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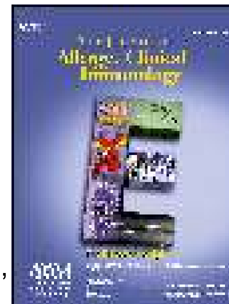

Synchronous Immune Alterations Mirror Clinical Response During Allergen Immunotherapy

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1 **[Title Page:]**

2 # Title: Synchronous Immune Alterations Mirror Clinical Response During
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33 **Abstract**

34 **Background.** Three years treatment with either sublingual or subcutaneous allergen
35 immunotherapy has been shown to be effective and to induce long-term tolerance. The GRASS*
36 trial demonstrated that two years treatment via either route was effective in suppressing the
37 response to nasal allergen challenge, although was insufficient for inhibition one year after
38 discontinuation.

39 **Objective.** To examine in the GRASS trial the time-course of immunologic changes during two
40 years sublingual and subcutaneous immunotherapy and for one year after treatment
41 discontinuation.

42 **Methods.** We performed multi-modal immunomonitoring to assess allergen-specific CD4 T cell
43 properties, in parallel with analysis of local mucosal cytokine responses induced by nasal
44 allergen exposure and humoral immune responses that included IgE-dependent basophil
45 activation and measurement of serum inhibitory activity for allergen-IgE binding to B cells (IgE-
46 Facilitated Allergen Binding).

47 **Results.** All three of these distinct arms of the immune response displayed significant and
48 coordinate alterations during 2 years allergen desensitization, followed by reversal at 3 years,
49 reflecting a lack of a durable immunological effect. Whereas frequencies of antigen-specific Th2
50 cells in peripheral blood determined by HLA class II tetramer analysis most closely paralleled
51 clinical outcomes, IgE-antibody dependent functional assays remained partially inhibited one
52 year following discontinuation.

53 **Conclusion.** Two years of allergen immunotherapy were effective but insufficient for long-term
54 tolerance. Allergen-specific Th2 cells most closely paralleled the transient clinical outcome and

55 it is likely that recurrence of the T cell ‘drivers’ of allergic immunity abrogated the potential for
56 durable tolerance. On the other hand, persistence of IgE-blocking antibody one year after
57 discontinuation may be an early indicator of a pro-tolerogenic mechanism.

58 * **G**auging **R**esponse in **A**llergic rhinitis to **S**ublingual and **S**ubcutaneous immunotherapy

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60 **Key Messages**:

61 - Two years of grass pollen immunotherapy leads to decreased frequency of circulating
62 allergen-specific Th2 cells, suppressed increases in nasal cytokine response to allergen
63 challenge and decreased antigen-specific IgE activity.

64 **Capsule Summary:** During and after grass pollen immunotherapy, changes in peripheral
65 antigen-specific Th2 cells paralleled clinical outcomes, reflecting distinctive cellular response in
66 concert with changes in specific IgE antibodies and local tissue cytokines, suggesting
67 coordinated immune mechanisms.

68 **Keywords:** Allergy; Immunotherapy; Immune tolerance; Allergen desensitization; Th2 cells

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70 **Introduction**

71 Allergen immunotherapy is an effective treatment option for patients with allergic rhinitis
72 who do not respond adequately to usual anti-histamine and topical corticosteroid medications
73 (1). Subcutaneous immunotherapy involves weekly administration of incremental doses of
74 allergenic material by injection followed by monthly maintenance injections for several years (2-
75 4). Immunotherapy has been associated with overall changes in T cell function with cytokine
76 changes that suggest a shift from Th2 cells towards Th1 phenotypes or induction of regulatory T
77 cells (5, 6). These alterations are accompanied by decreases in recruitment and/or activation of
78 allergic effector cells including mast cells, eosinophils and basophils in target organs (7, 8).
79 Measurement of serum immunoglobulins directed against the allergen in such immunotherapy
80 studies indicates that specific IgG, particularly of the IgG4 subclass, can be induced by therapy
81 and is presumed to be mechanistically linked to clinical benefit by virtue of competitive
82 inhibition of allergic responses triggered by specific IgE directed to the same allergens (9-12).
83 Alternative routes of allergen administration for immunotherapy are now under active
84 investigation, including sublingual (13-15), and epicutaneous routes (16, 17). For food allergens,
85 the oral route has also shown promising results (18, 19). Since immunological properties at each
86 of these sites differ, the mechanisms through which these forms of allergen immunotherapy exert
87 their therapeutic effects may differ, as well.

88 The GRASS (Long-Term Effects of Sublingual Grass Therapy) clinical trial was a
89 randomized, placebo-controlled, double-blind study of 106 adults with a clinical history of
90 moderate to severe seasonal allergic rhinitis due to grass pollen. Study participants received two
91 years of subcutaneous immunotherapy, sublingual immunotherapy or placebo and were
92 extensively studied over three years for clinical and immunological parameters of response (20).

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93 Clinical assessments in this trial were recently reported, demonstrating successful suppression of
94 the nasal response to allergen challenge after two years of therapy for both the subcutaneous and
95 sublingual routes, with lack of sustained benefit in the subsequent untreated third year (20). We
96 now report immunological findings from this trial, including peripheral blood cellular and
97 humoral assessments, as well as local tissue responses to allergen: evaluation of antigen-specific
98 CD4+ T cells in peripheral blood, functional outcomes from changes in the humoral response
99 detected in serum and peripheral IgE-dependent basophil assays and cytokine responses to
100 allergen challenge in the nasal mucosa.
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102 **Methods**

103 *Sample collection*

104 Clinical characteristics of the subjects in the GRASS study and details of the protocol
105 have been previously reported (20). Subcutaneous alum-adsorbed grass pollen immunotherapy
106 (Alutard SQ Grass Pollen®, ALK, Horsholm, Denmark) or matched placebo subcutaneous
107 injections were given weekly for 15 weeks followed by monthly maintenance injections until 2
108 years. Freeze-dried grass pollen (*Phleum Pratense*) sublingual tablets (Grazax®, ALK,
109 Horsholm, Denmark) or matched placebo sublingual tablets were self-administered daily for 2
110 years. Timothy grass-specific IgE and specific IgG4 were quantified using the CAP FEIA system
111 (Phadia, Uppsala, Sweden). Peripheral blood lymphocytes were collected and prepared for
112 cryopreservation as previously described (20). Coded samples were provided to the operator.

113 *Tetramer assays and flow cytometry analysis*

114 Timothy grass specific CD4+ T cell epitopes were identified by Tetramer Guided Epitope
115 Mapping (21, 22). Epitope specific pMHC tetramer reagents were generated by loading specific
116 peptides onto biotinylated soluble DR monomers, and subsequently conjugated with PE-
117 streptavidin (23). These included HLA-DR04:01, DR03:01, DR04:01, DR07:01, DR10:01 and
118 DR11:01 tetramer reagents. For *ex vivo* tetramer staining, 20 to 40 million frozen PBMC from
119 subjects with HLA genotypes corresponding to these tetramers were thawed and re-suspended in
120 200 µl of T cell culture medium and, in order to enhance tetramer staining, were treated with
121 dasatinib (Sigma-Aldrich) for 10 minutes at 37°C before tetramer staining (24). PE-labeled,
122 pooled tetramers were then added to a final concentration of 20 µg/ml, and the staining was
123 carried out for 100 minutes at room temperature. 1/100 fraction of the cells were saved and the
124 rest of the PE-tetramer positive cells were then enriched by the anti-PE bead enrichment protocol

125 through a magnetic column according to the manufacturer's protocol (Miltenyi Biotec) (22, 25).
126 Cells in both the enriched fraction and the pre-column fraction were stained with a panel of
127 antibodies of interest, including CD14 (HCD14, Biolegend), CD19 (HIB19, Biolegend),
128 CD45RA (HI100, BD Biosciences), CD4 (RPA-T4, BD Biosciences), CRTH2 (CRTH2, BM16,
129 BD Biosciences), CD161 (PK136, Biolegend) and CD27 (O323, Biolegend); and were further
130 treated with BD Via-Probes™ (BD Biosciences), before flow cytometry. Frequencies of tetramer
131 positive cells were calculated by the formula n/N, where n is the number of tetramer positive
132 cells in the enriched fraction, and N is the total number of cells in the sample, which can be
133 calculated by counting the number of cells in the pre-column fraction x 100. Efficiency of
134 recovery was optimized by using less than 30 million cells as starting material on samples with
135 less than 300 tetramer-positive cells per million, capturing greater than 95% of the PE-tetramer-
136 stained populations.

