.6	10.7	.1859	.8228	27	20
	Year 3	30	7.3	3.0	17.9	.5696	27	5.7	1.9	16.7	.6707	.3074	26	17
Nasal fluid grass pollen–specific IgG_4 (mg/L)	Baseline	30	2.1	1.0	4.5		27	1.1	0.4	2.9			26	1.
	Year 2	30	1.0	0.4	2.6	.1055	27	198.6	93.6	421.4	<.0001	<.0001	27	48
	Year 3	30	3.8	1.6	8.9	.1858	27	31.6	9.9	101.3	<.0001	.0001	26	23
Nasal fluid grass pollen–specific IgG (AU/mL)	Baseline	30	1.7	0.8	3.5		27	1.9	0.8	4.6			25	2.
	Year 2	30	0.7	0.3	1.6	.0447	27	29.6	14.0	62.3	<.0001	<.0001	27	10
	Year 3	30	1.6	0.7	3.6	.9575	27	3.8	1.4	10.4	.1177	.1599	27	8.
Serum grass pollen–specific IgE (kU/L)	Baseline	30	24.7	17.7	34.5		27	16.8	9.2	30.6			27	21
	Year 1	29	32.0	22.2	46.0	.0435	27	27.6	16.6	45.9	<.0001	.3759	27	62
	Year 2	30	32.1	22.1	46.7	.0249	27	21.0	11.9	36.9	.0663	.4721	27	44
	Year 3	30	26.9	19.4	37.3	.4645	27	16.8	9.9	28.5	.9983	.3275	27	27
Serum grass pollen–specific IgG_4 (mg/L)	Baseline	30	0.2	0.2	0.3		27	0.2	0.2	0.3			27	0.
	Year 1	29	0.2	0.2	0.3	.4819	27	17.3	10.1	29.7	<.0001	<.0001	27	2.
	Year 2	30	0.3	0.2	0.4	.1010	27	55.6	32.3	95.5	<.0001	<.0001	27	2.
	Year 3	30	0.3	0.2	0.4	.1416	27	4.4	3.2	6.1	<.0001	<.0001	27	1.
Serum grass pollen–specific IgG (AU/mL)	Baseline	30	3.0	1.8	5.1		27	1.8	0.9	3.6			27	3.
	Year 1	30	4.7	3.1	7.2	.0454	27	29.9	15.3	58.3	<.0001	<.0001	27	19
	Year 2	30	4.5	2.8	7.2	.0799	27	37.9	23.1	62.2	<.0001	<.0001	27	17
	Year 3	30	4.0	2.4	6.9	.2079	27	12.3	8.0	18.9	<.0001	.0007	27	9.

LCLM, Lower confidence limit; *UCLM*, upper confidence limit.

TABLE E4.

Levels of specific IgE to grass pollen components

Grass pollen–specific IgE	Year	Placebo				*P* value (year vs baseline)	SCIT				*P* value (year vs baseline)	*P* value (SCIT vs placebo)	SL		
		n	Mean	LCLM	UCLM		n	Mean	LCLM	UCLM			n	Mean	L
Phl p1 (kU/L)	Baseline	30	6.90	4.21	11.31		26	7.76	4.62	13.04			26	5.77	
	Year 1	29	9.00	5.27	15.37	.0732	26	12.16	6.92	21.37	.0029	.3062	26	20.47	1
	Year 2	30	8.56	5.08	14.40	.1222	26	8.92	4.82	16.51	.3524	.7991	26	13.59	
	Year 3	30	6.40	3.45	11.88	.5942	26	7.51	4.21	13.39	.8236	.7666	26	9.52	
Phl p11 (kU/L)	Baseline	30	0.19	0.09	0.40		26	0.25	0.10	0.63			26	0.30	
	Year 1	29	0.19	0.09	0.42	.9804	26	0.37	0.15	0.96	.0004	.0212	26	0.51	
	Year 2	30	0.19	0.09	0.42	.7950	26	0.42	0.17	1.03	<.0001	.0063	26	0.45	
	Year 3	30	0.18	0.09	0.37	.7583	26	0.33	0.13	0.82	.0102	.0597	26	0.35	
Phl p12 (kU/L)	Baseline	30	0.13	0.07	0.22		26	0.11	0.06	0.21			26	0.07	
	Year 1	29	0.13	0.08	0.23	.7710	26	0.13	0.07	0.26	.1317	.4919	26	0.09	
	Year 2	30	0.14	0.08	0.24	.3859	26	0.12	0.06	0.23	.4152	.9559	26	0.09	
	Year 3	30	0.13	0.08	0.22	.8156	26	0.11	0.06	0.20	.9044	.7931	26	0.09	
Phl p2 (kU/L)	Baseline	30	1.11	0.48	2.61		26	1.54	0.66	3.62			26	1.67	
	Year 1	29	1.31	0.53	3.22	.1539	26	2.14	0.87	5.30	.0422	.6012	26	4.43	
	Year 2	30	1.27	0.53	3.03	.3997	26	1.80	0.75	4.31	.3345	.8301	26	3.48	
	Year 3	30	1.18	0.50	2.78	.7161	26	1.62	0.66	3.95	.771	.9408	26	2.69	
Phl p4 (kU/L)	Baseline	30	1.83	0.79	4.23		26	2.29	1.08	4.89			26	1.94	
	Year 1	29	1.74	0.73	4.13	.9333	26	3.53	1.60	7.82	.0031	.0363	26	6.24	
	Year 2	30	1.81	0.73	4.48	.9226	26	2.39	1.09	5.26	.7719	.7534	26	4.61	
	Year 3	30	1.38	0.56	3.41	.0378	26	1.99	0.91	4.34	.3277	.4798	26	3.18	
Phl p5b (kU/L)	Baseline	30	6.40	2.99	13.68		26	5.68	2.44	13.23			26	7.40	
	Year 1	29	8.47	3.75	19.12	.0389	26	8.53	3.76	19.36	.0039	.6475	26	25.51	1
	Year 2	30	8.70	3.94	19.24	.0187	26	5.26	2.37	11.70	.5846	.0832	26	14.30	
	Year 3	30	7.93	3.63	17.31	.1003	26	4.62	2.12	10.10	.1414	.0594	26	10.71	
Phl p6 (kU/L)	Baseline	30	2.08	0.99	4.37		26	1.54	0.65	3.63			26	2.58	
	Year 1	29	2.95	1.37	6.36	.0342	26	3.10	1.32	7.24	<.0001	.1596	26	9.15	
	Year 2	30	3.06	1.42	6.58	.0062	26	2.17	0.97	4.87	.0227	.7094	26	5.35	
	Year 3	30	2.91	1.37	6.16	.0170	26	1.46	0.62	3.47	.7363	.0763	26	3.92	
Phl p7 (kU/L)	Baseline	30	0.05	0.05	0.06		26	0.05	0.05	0.06			26	0.06	
	Year 1	29	0.05	0.05	0.06	.8814	26	0.07	0.05	0.09	<.0001	.0051	26	0.06	
	Year 2	30	0.05	0.05	0.06	.9166	26	0.08	0.05	0.11	<.0001	.0002	26	0.06	
	Year 3	30	0.05	0.05	0.05	.5578	26	0.06	0.05	0.06	.4784	.3642	26	0.06	

