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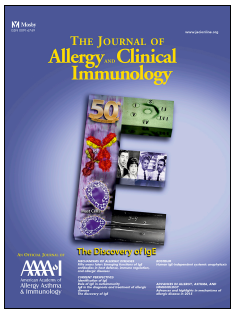

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INTRADERMAL GRASS POLLEN ALLERGEN IMMUNOTHERAPY FOR SEASONAL ALLERGY: A RANDOMIZED CONTROLLED TRIAL

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ABSTRACT

Background:

Repeated low dose grass pollen intradermal allergen injection suppresses allergen-induced cutaneous late phase responses, comparable with conventional subcutaneous and sublingual immunotherapy.

Objective:

To evaluate the efficacy and safety of grass pollen intradermal immunotherapy in the treatment of allergic rhinitis.

Methods:

We randomly assigned 93 adults with grass pollen allergic rhinitis to receive 7 pre-seasonal intradermal allergen injections (containing 7 nanograms of Phl p 5 major allergen) or histamine control. The primary endpoint was daily combined symptom-medication scores during the 2013 pollen season (area under curve). Analysis was by intention-to-treat. Skin biopsies were collected following intradermal allergen challenges and late phase responses measured four and seven, ten or thirteen months post-treatment.

Results:

There was no significant difference in primary endpoint between treatment arms (active n=46, control n=47, median difference, 14; 95% CI -172.5-215.1; P=.80). Among secondary endpoints, nasal symptoms were worse in the intradermal treatment group, measured by daily scores (median difference, 35; 95% CI 4.0-67.5; P=.03) and visual-analog scales (median difference, 53; 95% CI -11.6-125·2; P=.05). In a per protocol analysis, intradermal immunotherapy was further associated with worse asthma symptoms and fewer symptom free days. Intradermal immunotherapy increased serum Phl p-specific IgE (P=.001) compared to the control arm. T cells cultured from biopsies of intradermal immunotherapy subjects showed higher expression of Th2 surface marker CRTH2 (P=.04) and lower Th1 marker CXCR (P=.01), respectively. Late phase responses remained inhibited seven months after treatment (P=.03).

Conclusion:

Intradermal allergen immunotherapy suppressed skin late responses but was not clinically effective and resulted in worsening of respiratory allergic symptoms.

Clinical Implications

Repeated intradermal allergen exposure has the potential to exacerbate rather then ameliorate allergic airway disease, with possible implications for novel immunotherapy strategies that promote dermal allergen exposure.

Capsule Summary

Grass pollen intradermal allergen immunotherapy was not clinically effective, but worsened seasonal allergic rhinitis symptoms with implications for novel immunotherapy that targets allergen delivery to the skin.

Key Words:

Allergy immunotherapy, allergic rhinitis, grass pollen, Phleum Pratense, immunotherapy, intradermal, low-dose.

Abbreviations used:

ANCOVA: Analysis of covariance

ANOVA: Analysis of variance

ARIA: Allergic rhinitis and its impact on asthma

ITT: Intention-to-treat

MHRA: Medicines & Healthcare products Regulatory Agency

Mini-RQLQ: Mini-rhinoconjunctivitis quality of life questionnaire

SQ: Standardized quality

VAS: Visual analog scale

WAO: World Allergy Organization

INTRODUCTION

Immunotherapy with grass pollen for seasonal allergic rhinitis is a longstanding and clinically effective treatment.[1,2] Conventional immunotherapy vaccines involve administration of high doses of allergen (typically 10-20 microgram quantities of major allergens) by regular subcutaneous injection or as daily sublingual tablets, although both approaches have limitations. Subcutaneous immunotherapy is associated with a risk of systemic allergic reactions and therefore injections require specialist supervision. Sublingual immunotherapy requires self-daily dosing for 3 years and non-adherence is relatively commonplace.[3]

Intradermal allergen injection in sensitized subjects results in a localized wheal with erythema within 15 minutes (early phase response), followed by diffuse indurated swelling that persists for 24-36 hours (late phase response). The late phase response is accompanied by infiltration of activated Th2 cells, eosinophils and basophils, features that characterize chronic allergic inflammatory responses.[4] We previously reported that repeated intradermal injections of grass pollen extract every two weeks leads to a progressive and systemic attenuation of the macroscopic skin late phase responses induced by these injections.[5] After six intradermal injections - each containing the equivalent of 7 nanograms of the major allergen Phl p 5 - late phase responses were more than 90% suppressed, comparable to the degree of suppression achieved following conventional subcutaneous grass pollen immunotherapy containing over a thousand-fold greater cumulative allergen doses.