137 *Isolation of grass pollen allergen-reactive T cells with CD154 upregulation assay*

138 Global grass pollen-reactive CD4+ T cells were tracked using the CD154 assay (26, 27).
139 Briefly, frozen/thawed PBMC were cultured at a density of 10^6/ml with 1 µl/ml Timothy grass
140 pollen crude extract and 1 µg/ml of anti-CD40 blocking mAb (HB14, Miltenyi Biotec). After 18
141 hour stimulation at 37°C, cells were harvested and labeled with PE-Conjugated anti-CD154 mAb
142 for 10 minutes at 4°C. Cells were then washed, labeled with anti-PE magnetic beads and
143 enriched by using a magnetic column, according to the manufacturer's instructions (Miltenyi
144 Biotec). Magnetically enriched cells were next stained with antibodies against markers of interest
145 and analyzed on a FACSAria™ II flow cytometer (BD). Live memory CD45RO+ CD154+
146 CD4+ T cells were sort-purified for subsequent transcript analysis.

147 *Real-time PCR expression analysis*

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148 The Fluidigm BioMark 96.96 Dynamic Array (28) was used to measure the gene
149 expression in small cell populations. Ten cells per well were sorted by FACS in quadruplicate
150 into 96-well plates containing a reaction mix for reverse transcription (CellsDirect One-Step
151 qRT-PCR kit; Invitrogen) and pre-amplification with 96 selected gene primer pairs (DELTAgene
152 assays, Fluidigm). After sorting, samples were reverse-transcribed and pre-amplified for 18
153 cycles. Primers and dNTPs were removed by incubation with ExonucleaseI (NE Biolabs), and
154 samples were diluted (5×) with TE buffer and stored at −20°C. Samples and assays (primer
155 pairs) were prepared for loading onto 96.96 Fluidigm Dynamic arrays according to the
156 manufacturer's recommendations. The 96.96 Fluidigm Dynamic Arrays (Fluidigm Corp.) were
157 primed and loaded on an IFC Controller HX (Fluidigm Corp.) and real-time PCR was run on a
158 BiomarkHD (Fluidigm Corp.). Data were collected and analyzed using Fluidigm Real-Time PCR
159 Analysis software (v4.1.2).

160 *Measurement of nasal cytokines*

161 Nasal challenge was performed using Aquagen® (ALK) *Phleum Pratense* (Timothy
162 grass) extract as described previously (20). Challenge dose was determined according to a dose-
163 titration challenge at screening. The same dose was then used at the baseline (pre-treatment)
164 nasal challenge visit and at each subsequent challenge visit. Dose range was 1,500 BU/ml
165 (equivalent to 1.0 µg/ml major allergen) to 30,000 BU/ml (equivalent to 20.2 µg/ml major
166 allergen).

167 Nasal secretions were collected using synthetic polyurethane sponges pre-cut to 20 x 15 x
168 15 mm (RG 27 grau; Gummi-Welz GmbH & Co., Neu-Ulm, Germany) and sterilized by
169 autoclaving. A single sponge was inserted into each of the participant's nostrils, posterior to the
170 muco-cutaneous junction, by a study physician under direct vision using croc forceps and a nasal

171 speculum (Phoenix Surgical Instruments Ltd, Hertfordshire, UK). Sponges were left in place for
172 2 minutes before removal and then added to 2-ml centrifuge tubes with indwelling 0.22 µm
173 cellulose acetate filters (Costar Spin-X; Corning, Corning, NY, USA). Tubes were kept briefly
174 on ice before being centrifuged. At baseline, sponges were centrifuged 'neat' without adding an
175 elution buffer. At years 2 and 3, 75 µl of elution buffer [Milliplex Assay Buffer; Millipore,
176 Darmstadt, Germany; PBS pH 7.4, BSA (1%), Tween-20 (0.05%), sodium azide (0.05%)] was
177 added to sponges within their centrifuge tubes before being centrifuged. The isolated fluid was
178 then pipetted into Eppendorf tubes and stored at -80°C.

179 After thawing, nasal fluid was analyzed for cytokines in yearly batches. Measurements of
180 IL-4, IL-5, IL-10, IL-13 and IFN-γ, were performed using MSD Human TH1/TH2 7-Plex, Ultra-
181 Sensitive Kit according to the manufacturer's instructions (MS6000 7 spot; Meso Scale
182 Discovery, Maryland, USA). Briefly, after incubation of plates with diluent, 25 µl of samples,
183 calibrators, and high and low standards were added to appropriate wells and incubated on a plate-
184 shaker for 2 hours. Plates were then washed in PBS plus 0.05% Tween-20 using an automated
185 washer (Aquamax 2000). Twenty-five microliters of detection antibody at 1µg/ml was added to
186 wells, followed by incubation on a plate-shaker for 2 hours in the dark. Plates were then washed
187 3 times as before. One hundred and fifty microliters of Read Buffer T were then added to each
188 well before plates were read on an MSD SECTOR® 6000 instrument. All measurements were
189 performed in duplicate and reported as mean values per standardized volume. The assay was
190 validated for analysis of nasal secretion and the level of quantification was 5-5000 pg/ml for all
191 cytokines.

192 *IgE-FAB and basophil activation*

193 Serum inhibitory activity for IgE-facilitated allergen binding and presentation was
194 measured by FAB assay (10, 29, 30). Briefly, an indicator serum containing high concentration
195 of Timothy grass pollen (*P. pratense*)-specific IgE (>100 IU/mL), was pre-incubated with 1
196 μg/mL allergen at 37°C for 1 hour to allow formation of allergen-IgE complexes. To test for
197 inhibition of facilitated allergen binding, indicator serum and test serum (baseline, year 1, year 2
198 and year 3) or RPMI alone as a control was mixed. During this step, CD23-enriched EBV-
199 transformed B cells were washed three times by centrifugation in RPMI-1640 at 423 x g for 7
200 minutes at 4°C. Cells were then re-suspended in FAB buffer (138.60 mM NaCL, 1.12 mM
201 NaH2PO4, 8.16 mM Na2HPO4 and 0.1% bovine serum albumin dissolved in 1 liter of distilled
202 H_2O, adjusted pH to 7.2) at $2x10^7$ cells/ml. 1×10^5 EBV-transformed B cells were added to the
203 IgE serum/allergen complexes and incubated for 1 hour at 4°C on ice. Cells were then washed
204 twice to remove any unbound allergen-IgE complexes and immunostained with PE-labelled anti-
205 human IgE (Miltenyi; Biotech, Woking, UK) for 45 minutes at 4°C on ice. The cells were then
206 washed and re-suspended in FAB buffer and the percentage of cells bound by allergen-IgE
207 complexes was assessed by flow cytometry (BD FACSCanto II; BD Biosciences, San Jose, CA)
208 and data analyzed with FACS DIVA software (BD Biosciences, San Jose, CA). Five thousand
209 gated cells were analyzed and all samples were measured in triplicate.