LCLM, Lower confidence limit; *UCLM*, upper confidence limit.

TABLE E5.

Levels of specific IgG_4 to grass pollen components

Grass pollen–specific IgG_4	Year	Placebo				*P* value (year vs baseline)	SCIT				*P* value (year vs baseline)	*P* value (SCIT vs placebo)	SL		
		n	Mean	LCLM	UCLM		n	Mean	LCLM	UCLM			n	Mean	L
Phl p1 (kU/L)	Baseline	30	0.06	0.05	0.08		26	0.07	0.05	0.09			26	0.08	
	Year 1	29	0.07	0.06	0.08	.943	26	0.59	0.39	0.87	<.0001	<.0001	26	0.26	
	Year 2	30	0.07	0.06	0.08	.705	26	0.84	0.52	1.38	<.0001	<.0001	26	0.28	
	Year 3	30	0.07	0.06	0.09	.290	26	0.18	0.12	0.26	<.0001	<.0001	26	0.15	
Phl p11 (kU/L)	Baseline	30	0.05	0.05	0.05		26	0.06	0.05	0.06			26	0.05	
	Year 1	29	0.05	0.05	0.05	1	26	0.12	0.07	0.19	<.0001	.0003	26	0.08	
	Year 2	30	0.05	0.05	0.05	1	26	0.14	0.08	0.25	<.0001	<.0001	26	0.08	
	Year 3	30	0.05	0.05	0.05	1	26	0.07	0.05	0.09	.1796	.675	26	0.06	
Phl p12 (kU/L)	Baseline	30	0.07	0.05	0.09		26	0.07	0.05	0.08			26	0.06	
	Year 1	29	0.06	0.05	0.08	.2385	26	0.10	0.07	0.15	.0002	.002	26	0.06	
	Year 2	30	0.07	0.05	0.09	.4048	26	0.11	0.07	0.18	<.0001	.0004	26	0.06	
	Year 3	30	0.07	0.05	0.10	.9516	26	0.08	0.05	0.12	.2152	.4861	26	0.06	
Phl p2 (kU/L)	Baseline	30	0.05	0.05	0.05		26	0.06	0.05	0.07			26	0.06	
	Year 1	29	0.05	0.05	0.05	.8644	26	0.95	0.49	1.82	<.0001	<.0001	26	0.21	
	Year 2	30	0.05	0.05	0.05	.8778	26	1.77	0.88	3.57	<.0001	<.0001	26	0.24	
	Year 3	30	0.05	0.05	0.06	.7727	26	0.40	0.24	0.68	<.0001	<.0001	26	0.11	
Phl p4 (kU/L)	Baseline	30	0.05	0.05	0.06		26	0.06	0.05	0.06			26	0.07	
	Year 1	29	0.05	0.05	0.06	.9061	26	0.32	0.19	0.53	<.0001	<.0001	26	0.13	
	Year 2	30	0.05	0.05	0.06	.9137	26	0.48	0.29	0.82	<.0001	<.0001	26	0.12	
	Year 3	30	0.05	0.05	0.06	.9664	26	0.14	0.09	0.22	<.0001	<.0001	26	0.08	
Phl p5b (kU/L)	Baseline	30	0.07	0.06	0.09		26	0.07	0.05	0.09			26	0.06	
	Year 1	29	0.08	0.06	0.10	.7447	26	5.03	3.05	8.29	<.0001	<.0001	26	0.41	
	Year 2	30	0.08	0.06	0.11	.4498	25	9.82	7.17	13.44	<.0001	<.0001	26	0.40	
	Year 3	30	0.09	0.06	0.12	.2896	26	1.45	0.89	2.36	<.0001	<.0001	26	0.20	
Phl p6 (kU/L)	Baseline	30	0.05	0.05	0.06		26	0.05	0.05	0.06			26	0.06	
	Year 1	29	0.05	0.05	0.05	.8284	26	3.96	2.62	5.99	<.0001	<.0001	26	0.58	
	Year 2	30	0.06	0.05	0.06	.6461	25	7.93	5.98	10.52	<.0001	<.0001	26	0.85	
	Year 3	30	0.06	0.05	0.07	.3034	26	1.07	0.69	1.66	<.0001	<.0001	26	0.25	
Phl p7 (kU/L)	Baseline	30	0.05	0.05	0.05		26	0.05	0.05	0.05			26	0.05	
	Year 1	29	0.05	0.05	0.05	1	26	0.08	0.06	0.11	<.0001	.0002	26	0.06	
	Year 2	30	0.05	0.05	0.05	1	26	0.10	0.07	0.15	<.0001	<.0001	26	0.05	
	Year 3	30	0.05	0.05	0.05	1	26	0.05	0.05	0.06	.4448	.5947	26	0.05	