The concept of intradermal grass pollen allergen inoculation as a treatment for allergic rhinitis is not without precedent. In 1926, Phillips, a physician in Arizona, published a preliminary account of his experiences with intradermal grass pollen immunotherapy in 29 patients,[6] extended to 322 patients by 1933,[7] reporting that over 90% obtained “satisfactory relief”. Here we report the findings of the first randomized placebo-controlled clinical trial of intradermal grass pollen injections for seasonal grass pollen allergy. The Pollen Low dose Intradermal Therapy Evaluation (PollenLITE) was conceived to test the hypothesis that skin late phase response suppression following intradermal grass pollen administration is associated with clinical improvement in adults with seasonal allergic rhinitis.

METHODS

Study design

PollenLITE was a single centre, randomized placebo controlled double-blind phase 2 trial conducted at Guy's Hospital in London, investigating the efficacy and safety of seven pre-seasonal intradermal injections of *Phleum pratense* (Timothy grass) pollen extract versus histamine control (Fig 1). The National Research Ethics Service Committee London–Harrow (12/LO/0941) and MHRA approved the study, with oversight by King's Health Partners Clinical Trial Office and an independent trial steering committee. The clinical trial protocol[8] was finalized prior to randomization and the statistical analysis plan finalized prior to unblinding and data analysis. All participants provided written informed consent in accordance with the Declaration of Helsinki.

Participant selection

Ninety-three participants were recruited using advertisements in press, online and on public transport and a dedicated trial website. Eligible participants were aged 18-65 years with moderate-severe grass pollen allergic rhinitis according to ARIA classification[9] positive skin prick test (at least 3 mm diameter) and specific IgE (at least class two) to *Phleum pratense*. Exclusion criteria included seasonal grass pollen-induced asthma requiring regular albuterol or inhaled corticosteroids; symptomatic seasonal allergic rhinitis and/or asthma due to tree or weed pollen overlapping the grass season requiring regular treatment; perennial rhinitis and previous life-threatening anaphylaxis. The full inclusion and exclusion criteria are described in the Online Repository.

Randomization

Participants were randomized 1:1 by King's Clinical Trial Unit by block randomization using a 24-hour web-based system, with stratification according to skin test response size to grass pollen and presence of rhinitis symptoms outside the grass pollen season.

Study Procedures

Seven intradermal active or control histamine forearm injections were administered 2-weekly before the 2013 grass pollen season (February 18-May 24, 2013). Each active injection contained 10 Biological Units (BU) (33.3 SQ-U; 7 nanograms major allergen Phl p 5) of *Phleum pratense* (Aquagen SQ Timothy, ALK Abello, Reading UK) in a 20 microliter volume. This regimen was chosen based on our previous study showing that 6 injections at the same dose and interval led to 90% suppression of the late phase response in the skin. Histamine

control was administered at 100 μg/ml for the first two injections, reduced to 30 μg/ml for the second two injections, and then 10 μg/ml for the final injections, to help preserve blinding. Details of active and placebo manufacture are supplied in the Online Repository. Antihistamines were avoided 5 days prior to intradermal injections, so that a wheal in response to the injection could be confirmed. All participants were observed for systemic reactions after the first injection for one hour, and for 30 minutes after subsequent injections. Participants completed diary cards during the 2013 grass pollen season, recording symptoms and rescue medication usage.

Study Outcomes

The primary outcome was a combined symptom and medication score during the grass pollen season (May 13-August 31, 2013; 111 days) as recommended by World Allergy Organization (WAO) guidelines for allergic rhinitis immunotherapy trials.[10] (see Online Repository for details of symptom and medication scoring).