210 Assessment of *ex-vivo* allergen-induced basophil responsiveness by flow cytometry was
211 performed on heparinized whole blood (47). Briefly, whole blood was incubated with or without
212 100 ng/ml of *P. pratense* extract (ALK-Abelló) in a 37°C water bath for 15 minutes. Cells were
213 immunostained with anti-human CD3, CD303, CD294 (CRTh2), CD63 (all BD Biosciences, San
214 Jose, CA). Erythrocytes from whole blood were lysed with BD lysing solution (BD Biosciences,
215 San Jose, CA) for 10 minutes at room temperature in the dark, samples were centrifuged (5 min,

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216 200 x g) and the supernatants discarded. The resulting cell pellets were washed in 3 ml PBS
217 (without Ca2+ and Mg2+) and re-suspended in 450 µl ice-cold fixative solution (CellFix, BD
218 Biosciences, San Jose, CA) prior to acquisition on the BD FACSCanto II flow cytometer (BD
219 Biosciences, San Jose, CA). Activated cells were also identified as CD63+CRTh2+ basophils.
220 Analyses were performed using FlowJo v10.2 (FlowJo, LLC, Oregon).

221 *Statistics*

222 In the GRASS trial, the intention to treat population included 106 randomized
223 participants of whom 92provided an evaluable primary endpoint at 3 years. (20). The per-
224 protocol (PP) population included 84 participants who remained in the study 3 years, were
225 compliant with study medications, defined as taking 50% or more of their study medication for
226 the duration of the study, and had an evaluable primary endpoint. All mechanistic data were
227 assessed in the PP population using a linear mixed model adjusted for baseline values. To be
228 consistent, clinical endpoints, nasal challenge induced total nasal symptom score (TNSS) area
229 under curve (AUC) and peak nasal inspiratory flow (PNIF) change from pre-challenge AUC,
230 were re-analyzed in the PP population using a linear mixed model adjusted for baseline values.
231 The threshold for significance was $p<0.05$ (two-sided). Since all analyses were considered
232 exploratory, p-values were not adjusted for multiple comparisons. Analyses were performed with
233 SAS Version 9.4 (SAS Institute Inc., Cary, NC) and R version 3.2.4 (R Foundation for Statistical
234 Computing).

235 *Data and materials availability*

236 Datasets and Figures from this study, along with clinical data from the GRASS trial, are
237 available on TrialShare, the Immune Tolerance Network data visualization portal, at:
238 https://www.itntrialshare.org/GRASSmech.url.

239 *Study approval*

240 Written informed consent was received from participants prior to inclusion in the study.

241 The study was approved by the National Research Ethics Committee in the UK.

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242 **Results**

243 *Peripheral antigen-specific T cells*

244 Allergen-specific T cell responses were assessed by direct *ex vivo* HLA class II tetramer
245 staining of CD4+ lymphocytes from peripheral blood. Peptide epitopes from the major Timothy
246 grass pollen allergens *Phl p* 1 and *Phl p* 5 that were used for tetramer production are shown in
247 Table I; HLA genotypes in the Table were matched for each subject analyzed. Figure 1 shows
248 representative flow cytometry analysis of these allergen-specific T cells from a study participant
249 receiving subcutaneous grass allergen immunotherapy. Detection of lymphocytes that bind the
250 tetramers—consisting of timothy grass peptides bound to HLA class II molecules—identifies
251 CD4 T cells specific for the grass pollen allergen. As shown in Figure 1A, flow cytometry
252 profiles prior to therapy identify grass pollen-specific T cells in peripheral blood that display a
253 typical allergic profile, with expression of CRTH2, CD161 and CCR4 surface markers
254 characteristic of Th2 lymphocytes involved in allergic diseases, and few cells expressing CD27.
255 Two years after continuous subcutaneous immunotherapy (SCIT), profiles of remaining allergen-
256 specific T cells have shifted as characterized by reduced frequencies of the
257 CCR4/CRTH2/CD161-positive cells, and increased frequencies of CD27-positive cells. This
258 altered pattern of cell-surface markers indicates a phenotypic change consistent with a loss of
259 Th2 cells in the circulating allergen-specific compartment. However, after one additional year off
260 therapy, this phenotypic profile has reverted back towards the original distribution of allergic
261 markers, once again displaying increased frequencies of CRTH2- and CD161-positive cells and
262 reduced frequency of CD27-positive cells in the allergen-specific CD4 T cell compartment.

263 Figure 1B summarizes these profiles for 53/84 Per Protocol population (SCIT: 16, SLIT:
264 21, placebo: 16) enrolled in the GRASS clinical trial in whom there was adequate HLA-

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265 matching for tetramer-profiling of allergen-specific T cells. In placebo-treated participants,
266 allergen-specific CD4 T cell frequencies were stable over 3 years. Participants in both the
267 subcutaneous and the sublingual therapy arms of the trial showed significant decreases compared
268 to placebo in the frequencies of allergen-specific cells, during the first year of treatment, a
269 timepoint where clinical parameters in the two arms were also similar (Fig 1C). This downward
270 trend continued in the group receiving a 2nd year of subcutaneous therapy and remained
271 significant compared to placebo, while the decline appeared to plateau in the sublingual group
272 compared to placebo at year 2 (Fig. 1B) The decrease in overall CD4 antigen-specific cells (Fig.
273 1B [left panel]) reflects the specific decrease in Th2 cells (Fig. 1B [right panel]). After two years
274 of therapy, subjects receiving subcutaneous therapy had fewer allergen-specific Th2 cells in the
275 peripheral blood compared to the sublingual therapy group. Notably, one year after
276 discontinuation of the allergen therapy, specific CD4 T cell numbers in both treatment groups
277 returned to the baseline frequencies, indicating a lack of a durable immunological effect.

278 These antigen-specific T cell profiles are remarkably concordant with the clinical
279 parameters measured in the GRASS trial (20). This is illustrated in Figure 1C for symptom-
280 related outcomes of the per-protocol subjects studied in the trial, with clinical outcomes
281 measured on the same day as collection of samples used for the T cell analysis: the total nasal
282 symptom score (TNSS), a composite clinical index (scale 0-12) of the severity of nasal
283 symptoms after nasal allergen challenge (left panel), and the peak nasal inspiratory flow (PNIF),
284 an objective measurement of nasal airflow obstruction (right panel), for the same subjects and
285 time-points. Clinical improvement was seen with both forms of immunotherapy over the two
286 year period of treatment, but reverted back to the baseline allergic parameters at year 3, one year

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287 after discontinuation of treatment. Thus, the allergen-specific CD4+ T cell frequencies measured
288 by flow cytometry of tetramer-binding cells closely paralleled clinical outcomes.

289 *Effects of therapy on local Th2 cytokines*

290 Nasal allergen challenges were performed before treatment and at yearly intervals, with
291 collection and cytokine analysis of nasal fluids. Nasal allergen challenge-induced increases in
292 interleukin (IL)-4, IL-5 and IL-13 in nasal fluids were significantly suppressed after 2 years
293 treatment with either subcutaneous or sublingual immunotherapy, as shown in Figure 2. There
294 was no treatment effect on IFN-γ or IL-10 responses, indicating that both treatment modalities
295 selectively reduced local Th2 cytokines. Similar to what we observed with allergen specific Th2
296 cells in peripheral blood, and in parallel with clinical outcomes in Figure 1C, suppression of
297 these local Th2 cytokine responses to nasal allergen provocation was not maintained one year
298 after therapy cessation at year 3.