LCLM, Lower confidence limit; *UCLM*, upper confidence limit.

TABLE E6.

Induction of blocking antibody *in vitro* allergen-IgE complex binding to B cells in nasal fluid and serum

Measurements (unit)	Year	Placebo				SCIT				*P* value (SCIT vs placebo)	SLIT			
		n	Mean	LCLM	UCLM	n	Mean	LCLM	UCLM		n	Mean	LCLM	UCLM
Nasal fluid IgE-FAB (change in allergen binding, fold change from placebo)	Baseline	29	1	0.81	1.19	26	1.08	0.86	1.30		27	1.32	1.12	1.53
	Year 2	29	1	0.66	1.34	26	0.43	0.14	0.72	.013	26	0.43	0.19	0.67
	Year 3	28	1	0.64	1.36	26	1.04	0.01	2.07	.207	25	0.66	0.02	1.30
Serum IgE-FAB (change in allergen binding, fold change from placebo)	Baseline	30	1	0.95	1.05	*27*	0.94	0.86	1.02		27	1.03	0.97	1.09
	Year 2	30	1	0.94	1.06	27	0.45	0.33	0.58	<.0001	27	0.53	0.38	0.69
	Year 3	30	1	0.92	1.08	27	0.61	0.50	0.72	.0007	27	0.73	0.61	0.84

IgE-FAB, IgE-facilitated allergen binding; *LCLM*, Lower confidence limit; *UCLM*, upper confidence limit.

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Abbreviations:

SCIT	subcutaneous immunotherapy
SLIT	sublingual immunotherapy
IgG_4	timothy grass pollen - specific immunoglobulin G_4
IgA	timothy grass pollen - specific Immunoglobulin A

Abbreviations used

AIT	Allergen immunotherapy
AUC	Area under the curve
GRASS	Gauging Response in Allergic Rhinitis to Sublingual and Subcutaneous Immunotherapy
NAC	Nasal allergen challenge

Phl p	Phleum pratense
PP	Per-protocol
SCIT	Subcutaneous immunotherapy
SLIT	Sublingual immunotherapy
TGP	Timothy grass pollen
TNSS	Total nasal symptom score

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Key messages

- Grass pollen–specific IgA antibodies were elevated in nasal fluid and serum in SLIT-compared with SCIT- and placebo-treated participants.
- In hierarchical clustering analysis, grass pollen–specific IgA, IgE, and IgG4 grass pollen molecular component data enhanced the ability to cluster subjects by treatment groups and response.