Pre-defined secondary clinical endpoints were overall symptom scores, individual nose, mouth, eye and lung symptom scores, overall medication scores, combined symptom and medication scores during the peak season, visual-analog scale (VAS) scores for nose and eye symptoms (two-weekly), mini-Rhinitis Quality of Life Questionnaire scores (mini-RQLQ) and health related quality of life scores (EQ-5D-5L) (four time points) a global evaluation of symptoms (at end of season), number of symptom and medication free days and number of days prednisone was used. Adverse events were recorded for all patients who received at least one dose of study drug (see Online Repository). To verify blinding, participants guessed whether they had received the active or control intervention after the 2013 pollen season.

In September 2013, i.e. 4 months after completion of intradermal treatment injections, cutaneous early (15 minutes) and late phase responses (24 hours) were measured after intradermal injections of grass pollen (identical to treatment dose) and diluent (ALK Abelló). Twenty participants per treatment arm were also randomized to undergo 3mm punch biopsies from these sites after 24 hours. Biopsies were all analyzed by immunohistochemistry for numbers of eosinophils, neutrophils, CD3+ T cells and CD4+ T cells. In half of participants who underwent biopsy, the biopsies were divided into two fragments, with the second fragment used for T cell expansion, flow cytometric evaluation of Th1/Th2 markers and microarray analysis. Blood specimens were collected for *Phleum pratense*-specific IgE and IgG levels, and basophil activation studies. Subjects were also randomized for repeat late phase response measurements at either seven, ten or thirteen

months following treatment completion. Further methodological information is provided in the Online Repository.

Statistical Analysis

Details of the power calculation are provided in the Online Repository. All analyses were pre-defined in a detailed statistical analysis plan and overseen by a data monitoring committee. Primary outcome analysis, performed on an intention-to-treat basis, included all participants who were randomized without imputation for missing data. Differences between the groups in AUC of the combined symptom and medication scores, the primary outcome, were assessed using a stratified Mann-Whitney U test (van Elteren test), adjusted for baseline stratification factors. The stratified Hodges-Lehmann estimation was used to calculate median differences with confidence intervals. Similar analyses were conducted for total and organ symptom scores, medication scores and VAS scores. Mini-RQLQ and EQ-5D-5L scores were evaluated by linear mixed models with 95% confidence intervals. Sensitivity analyses were performed with missing data imputed, utilizing mean scores on the day concerned and in the relevant trial arm, for primary and secondary outcomes in the intention-to-treat population. Analyses were also performed in the pre-defined per-protocol population. All mechanistic analyses were by Mann Whitney U Test, except serology and immunohistochemistry, which were analyzed by ANCOVA. Wilcoxon signed-rank test was used to compare pre- versus post-treatment serology, and diluent control versus allergen challenge immunohistochemistry results.

The principal software package was SAS/STAT®, with verification of results from Syntax for selected analyses analyzed in Stata®. This trial was registered with Current Controlled Trials, number ISRCTN 78413121.

RESULTS

Study participants

A total of 93 participants were randomized. All could be evaluated for the primary outcome in the intention-to-treat analysis (Fig 2). Baseline characteristics were well balanced between groups (Table I). All 46 participants receiving intradermal allergen immunotherapy completed the treatment course; one delayed an injection by one day due to a scheduling conflict. One of 47 participants assigned to control injections withdrew after the second injection due to work commitments, and another delayed an injection by four days due to an upper respiratory tract infection. Missing diary data for the primary endpoint were few, with 94% of participants supplying over 90% of daily data. One patient completed less than the pre-determined per-protocol 50% threshold of daily data and was excluded from the per-protocol population. Five participants, all in the control arm, significantly deviated from protocol-specified use of rescue medications. After the pollen season, participants were unable to identify if they had received active allergen or histamine control treatment (Table E1, Online Repository).

Primary Outcome

There was a clear temporal relationship between the combined symptom and medication scores and daily pollen counts (Fig 3, *A*), which peaked at above-average levels. Intradermal immunotherapy did not significantly affect the primary endpoint, i.e. the combined symptom and medication score over the entire grass pollen season (111 days) (difference in median AUC, 14; 95% confidence interval [CI], -172.5 to 215.1; P = .80) (Fig 3, *B*; Table II).