299 These changes in mucosal cytokines together with fewer circulating allergen-specific Th2
300 cells, suggested an overall decrease of Th2 immunity in desensitized subjects. We confirmed this
301 interpretation through transcriptional analysis of antigen-reactive memory CD4+ T cells at
302 baseline and at year 2 (Supplemental Figure 1A) (27, 31). For these experiments, cryopreserved
303 PBMC were stimulated with Timothy grass extract overnight in the presence of anti-CD40, and
304 upregulation of CD154 on memory CD4+ T cells was used to identify allergen-reactive T cells.
305 Similar to the tetramer assay, both subcutaneous and sublingual treatment modalities reduced the
306 frequency of allergen-reactive memory CD4+ T cells in peripheral blood at year 2, but this
307 reduction was only statistically significant for subcutaneous immunotherapy. These CD154-
308 positive cells were isolated using fluorescence-activated cell sorting and RNA extracted for
309 transcriptional analysis; as shown in Supplemental Figure 1B. The CD27-negative population of

310 allergen-reactive cells displayed a characteristic Th2 transcript profile with increased IL-4, IL-5,
311 IL-13, IL-31 and ST2 expression, consistent with a Th2-predominant phenotype of the allergen-
312 specific CD4+ T cells impacted by specific immunotherapy.

313 *Humoral immunological outcomes*

314 As previously reported, Timothy grass pollen-specific IgG4 levels increased during
315 allergen immunotherapy, in parallel with the therapeutic response (9, 10). In order to assess the
316 functional capacity of this induced IgG, we measured the ability of post-immunotherapy serum
317 to inhibit the binding of allergen-IgE complexes to B cells (IgE-FAB), an *in vitro* surrogate of
318 IgE-facilitated antigen presentation. In this assay, serum from patients who have received
319 allergen-specific immunotherapy was evaluated for its ability to inhibit this allergen-IgE
320 complex binding (10). As shown in Figure 3A, there was a marked decrease in IgE-FAB during
321 allergen immunotherapy, for both subcutaneous and sublingual-treated subjects. Interestingly,
322 after 2 years therapy, the changes in allergen specific IgG4/IgE ratios were 10-fold higher after
323 subcutaneous compared to sublingual immunotherapy (Fig. 3B), whereas the changes in IgG-
324 associated inhibitory activity for IgE-FAB were comparable for the 2 groups. At 3 year follow
325 up, one year off therapy, the IgE-FAB binding trended back towards baseline values, but
326 remained significantly depressed relative to placebo-treated participants. This pattern was similar
327 to the profile reflected in the allergen-specific IgG4-to-IgE ratio (Fig. 3B).

328 Basophil activation is another indicator of IgE-mediated allergic response that can be
329 monitored *ex vivo* by detection of basophil surface activation markers following incubation of
330 grass pollen allergen with whole blood (32, 33). Similar to what was observed for serum
331 inhibitory activity for IgE, both therapies significantly suppressed grass pollen allergen-induced

332 basophil hyper-responsiveness as measured by surface CD63 expression. This effect persisted at
333 year 3 follow up, one year after withdrawal of treatment (Fig. 3C).

334 *Relationships between local tissue and systemic immunological parameters*

335 The overall concordance between decreased nasal Th2 cytokine measurements, lower
336 peripheral blood antigen-specific CD4+ T cells, and antigen-specific IgE activity after 2 years of
337 allergen desensitization therapy suggested coordinated immune mechanisms. To explore the
338 relationship between the treatment effect on these immunological parameters, each was plotted
339 for individual subjects as year 2 fold-change from baseline in a 3-D scatter plot. Indeed, we
340 identified a distinct co-clustering of study participants within the immunotherapy treatment arms,
341 well-demarcated from the placebo-treated controls (Fig. 4). Many participants cluster near the
342 origin of the graph, indicating synchronous allergen-specific CD4+ T cell, nasal Th2 cytokine,
343 and antibody changes, suggesting that the changes observed are reflecting the same shift in the
344 immune responses even though differences in the significance compared to placebo are
345 observed. There was no difference between the subcutaneous and sublingual treatment groups,
346 which cluster together in this analysis at the end of two years of desensitization therapy.
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348 **Discussion**

349 Allergic manifestations are largely driven by Th2-mediated immune mechanisms, and
350 allergen-specific immunotherapy may inhibit, deviate and/or delete these effector responses. In
351 the GRASS clinical trial, immunological assays were utilized to directly compare different
352 immune modalities, monitoring subjects using a highly sensitive and specific tetramer assay to
353 identify and phenotype allergen-specific CD4+ T cells, a sensitive and quantitative measurement
354 of *in vivo*, allergen-provoked nasal cytokines to measure local mucosal immunity, and two
355 assessments of peripheral allergen-specific IgE reactivity, namely inhibitory activity for IgE-
356 FAB and inhibition of allergen-stimulated peripheral basophil activation. The distinctive
357 opportunity to conduct this study in the context of a randomized, placebo-controlled trial
358 comparing two different forms of allergen administration in humans is unique, as is the
359 integration of immunobiology for antigen-specific peripheral blood T cells and humoral
360 immunity with *in vivo* target organ immune response following allergen exposure.

361 The three modalities tested—specific T cells, local cytokines, and specific humoral
362 immunity—were generally concordant while on treatment, as demonstrated by the movement
363 toward the origin of both subcutaneous and sublingual treated populations in Figure 4. Thus,
364 despite the fundamentally different routes of allergen administration and treatment between
365 sublingual and subcutaneous therapy, with corresponding site-specific differences in antigen
366 presentation, allergen immunotherapy via either route of exposure similarly reduced the
367 immunological effectors of the allergic response in each case. However, differences were evident
368 in the magnitude, timing and duration of effect: Allergen-specific CD4+ T cells were decreased
369 in frequency after one year of treatment via either the subcutaneous or sublingual route, and
370 continued to decrease during therapy for a second year only in subjects receiving subcutaneous

371 allergen immunotherapy. This decrease occurred within the Th2 subpopulation of allergen-
372 specific memory T cells, characterized by expression of CRTH2 and CD161, and lack of
373 expression of CD27 (22). Transcript analysis of allergen-reactive CD4+ T cells, purified using
374 the parameter of CD154-upregulation after allergen exposure *in vitro,* confirmed the loss of Th2
375 phenotype (Supplemental Figure 1). Similar kinetics were found in the serum immunoglobulin
376 compartment: Therapeutic elevations of the ratio of specific IgG4/IgE, as well as decreases in
377 facilitated IgE binding assays were seen with both forms of specific immunotherapy, but were
378 more prominent earlier—at one year after initiation of therapy—in the subcutaneous treatment
379 arm.

380 Clinical symptoms improved in the GRASS study participants during therapy, but this
381 response was not durable. When assessed one year after discontinuation of treatment, subjects
382 receiving either form of immunotherapy regained their responses to allergen challenge,
383 indicating a lack of immune tolerance (20). Of the three types of immunological characteristics
384 studied—circulating T cells, local tissue cytokines and systemic immunoglobulins—the allergen-
385 specific CD4+ T cell frequencies and nasal Th2 cytokine levels showed a close temporal
386 relationship with clinical outcome, reverting back to baseline values after cessation of therapy at
387 year three. This supports the concept that future clinical studies for allergen tolerance may need
388 to focus on more durable strategies for deleting or deviating the allergen-specific T cell
389 compartment; an example is the current CATNIP clinical trial (NCT 02237196) combining anti-
390 TSLP with allergen-specific immunotherapy.

391 IgG4/IgE and functional assays of IgE-FAB and basophil activation showed a slightly
392 different pattern compared to the T cell responses (Fig. 3). Increases in specific IgG4/IgE ratio
393 and inhibition of IgE-FAB (which is known to be largely IgG4-associated (34)) were less marked

394 for sublingual compared to subcutaneous immunotherapy at year 1. In contrast to the T cell
395 response, changes in IgG4/IgE-blocking activity in the intervention groups persisted until year 3,
396 one year after discontinuation, although the magnitude was reduced compared to the year 2
397 values. This could simply reflect a slower change in immunoglobulin levels compared to the T
398 cell profiles over time, or alternatively might indicate the potential for uncoupling of B cell
399 associated responses—raising the possibility that B cells might be more amenable to long-term
400 tolerance effects of specific immunotherapy.