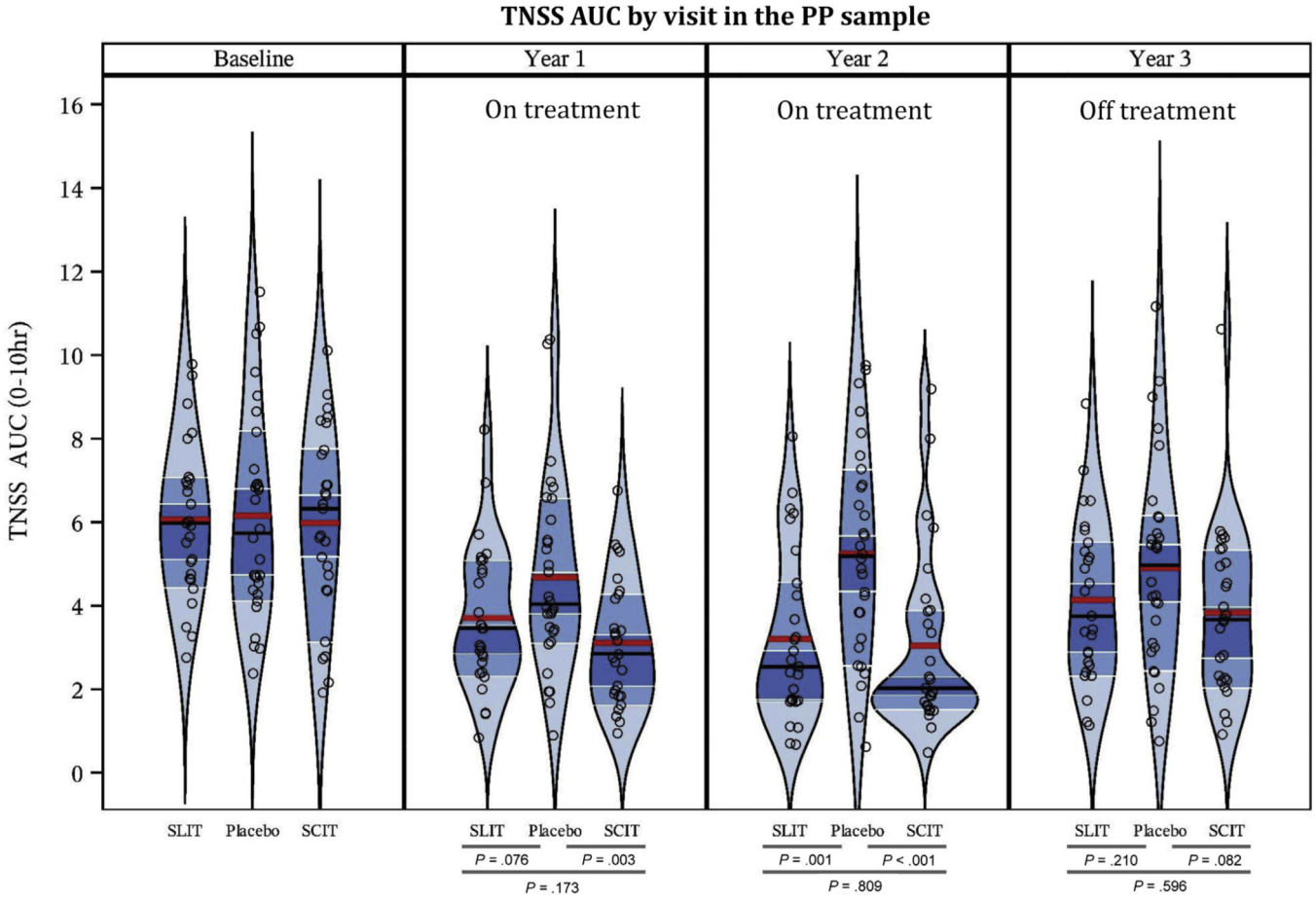

FIG 1.

TNSSs following SLIT, SCIT, or placebo. TNSS AUCs of SLIT-, SCIT-, or placebo-treated patients were measured at baseline, year 1 (on treatment), year 2 (on treatment), and year 3 (off treatment). Multicolored bands, quintiles; red line, mean; black lines, median. $P < .05$ was considered as significant. *P* values calculated using an ANCOVA model adjusted for baseline AUC (https://www.itntrialshare.org/GRASS_antibody_fig1.url).