Secondary Outcomes

No significant group differences were also seen in secondary endpoints of overall symptom scores (P = .24) and rescue medication use (P = .44) during the whole season and combined symptom and medication scores during the peak season (June 12–July 26, 2013) (P = .90) (Table II).

Amongst other secondary endpoints, allergic rhinitis symptoms measured by daily nasal symptom scores were 44% higher in the intradermal allergen immunotherapy group, with a difference in median AUC of 35 (95% CI, 4.0 to 67.5; P = .03) (Fig 3, *C*). Rhinitis symptoms measured by VAS were 28% higher in the intradermal allergen immunotherapy group, with a difference in median AUC of 53 (95% CI, -11.6 to 125.2; P = .05) (Fig 3, *D*). No significant differences were seen between groups in daily eye or lung symptoms (Table II),

although mouth symptoms tended to be higher in the intradermal allergen group (median difference of AUC = 10.0; 95% CI, 3.8 to 24; P = .05). No significant group differences were observed in eye symptoms measured by VAS, mini-RQLQ scores, EQ-5D-5L scores, global evaluation of symptoms scores, or number of symptom or medication free days or number of days prednisone was taken.

In the per-protocol analysis (Table III) the individual nasal (P = .02) and mouth (P = .02) daily symptom scores were significantly higher in the active group, whilst lung daily symptom scores (P = .05) and overall symptom score (P = .09) tended to significance. Active group participants also had significantly worse nasal symptoms measured using visual-analogue scales (P = .008) and recorded fewer symptom free days than subjects in the control group (P = .04). In the intention-to-treat analysis, when missing data were imputed (Table E2, Online Respository), nasal daily symptoms scores (P = .03) and VAS nasal symptoms were statistically significant (P = .02) and mouth symptoms tended to be higher (P = .05).

As allergic rhinitis nasal symptoms were unexpectedly worse in intradermal immunotherapy participants, we performed *post-hoc* analyses comparing daily data for each individual allergic symptom between groups (Table IV). In the active group, sneezing (P = .01), cough (P = .02), chest tightness (P = .08) and mouth itching (P = .06) were higher, whilst eye swelling was lower (P = .03). Individual nasal symptoms measured by VAS also revealed higher scores after intradermal immunotherapy for rhinorrhoea (P = .006), sneezing (P = .006) and nasal itching (P = .003) (Table 4).

The frequency of adverse events was similar between groups. The frequency of treatment-related adverse events was low: 3 (6.5%) and 6 (13%) participants in the intradermal immunotherapy and control group, respectively, experienced mild systematic reactions manifested as generalized pruritus only, except for one intradermal allergen participant who developed erythema tracking from the injection site in a lymphatic distribution ('IgE-mediated lymphangitis') 20 minutes after each injection. There were 3 serious adverse events all unrelated to treatment: 1 (2.2%) in the active and 2 (4.3%) in the control group. (Table E3, Online Repository).

Immunologic findings

Serological assessments pre- (October 2012) and post-treatment (May 2013) showed a typical seasonal fall in allergen-specific IgE in the control group (P < .001), which was significantly less in the intradermal allergen immunotherapy group (P = .001), indicating a treatment-induced relative increase in allergen-specific IgE (Fig 4, *A*). A treatment effect was also seen on *Phleum pratense*-specific IgG (P = .03) (Fig 4, *B*) and IgE

titres to major grass allergens Phl p 5 and Phl p 1 (Fig E1, Online Respository), although no effect was seen on IgG_4 responses (not shown).