401 Previous studies of allergen-responsive T cells during immunotherapy have revealed
402 immune deviation away from Th2 in favor of Th1 responses (35, 36) whereas others have shown
403 no change in T cell phenotype (37, 38). PBMCs harvested during immunotherapy have shown
404 suppression of allergen-stimulated proliferation, accompanied by increases in TGF-beta (39, 40)
405 and/or IL-10 (39, 41-43) in culture supernatants or by ELIspot assay (44). These changes were
406 accompanied by increases in phenotypic Tregs as determined by flow cytometry (36, 39-41). In
407 two studies, immunotherapy-induced suppression of allergen-stimulated T cell proliferation was
408 reversed by the addition of either anti-IL-10 (36) or TGF-beta soluble receptor (40) to the
409 cultures. Suppression of allergen-reactive Th2 cells by measurement of allergen-stimulated
410 CD154+ CD4+ T cells has previously been shown during subcutaneous immunotherapy (45),
411 whereas studies of class II tetramer-positive T cells have been variable, with trials suggesting
412 either decreases (22, 46, 47) or no change (48) in tetramer-positive cells after immunotherapy.
413 The present study demonstrates clear decreases in tetramer-positive phenotypic Th2 cells that
414 parallel the clinical response during and following specific immunotherapy, similar for both
415 subcutaneous and sublingual modes of allergen administration.

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416 Transient increases in specific IgE and IgG4/IgE ratios have previously been
417 demonstrated (9, 10) but not in a long-term comparison of sublingual and subcutaneous
418 immunotherapy (20). In this study, while the onset of blocking antibody activity was slower for
419 sublingual immunotherapy, it persisted at 3 years follow up and was equivalent to that observed
420 for subcutaneous immunotherapy despite a 10-fold lower increase in specific IgG4/IgE ratio for
421 sublingual immunotherapy after two years desensitization (Figure 3). This highlights likely
422 differences between the two routes of delivery where local antigen processing via the sublingual
423 route may possibly result in fewer IgG4 antibodies but with higher avidity and/or affinity and
424 greater IgE-blocking activity.

425 The transient clinical benefit from two years of immunotherapy in the GRASS study
426 stands in contrast to previous allergy clinical trials (49, 50) that demonstrated more durable
427 benefit from three years of treatment, suggesting that 3 years of desensitization may be required
428 for sustained effects. Our finding of a close temporal relationship between the frequency of
429 allergen-specific circulating Th2 cells and this transient clinical outcome could represent a causal
430 relationship, in that recurrence of the T cell 'drivers' of allergic immunity may have abrogated
431 the potential for durable tolerance. On the other hand, persistence of IgE-blocking antibody (9,
432 20) may be an early indicator of a pro-tolerogenic mechanism. If this hypothesis is correct, then
433 future strategies for allergen immunotherapy could be directed both at enhancing persistent
434 depletion of the allergen-specific Th2 cell population for optimal induction of tolerance and
435 augmenting the antigen-specific therapeutic B cell response for maintenance of long-term effect.
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437 **Author contributions**

438 S.R.D, S.J.T., G.T.N., K.M.H. W.W.K. and A.T. contributed to concept development and
439 experimental design, A.R., M.H.S, G.W.S, P.A.W. and E.W. collected data and performed
440 experiments. T.Q. helped with data analysis and visualization. M.H.S., K.M.H, T.Q., G.W.S,
441 A.T, G.T.N., W.W.K. and S.R.D. aided in interpretation of the data. K.M.H, G.T.N and S.R.D
442 wrote the manuscript. All authors made contributions to the final manuscript prior to submission.

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601 **Figures**

602 **Fig. 1**. Analysis of allergen-specific CD4 T cells and relationship to the clinical parameters
603 measured during the GRASS trial. (**A**) Representative flow cytometry analysis of
604 tetramer bound allergen-specific T cells from a study subject receiving subcutaneous
605 allergen immunotherapy. Lymphocytes that bind the pooled tetramers are displayed on
606 the y axis and expression of phenotypic cell surface markers CD4, CRTH2, CD27,
607 CD161 and CCR4 are displayed on the x axis at baseline, at 2 years after continuous
608 SCIT therapy and at 3 years after one year off therapy. (**B**) Frequencies of allergen-
609 specific CD4 T cells were determined for 53 HLA-DR4 subjects in the GRASS trial, by
610 tetramer binding (left panel). Frequencies of Th2 cells are identified by
611 CD161+CRTH2+CD27-CCR4+T4+/CD45RA-T4+ phenotypic marker expression (right
612 panel). (**C**) The total nasal symptom score (TNSS) average AUC (left panel) and the peak
613 nasal inspiratory flow (PNIF) average AUC (right panel) for 0-10 hours following
614 allergen challenge were measured at baseline and years 1-3 for all participants treated
615 with sublingual immunotherapy (green), subcutaneous immunotherapy (red), and placebo
616 (blue). Significant differences are indicated by * ($p<.05$), ** ($p<.01$). Data are shown as
617 means with 95% confidence intervals, for the 84 per-protocol subjects enrolled in the
618 GRASS trial.

619

620 **Fig. 2.** Cytokine levels of nasal fluids following nasal allergen challenge. The levels of cytokines
621 IL-4, IL-5 and IL-13 in nasal fluids for 10 hours following nasal allergen challenge are
622 displayed from study subjects at baseline, after 2 years of desensitization and at 3 years,
623 one year after discontinuation of desensitization therapy. Participants treated with
624 sublingual immunotherapy are displayed in green, subcutaneous immunotherapy in red
625 and placebo in blue. Data are shown as means with 95% confidence intervals.
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627 **Fig. 3.** Allergen-specific IgE-dependent functional assays. (**A**) The impact of the IgG4 increase on the ability of
628 allergen-IgE complexes to bind B cells was measured by IgE-FAB assay. The serum from patients was evaluated for
629 ability to inhibit allergen-IgE complex binding at baseline and years 1-3. (**B**) Grass pollen-specific IgG4 and IgE
630 were monitored and the IgG4/IgE ratio is displayed for subjects at baseline, after years 1 and 2 during
631 desensitization therapy and at 3 years after discontinuation of therapy. (**C**) Basophil surface activation markers from
632 whole blood of participants treated with sublingual immunotherapy (green), subcutaneous immunotherapy (red) and
633 placebo (blue) at baseline and years 1-3 after incubation with grass pollen allergen. Significant differences are
634 indicated by * ($p<.05$), ** ($p<.01$). Data are shown as means with 95% confidence intervals.

635

636 **Fig. 4.** Relationship between nasal cytokine measurements and lower peripheral blood antigen-specific T cells
637 (memory tetramer + cells) and serum antigen-specific IgE-FAB for each individual studied. Data displayed are
638 expressed as fold changes from the baseline at year 2 (on treatment) for each immunological parameter measured for
639 participants treated with Sublingual immunotherapy (green diamonds), Subcutaneous immunotherapy (red
640 triangles), and placebo (blue circles), so values <1 on each axis represent reduction (improvement) in the parameters
641 shown. Nasal cytokine measurements are the summation of area under curve (AUC) from 2 to 10 hours post-
642 challenge for Th2 cytokines (IL-4, IL-5 and IL-13). Lower peripheral blood antigen-specific T cells are measured as
643 the frequency of memory tetramer + cells per million CD4+ cells. Serum antigen-specific IgE-FAB is measured as
644 the percentage of allergen-IgE binding to B cells. Cluster distributions were compared using a Hotelling T-square
645 test, as follows: Placebo vs. SCIT: $p<0.001$; Placebo vs. SLIT: $p<0.001$; SLIT vs. SCIT: $p=0.31$. An online
646 interactive version of this Figure is available at https://www.itntrialshare.org/GRASSmech_fig4.url.