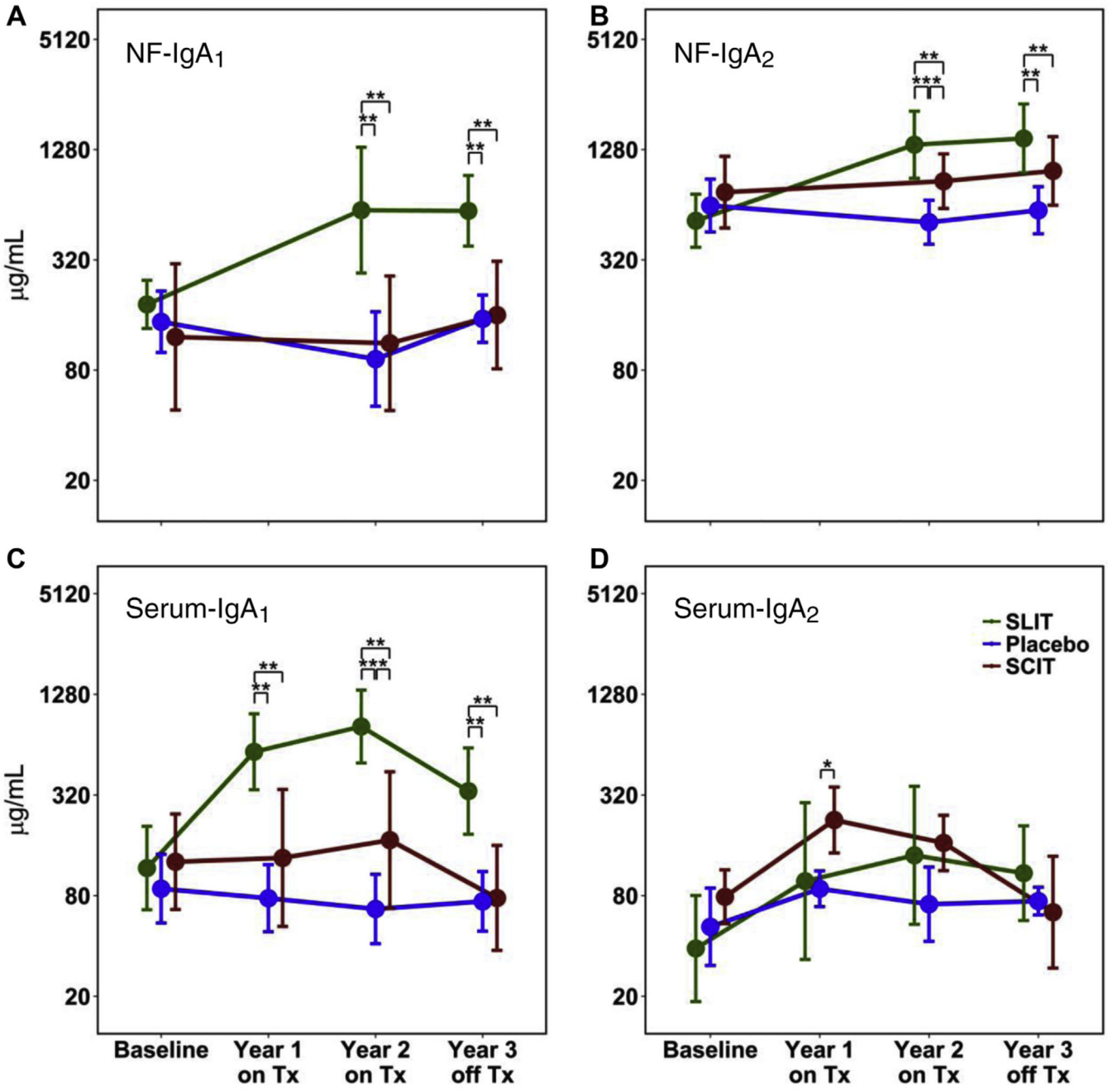

FIG 2.

TGP-specific IgA_1 and IgA_2 following SLIT, SCIT, or placebo. Nasal fluid and serum samples were collected from SLIT-, SCIT-, and placebo-treated groups. TGP-specific (**A**) IgA_1 (µg/mL) and (**B**) IgA_2 (µg/mL) in nasal fluid was measured by ELISA. Serum grass pollen–specific (**C**) IgA_1 and (**D**) IgA_2 was measured by ELISA. *NF*, Nasal fluid; *Tx*, treatment. Data are presented as mean ± 95%. A linear mixed model was used with adjustment for baseline value. **P* < .05. ***P* < .01 (https://www.itntrialshare.org/GRASS_antibody_fig2.url).

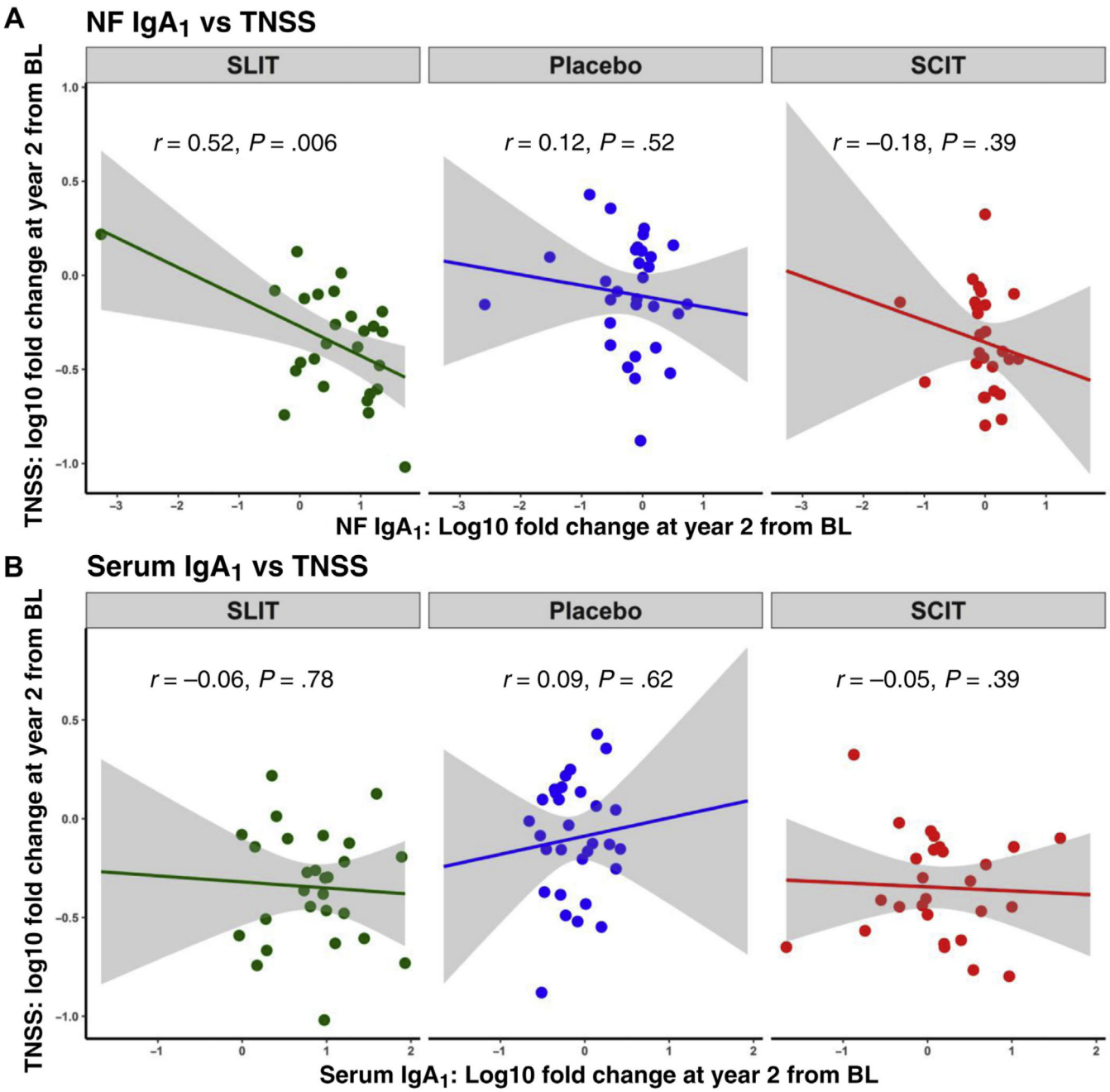

FIG 3.