CD4+ T cells expanded from 19 of 20 skin biopsies collected post intradermal grass pollen challenge after the 2013 grass pollen season, showed higher expression of Th2 marker CRTH2 in the active group (median 13.4% (IQR 6.3 to 25.4)) compared to the control group (6.3% (IQR 1.9 to 7.6)) (P = .04), whereas expression of Th1 cell marker CXCR3 was lower (33.5% (IQR 24.7 to 47.3) vs. 56% (IQR 45.8 to 63.8); P = .01) (Fig 4, *B* and Fig E2, Online Respository). No differences were seen in expression of Th17 marker, CCR6 (data not shown). Insufficient T cells could be expanded from diluent challenged skin biopsies for analysis. Microarray transcriptional profiling performed on cultured T cells from 15 allergen-challenged skin biopsies, showed only 14 genes that were significantly over-expressed in the active group, (defined as >1.5-fold higher expression than control group and P < .05 using a 3 way-ANOVA model), including interleukin-5, but no other Th2- or Th1-related genes (Table E4, Online Repository; microarray Gene Expression Omnibus Accession number GSE72324; Fig. E3, Online Repository for heat map of cytokines and relevant transcription factors). GO analysis did not highlight a broader effect on Th2 or inflammation-related genes. No significant treatment effect was seen on surface expression of peripheral blood basophil activation markers (Fig E4, Online Repository) or on numbers of eosinophil, neutrophil, CD3+ T cells and CD4+ T cells following immunohistochemical staining of diluent and allergen challenged skin biopsies (Fig E5, Online Repository).

Skin challenge results

Early (15 minutes) and late phase (24 hour) skin responses could be measured in 86 participants four months after the final intradermal allergen injection (September 2013), and then repeated at either seven, ten or thirteen months. The size of late phase responses in the control group was consistent with that reported in our previous study under the same conditions[5] (shown for comparison in Fig 4, *C*). In the present trial, the late phase response was still suppressed four and seven months after completing intradermal allergen treatment (P = .03 for both time points), but not at 10 or 13 months. In comparison with the historical data however, suppression at these times was less than that which we observed immediately after completing six injections (Fig 4, *C*), suggesting that the suppressive effect on late phase responses was wearing off within four months**.**

DISCUSSION

In this phase 2, randomized, double-blind, placebo-controlled trial in adults with moderate-severe allergic rhinitis, pre-seasonal treatment with intradermal grass pollen injections did not affect the primary endpoint (combined symptom and medication scores during the 2013 grass pollen season). These findings repudiate our hypothesis that suppression of cutaneous late phase responses following repeated intradermal low dose grass pollen injections[5] would be associated with clinical improvement of allergic rhinitis. Intradermal allergen immunotherapy was associated with 44% worse allergic rhinitis nasal symptoms as measured by daily symptom scores and 28% worse symptoms as measured by VAS, although the trial was neither designed nor powered to detect deterioration of symptoms. These findings were consistent when missing data were imputed. In the per-protocol population, in addition to worsening of nasal symptoms measured both daily and by VAS, there were worsening of lung and mouth symptoms and significantly fewer symptom-free days.

No serious adverse events attributable to grass pollen intradermal allergen immunotherapy occurred. 92 of the 93 participants completed the full injection course; one withdrew for unrelated reasons. Five participants deviated significantly from the protocol in use of rescue medications, mainly using excessive antihistamines, topical nasal steroid or eye drops. Two of these participants also used prednisone without study physician guidance. We are unable to account for why these five participants were all in the control arm, although their exclusion from the per-protocol population did not affect the conclusions of the study.

Strengths of this first randomized controlled trial of low dose intradermal immunotherapy include: recruitment of moderate-severe participants in accordance with ARIA classification; use of primary outcome combined symptom and medication scores during the grass pollen season in accordance with WAO guidance for allergic rhinitis trials; a low level of missing daily diary card data and the successful blinding of the active treatment. This was achieved through participant daily data entry, text reminders and regular data collection throughout the season.

The rationale for a trial of intradermal immunotherapy was based on our previous study,[5] showing that this regimen systemically abrogated allergen-induced skin late responses, and also previous clinical studies suggesting that epicutaneous[11-13] and intralymphatic[14,15] immunotherapy may be clinically effective. We hypothesized that intradermal injection of allergen might promote tolerogenic pathways through rapid uptake to regional lymph nodes, or possibly, by dermal dendritic cell populations which are relatively abundant compared to subcutaneous tissue.[16] Indeed, one of our active group participants reproducibly demonstrated lymphangitis (Fig E6, Online repository) within 30 minutes of each injection, suggestive of rapid lymphatic uptake of