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648 **Table I.**

649 Peptide epitopes from the major grass allergens used for HLA class II tetramer production.

HLA	Class II tetramers	AA sequences
DRB1*0101	Phl p 1 153-172	KGSNPNYLALLVKYVNGDGD
	Phl p 5b 26-45	KLIEDINVGFKAAVAAAASV
DRB1*0301	Phl p 1 169-188	GDGDVVAVDIKEKGKDKWIE
DRB1*0401	Phl p 1 97-116	EEPIAPYHFDLSGHAFGAMA
	Phl p 1 221-240	TEAEDVIPEGWKADTSYESK
DRB1*0701	Phl p5a 119-138	PEAKYDAYVATLSEALRIIA
	Phl p5b 90-109	ATPEAKFDSFVASLTEALRV
DRB1*1001	Phl p5a 32-51	GKATTEEQKLIEKINAGFKA
	Phl p5a 103-122	LDAAYKLAYKTAEGATPEAK
	Phl p5a 167-186	VDAAFKVAATAANAAPANDK
DRB1*1101	Phl p 1 153-172	KGSNPNYLALLVKYVNGDGD
	Phl p 1 185-204	KWIELKESWGAIWRIDTPDK
	Phl p5a 79-98	FAEGLSGEPKGAAESSSKAA

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651 **Supplementary Materials:** **Supplemental Figure 1**

652 **Fig. S1.** Antigen reactive memory CD4+ T cells and mRNA expression profiles. (**A**)
653 Frequency of antigen reactive cells after 2 years of desensitization treatment based on CD154 up-
654 regulation after allergen stimulation. Participants treated with sublingual immunotherapy are
655 displayed in green, subcutaneous immunotherapy in red and placebo in blue. An Analysis of
656 Covariance (ANCOVA) model with baseline adjustment was used for analyzing CD154 assay
657 data. Data are shown in the plot as means of log10 fold change from baseline with 95%
658 confidence intervals.

659 (**B**) Transcriptional analysis of extracted RNA from CD27+CD154+ and CD27- CD154+ cells
660 at the 2-year timepoint. Transcript levels are displayed in color scale from high (yellow) to low
661 (blue). Genes are grouped by category with Th2 in red, apoptosis in grey and Th1 related genes
662 in purple.

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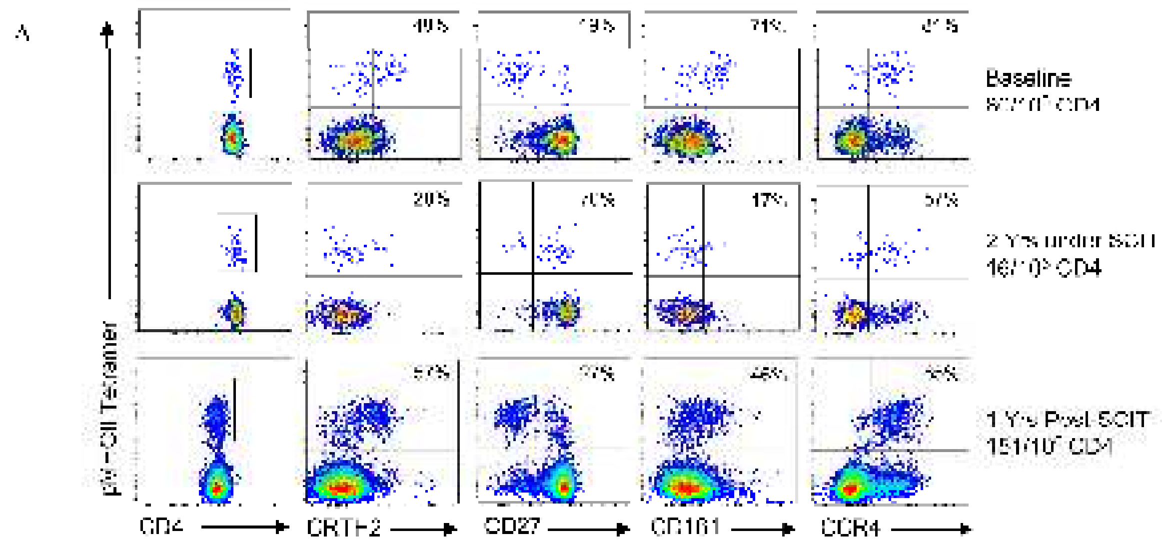
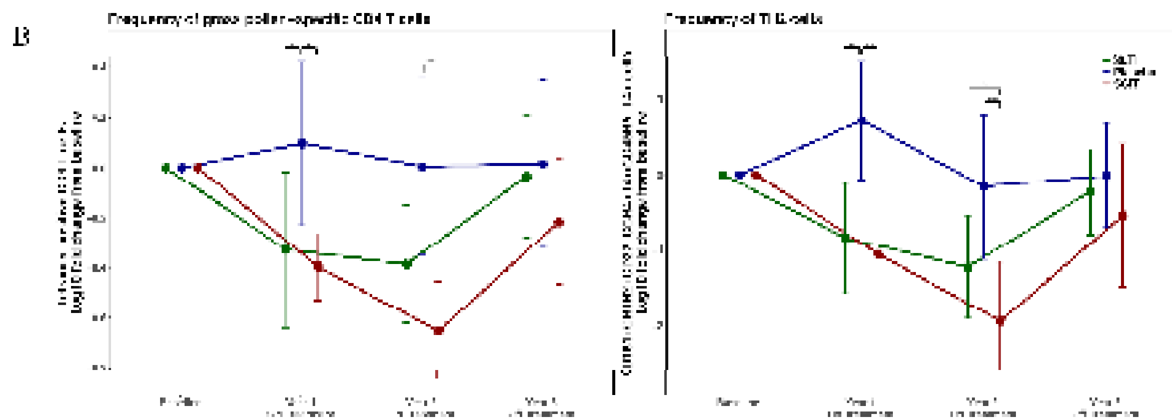
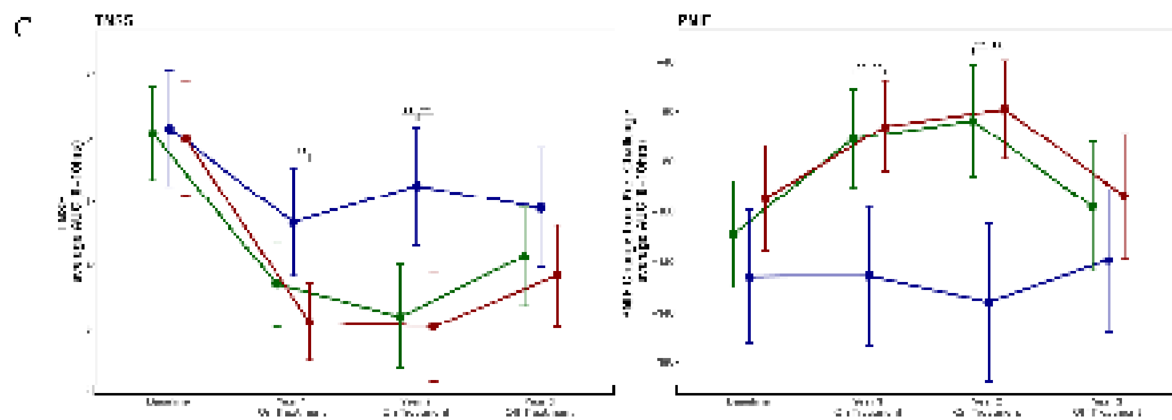
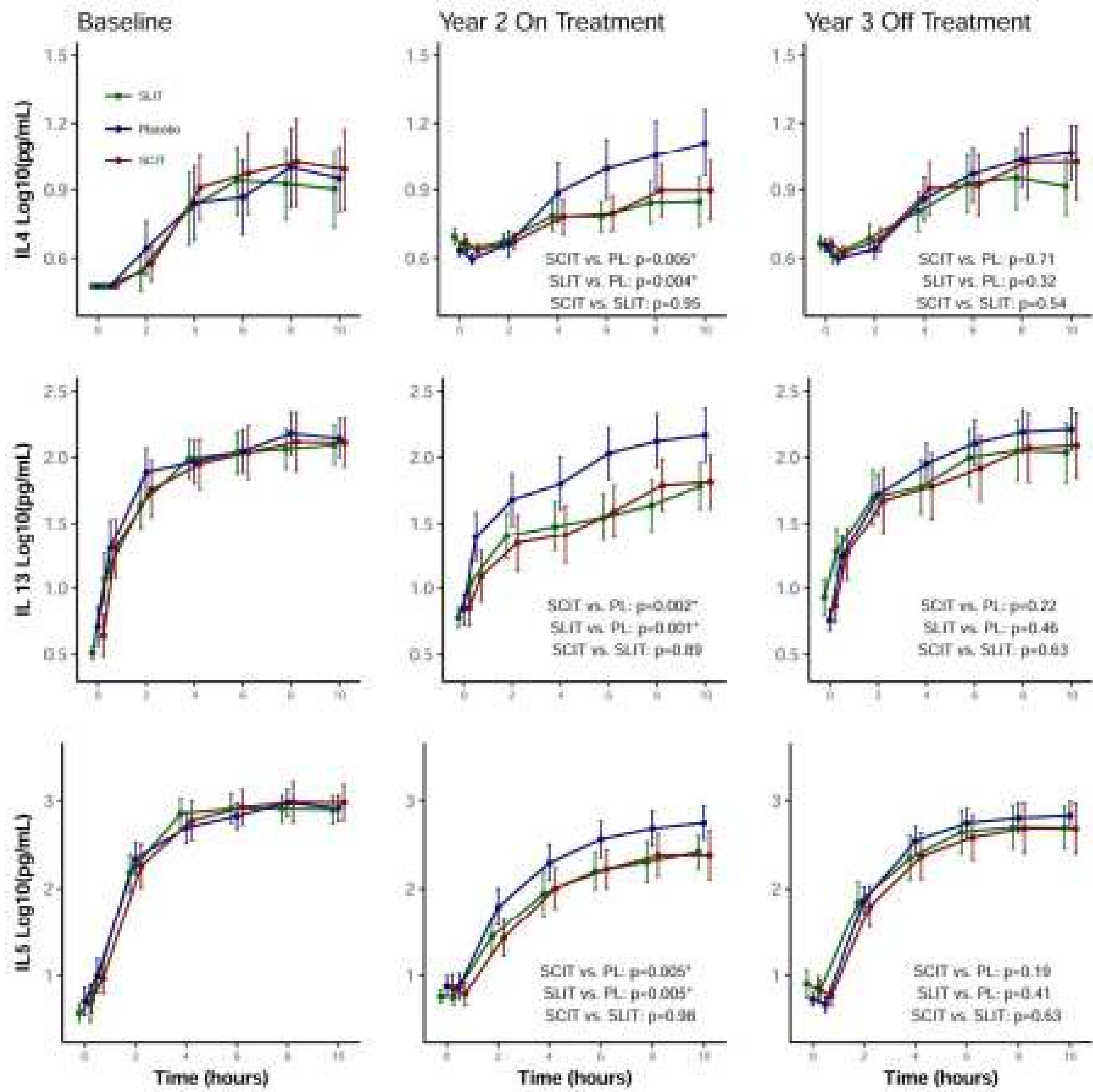