Nasal IgA_1 measured at year 2 correlates with TNSSs in SLIT but not placebo and SCIT. Correlation of log_{10}change from baseline of TNSSs with log_{10}change from baseline of (**A**) nasal or (**B**) serum IgA_1 of SLIT-, placebo-, and SCIT-treated groups. *BL*, Baseline; *NF*, nasal fluid. Correlations were obtained using Pearson correlation method (https://www.itntrialshare.org/GRASS_antibody_fig3.url).

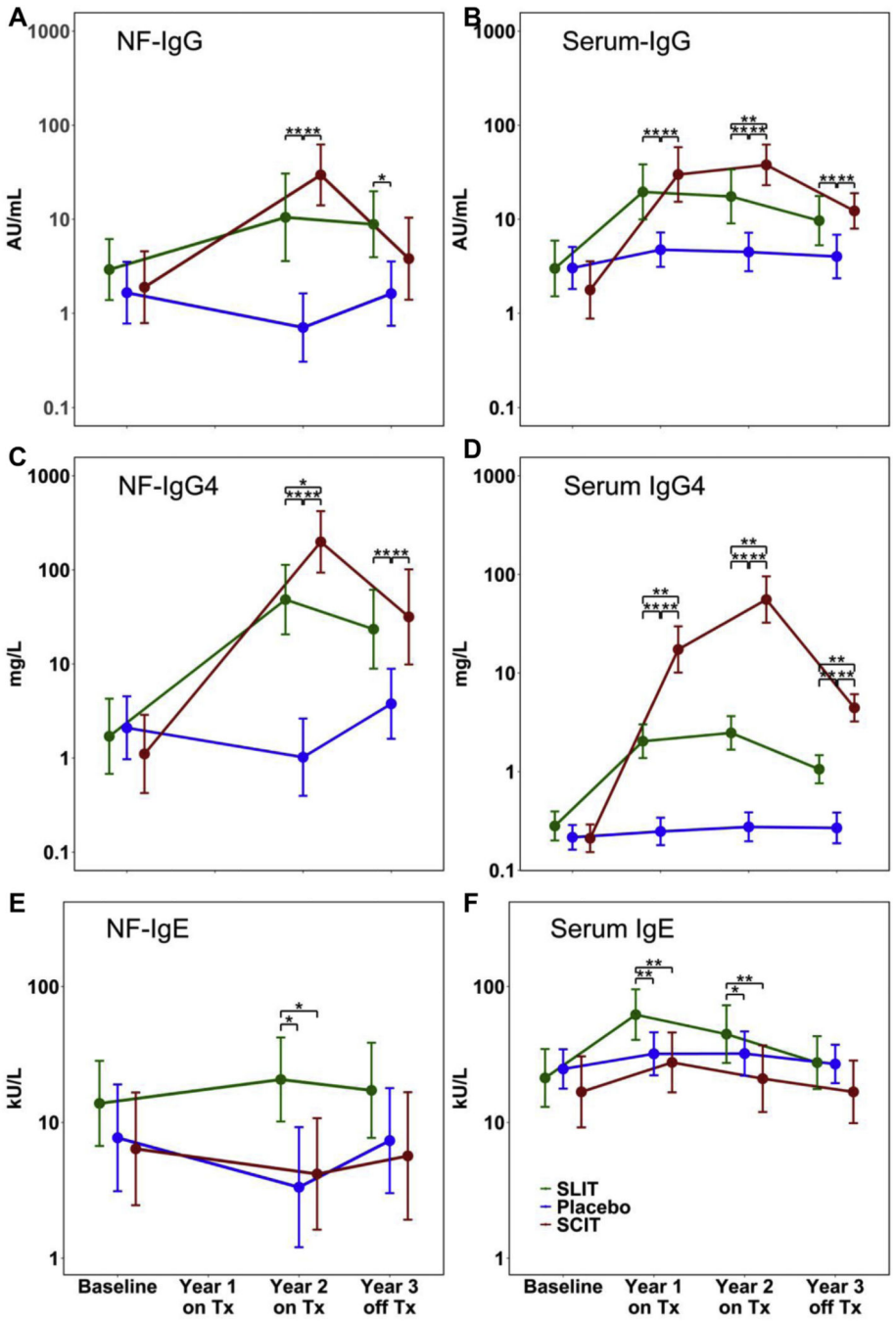

FIG 4.

Longitudinal changes in IgG, IgG_4, and IgE in nasal lining fluid and serum following SLIT, SCIT, or placebo. Nasal and serum (**A** and **B**) specific IgG (AU/mL), (**C** and **D**) IgG_4 (mg/mL), and (**E** and **F**) IgE (kU/L) to grass pollen allergen. Nasal-specific (Fig 4, *A*) IgG, (Fig 4, *C*) IgG_4, (Fig 4, *E*) IgE, and serum (Fig 4, *B*) IgG levels were measured by ELISA. Serum (Fig 4, *D*) IgG_4 and (Fig 4, *F*) IgE levels were measured by ImmunoCAP in SCIT-, SLIT-, and placebo-treated groups. *Note:* Different y-axis scale for Fig 4, *E* and *F*. Data are

presented as mean ± 95% CI. A linear mixed model was used with adjustment for baseline value. *$P < .05$. **$P < .01$ (https://www.itntrialshare.org/GRASS_antibody_fig4.url).