allergen. We selected an allergen dose equivalent to 7 nanograms of the major Timothy grass pollen allergen Phl p 5 for several reasons. Firstly, we previously reported in a proof of concept study conducted in a similar population that six two-weekly injections at the same dose led to almost complete attenuation of the cutaneous late phase response induced by these injections. This is comparable to the effect on cutaneous late phase responses seen following high-dose subcutaneous immunotherapy[17] and exceeds that following treatment with sublingual grass pollen vaccines.[18] Secondly, the average late response induced by this dose was approximately 10 cm diameter, which we considered to be at the limits of tolerability for patients. Although precise intradermal grass dosages used in the uncontrolled historic studies of Phillips are unknown,[6,7] his aim during treatment was to induce "a local reaction about the size of the patient's palm, which should begin to subside within twenty four hours". Our study has possible limitations. Firstly, grass pollen doses were not increased during the treatment course. This treatment protocol was chosen because of our previous observation that repeating the same dose was sufficient to achieve almost complete suppression of the late phase response. Secondly, injections were not continued throughout the grass pollen season, although previous randomized controlled trials of subcutaneous grass pollen immunotherapy have demonstrated efficacy for pre-seasonal regimens.[19]

Late phase skin responses were first measured at the end of the 2013 grass pollen season because performing such measurements before or during collection of clinical outcome data would have risked unblinding the trial. Late phase responses still appeared partially suppressed at this and the subsequent 7-month time points. Nonetheless, this difference was less than we observed immediately after completion of six intradermal injections in the proof of concept study, suggesting that suppression is transient and mostly reversed within four months. This effect might therefore be similar to that seen with transient desensitization during food oral immunotherapy. The late cutaneous response is considered to be at least partially T cell-dependent and has been extensively used as an experimental model for exploring mechanisms of allergic disease.[4,20] Our data suggest that either the late skin response is not relevant for disease expression or, more likely in our view, that suppression of the late phase response may be necessary but not sufficient for clinical improvement following allergen-specific immunotherapy.

The fall in Phl p-, Phl p 1- and Phl p 5-specific IgE in the placebo group between the baseline (October 2012) and the follow up measurement after 7 injections (May 2013) was consistent with natural seasonal variation as described in previous studies; levels of pollen-specific IgE rise during the grass pollen season and then gradually decline over the following winter months.[21,22] Similar changes also occur in pollen-specific IgG

antibodies.[22] Intradermal immunotherapy arrested the anticipated winter decline, which was seen in the placebo group. Therefore, taking into account the seasonal changes, intradermal allergen immunotherapy stimulated IgE production. In keeping with this, and the exacerbation of nasal symptoms (and other clinical parameters in the per protocol population), T cells cultured from skin punch biopsy explants in the intradermal immunotherapy group expressed higher levels of Th2 marker CRTH2 and lower surface expression of Th1 marker CXCR3 than biopsies from placebo subjects. Exploratory microarray analysis of these T cells was performed in a subgroup only due to limited cell numbers. Although IL-5 was one of only 14 genes overexpressed according to pre-specified criteria, GO analysis did not highlight an effect on other Th2 or inflammation-related genes. Also, post hoc analysis using less stringent criteria did not highlight additional Th2 or Th1-related genes. Therefore, although the clinical and other immunologic findings indicate a priming effect, we interpret the IL-5 microarray data in isolation with caution. An intradermal priming effect could be consistent with observational human studies linking cutaneous exposure to peanut protein in children with atopic dermatitis with development of peanut allergy, an effect more apparent in those with impaired skin barrier function, which may promote dermal allergen exposure.[23,24] Our findings also raise the possibility that intracutaneous exposure to aeroallergens, for example in atopic dermatitis patients with disrupted skin barrier function, might have potential to promote or exacerbate respiratory allergic disease. Such a link has been hypothesized as the basis of so-called 'atopic march' from atopic dermatitis to later development of respiratory allergies.[25]