A
Baseline
2 Yrs under SCIT
1 Yrs Post SCIT
CD4
CRTH2
CD27
CD161
CCR4

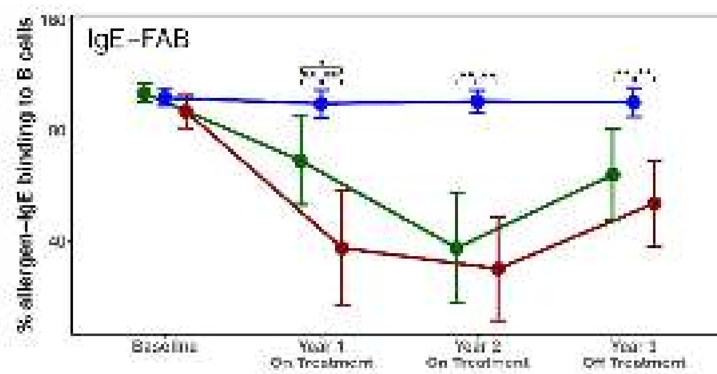

B
Frequency of grass pollen-specific CD4 T cells
Frequency of TH2A cells


C
TNSS

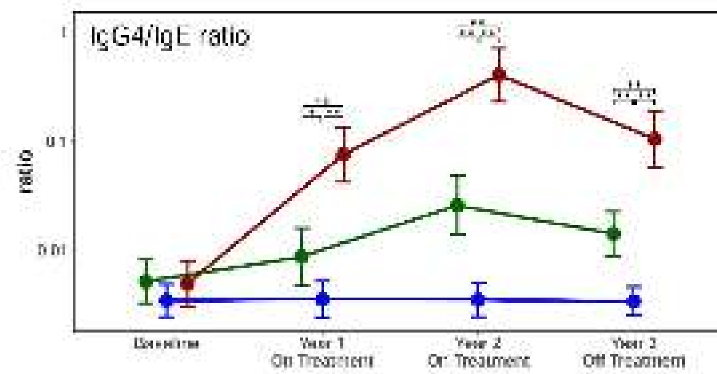


Baseline
Year 2 On Treatment
Year 3 Off Treatment
SLIT
Placebo
SCIT
IL4 Log10(pg/mL)
IL 13 Log10(pg/mL)
IL5 Log10(pg/mL)
SCIT vs. PL: p=0.006*
SLIT vs. PL: p=0.004*
SCIT vs. SLIT: p=0.95
SCIT vs. PL: p=0.71
SLIT vs. PL: p=0.32
SCIT vs. SLIT: p=0.54
SCIT vs. PL: p=0.002*
SLIT vs. PL: p=0.001*
SCIT vs. SLIT: p=0.89
SCIT vs. PL: p=0.22
SLIT vs. PL: p=0.46
SCIT vs. SLIT: p=0.63
SCIT vs. PL: p=0.005*
SLIT vs. PL: p=0.005*
SCIT vs. SLIT: p=0.98
SCIT vs. PL: p=0.19
SLIT vs. PL: p=0.41
SCIT vs. SLIT: p=0.63
Time (hours)