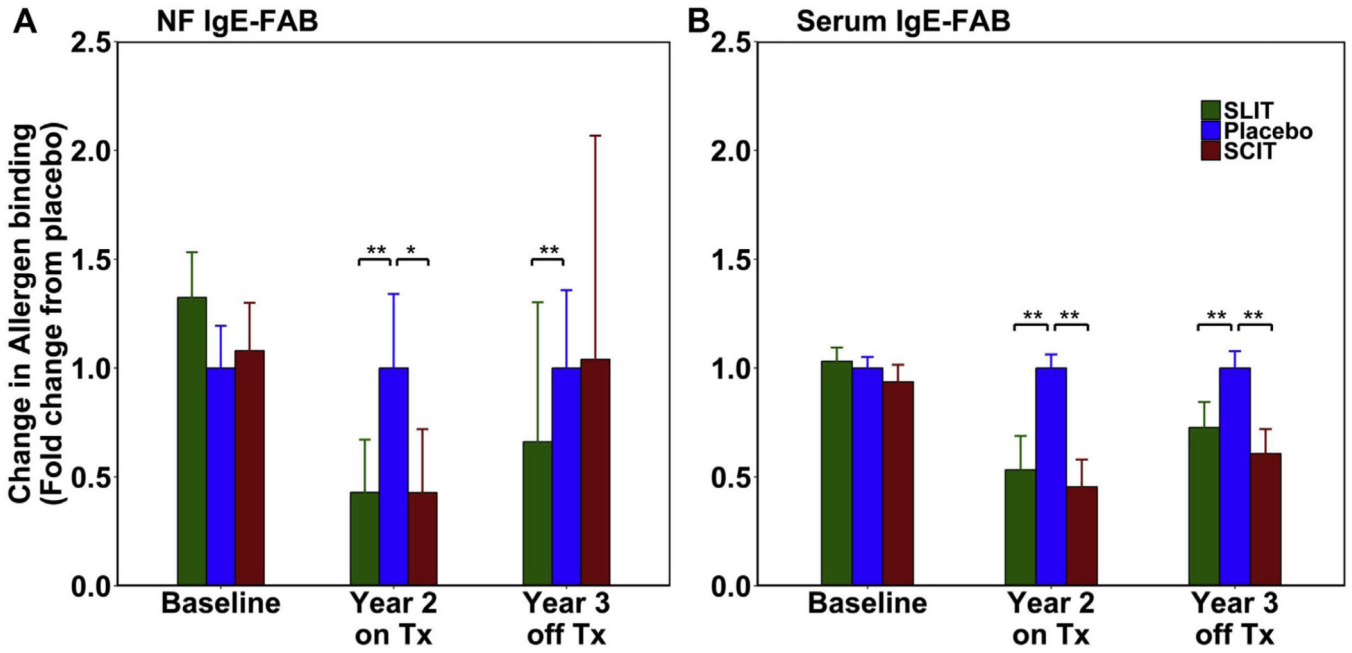

FIG 5.

Time course of allergen-neutralizing blocking antibody responses in nasal fluid and peripheral blood in SLIT, SCIT, and placebo. (**A**) The co-operative allergen-IgE binding to B cells and inhibitory activity for IgE-FAB was measured in nasal fluid and (**B**) serum obtained from SLIT-, SCIT-, and placebo-treated patients. *IgE-FAB*, IgE-facilitated allergen binding. Data are presented as mean ± 95%. A linear mixed model was used with adjustment for baseline value. **P* < .05. ***P* < .01 (https://www.itntrialshare.org/GRASS_antibody_fig5.url).

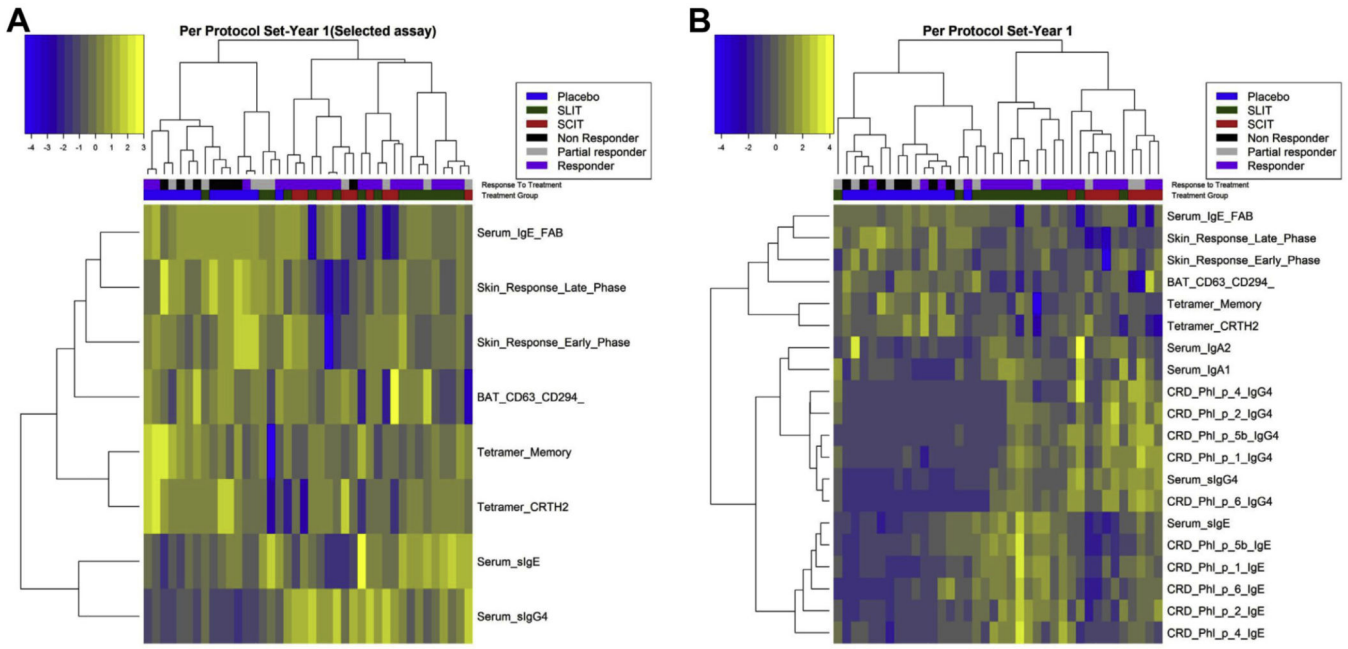

FIG 6.

Linkage hierarchical clustering analysis using immune monitoring biomarker assays with or without IgE, IgG_4 to grass pollen components, and IgA to grass pollen. Log_{10}fold changes in variables from baseline to year 1 were used to perform complete linkage hierarchical clustering using (**A**) selected biomarker assays or (**B**) with the addition of serum IgA data and serum component IgE and IgG_4 data. Yellow color indicates large log_{10} increases from baseline to year 1, and blue color indicates large log_{10} decreases from baseline to year 1. Inclusion of IgE/IgG_4 component data and serum IgA data leads to improved clustering by treatment group (SLIT = green, n = 15; placebo = blue, n = 14; SCIT = red, n = 9) and response to treatment (Nonresponder: <10% reduction in TNSS from baseline to year 2 = black, Partial Responder: 10%−40 % reduction in TNSS from baseline to year 2 = white, Responder: >40% reduction in TNSS from baseline to year 2 = purple) (https://www.itntrialshare.org/GRASS_antibody_fig6.url).

TABLE I.

Participants' demographic and baseline characteristics in the PP population

Characteristic	SLIT (N = 27)	Placebo (N = 30)	SCIT (N = 27)
Age (y), mean (95% CI)	36.5 (32.66–40.39)	32.7 (29.76–35.59)	33.5 (29.48–37.60)
Sex: male, n (%)	22 (81.5)	20 (66.7)	16 (59.3)
Ethnicity, n (%)			
Asian	5 (18.5)	4 (13.3)	1 (3.7)
Black	2 (7.4)	2 (6.7)	2 (7.4)
Chinese	0 (0.0)	1 (3.3)	1 (3.7)
Middle Eastern	1 (3.7)	0 (0.0)	0 (0.0)
Mixed	1 (3.7)	1 (3.3)	0 (0.0)
White	18 (66.7)	22 (73.3)	23 (85.2)
Grass skin prick test wheal diameter (mm), mean (95% CI)	10.6 (9.29–11.89)	8.7 (7.50–9.97)	8.7 (7.25–10.16)
*Total IgE (kU/L), median (Q1, Q3)	78.9 (26.70, 258.00)	129.0 (65.40, 290.00)	84.4 (39.20, 261.00)
IgE component samples, n	26	30	26
Phl P1 IgE (kU/L)			
Median (Q1, Q3)	6.1 (2.54, 14.60)	6.9 (3.82, 17.20)	10.2 (2.21, 17.60)
Sensitized, n (%)	25 (96.2)	29 (96.7)	26 (100)
Phl P2 IgE (kU/L)			
Median (Q1, Q3)	1.5 (0.40, 8.01)	2.0 (0.05, 6.53)	2.2 (0.48, 7.02)
Sensitized, n (%)	22 (84.6)	21 (70.0)	21 (80.8)
Phl P4 IgE (kU/L)			
Median (Q1, Q3)	2.9 (0.78, 4.38)	5.2 (0.90, 9.55)	2.8 (1.05, 8.85)
Sensitized, n (%)	22 (84.6)	23 (76.7)	23 (88.5)
Phl P5b IgE (kU/L)			
Median (Q1, Q3)	8.5 (4.20, 19.90)	10.5 (3.20, 25.00)	10.1 (2.79, 26.10)
Sensitized, n (%)	25 (96.2)	27 (90.0)	23 (88.5)
Phl P6 IgE (kU/L)			
Median (Q1, Q3)	3.3 (0.79, 5.88)	3.0 (0.66, 11.40)	1.8 (0.27, 9.77)
Sensitized, n (%)	25 (96.2)	26 (86.7)	23 (88.5)
Phl P7 IgE (kU/L)			
Median (Q1, Q3)	0.05 (0.05, 0.05)	0.05 (0.05, 0.05)	0.05 (0.05, 0.05)
Sensitized, n (%)	1 (3.8)	1 (3.3)	1 (3.8)
Phl P11 IgE (kU/L)			
Median (Q1, Q3)	0.05 (0.05, 2.75)	0.05 (0.05, 0.91)	0.05 (0.05, 2.74)
Sensitized, n (%)	12 (46.2)	10 (33.3)	9 (34.6)
Phl P12 IgE (kU/L)			
Median (Q1, Q3)	0.05 (0.05, 0.05)	0.05 (0.05, 0.71)	0.05 (0.05, 0.05)
Sensitized, n (%)	4 (15.4)	9 (30.0)	6 (23.1)

*Total IgE measurements were performed in samples collected during the grass pollen season.