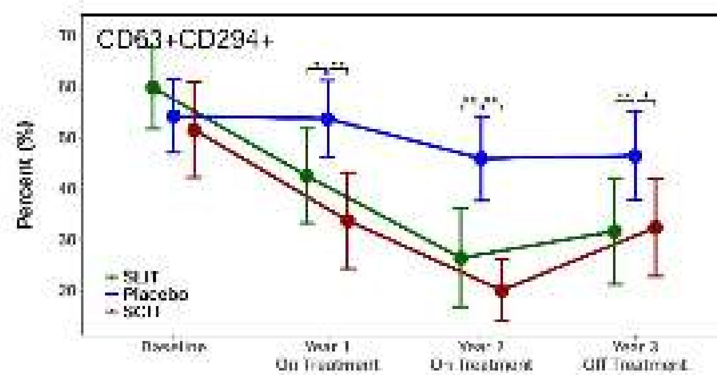
A



B



C



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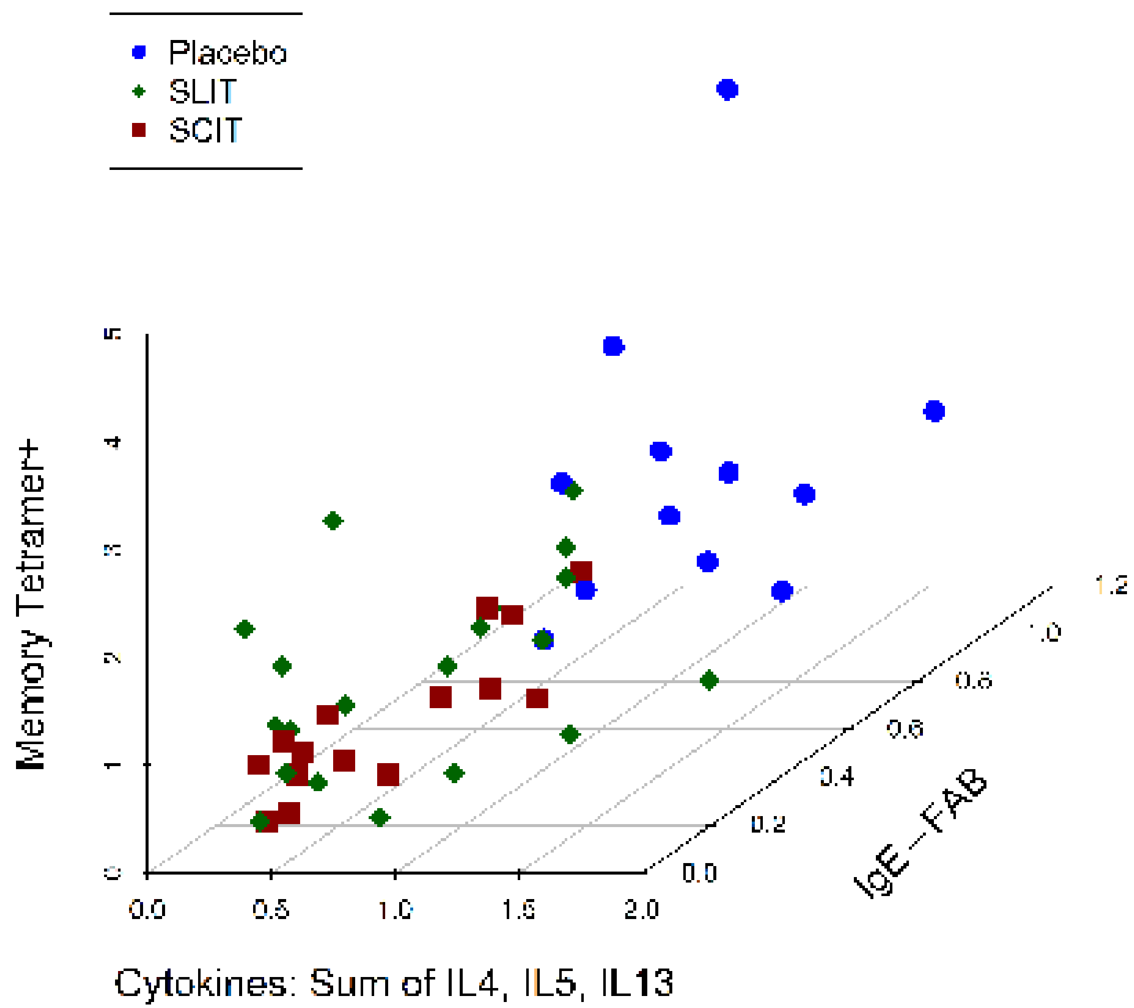

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1 **Supplementary Materials: Supplemental Figure 1**

2 **Fig. S1.** Antigen reactive memory CD4+ T cells and mRNA expression profiles. (**A**)
3 Frequency of antigen reactive cells after 2 years of desensitization treatment based on CD154 up-
4 regulation after allergen stimulation. Participants treated with sublingual immunotherapy are
5 displayed in green, subcutaneous immunotherapy in red and placebo in blue. An Analysis of
6 Covariance (ANCOVA) model with baseline adjustment was used for analyzing CD154 assay
7 data. Data are shown in the plot as means of log10 fold change from baseline with 95%
8 confidence intervals.

9 (**B**) Transcriptional analysis of extracted RNA from CD27+CD154+ and CD27- CD154+ cells
10 at the 2-year timepoint. Transcript levels are displayed in color scale from high (yellow) to low
11 (blue). Genes are grouped by category with Th2 in red, apoptosis in grey and Th1 related genes
12 in purple.
13

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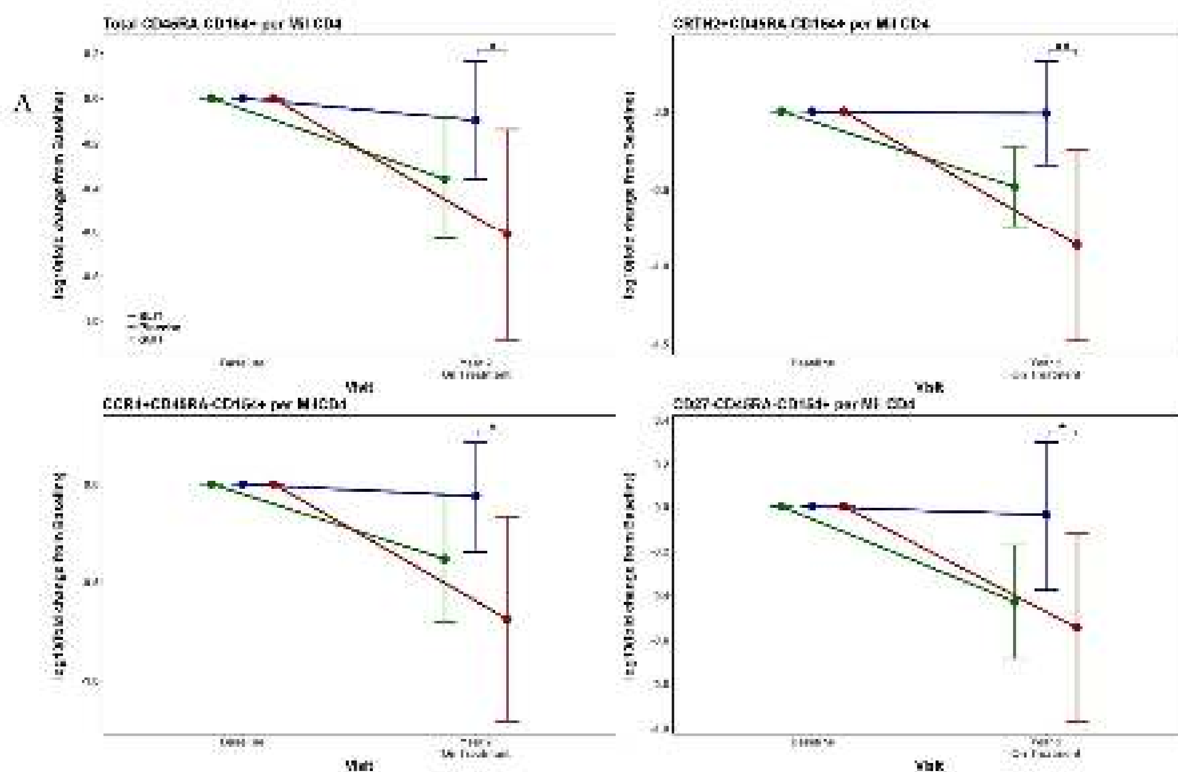
A



B