Previous attempts to develop novel immunotherapy approaches based on epicutaneous allergen application have shown some initial promise. Early phase clinical trials have provided evidence that this may be effective for treatment of grass pollen allergy[13] and similar patches are also under investigation for peanut allergy.[11,12] A potentially important immunological difference between epicutaneous and intradermal allergen immunotherapy is in the types of antigen presenting cells – particularly DC populations – likely to be encountered by allergen.[16] In the epidermis, Langerhans cells predominate, although atopy patch tests also induce infiltration by inflammatory dendritic epidermal cells[26] whereas in the dermis three major DC subtypes have been identified.[27] Recent attention has focused on methods that enhance keratinocyte activation and skin penetration by epicutaneous allergen, such as skin stripping[28] or use of microneedles.[29] Skin barrier disruption appears to promote dermal allergen exposure[30] and in some animal models epicutaneous immunotherapy on stripped skin has appeared to potentiate pre-existing systemic Th2 responses.[31] More recently, dermal DC, but not Langerhans cells were found to elicit murine Th2 responses in response to epicutaneous antigen.[32]

In conclusion, this is the first randomized controlled trial to directly evaluate the efficacy of intradermal grass pollen immunotherapy and the results suggest that this approach is not clinically effective, despite local suppression of skin late phase responses. Moreover, the data suggest that this resulted in immunological priming and worsening of allergic rhinitis symptoms, providing direct evidence that dermal allergen exposure has the potential to exacerbate rather than ameliorate allergic disease, with implications for novel immunotherapy delivering allergen to the skin.

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Declaration of Interests

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Figure Legends

FIG 1. Study design.

FIG 2. CONSORT diagram. All randomized participants were included in the intention-to-treat analysis. Only participants who adequately adhered to treatment and rescue medications were included in the per-protocol analysis.

FIG 3. Primary Outcome and Nasal Symptoms. Daily grass pollen counts in central London during the 2013 grass pollen season (A). Broken vertical lines indicate beginning and end of the peak pollen season (12 June–26 July 2013). Mean daily combined symptom and medication scores in the primary intention-to-treat analysis (B). Mean daily nasal symptom scores (sum of scores for sneezing, blockage and running) (C). Mean nasal symptoms measured by visual-analog scales (VAS) (total of blockage, running, itching and sneezing) (D). Area under curve values for each participant were compared according to treatment arm. P values are based on the Mann-Whitney U test.

FIG 4. Immunological Outcomes. Levels of *Phleum pratense*-specific IgE and IgG before and after completion of seven intradermal allergen or histamine control injections (A). Expression of CRTH2 (Th2 marker) and CXCR3 (Th1 marker) on CD4+ cells expanded from skin biopsies (24hours post-skin challenge) (B). Areas of cutaneous late phase responses (24 hours after intradermal skin challenge) 4 months and either 7, 10 or 13 months post-treatment (September 2013). Late response suppression shown from our previous study (Rotiroti et al.[5]) immediately after six 2-weekly intradermal injections.. Solid bars represent median values (C). P values for pre- and post-treatment serology comparisons based on the Wilcoxon signed-rank test and for between group IgE and IgG are based on ANCOVA. P values in Panels B and C are based on the Mann-Whitney U test.

TABLE I. Baseline characteristics of study participants

	Control (n=47)	Intradermal Immunotherapy (n=46)
Age (y), mean (SD)	35 (10.8)	32 (9.9)
Female sex, no. (%)	12 (26)	19 (41)
Race, no. (%)		
White	37 (79)	37 (80)
Mixed	2 (4)	3 (7)
Asian	3 (6)	4 (9)
Black	3 (6)	0 (0)
Other	2 (4)	2 (4)
Allergy symptoms outside grass pollen season, no. (%)	18 (38)	16 (35)
Total IgE (kU_c/L), median (IQR)	121 (64-255)	160 (80-263)
Phleum pratense-specific IgE (kU_A/L), median (IQR)	27 (10-54)	22 (9-49)
Phleum pratense SPT weal diameter (mm), mean (SD)	12 (4.2)	11 (5.0)
SPT-positive, no. (%)		
Timothy grass	47 (100%)	46 (100%)
Mixed grass	47 (100%)	46 (100%)
Silver birch	19 (40%)	24 (52%)
Mugwort	11 (23%)	9 (20%)
House dust mite	28 (60%)	24 (52%)
Cat	24 (51%)	18 (39%)
Dog	41 (87%)	36 (78%)
Horse	4 (9%)	6 (13%)
Aspergillus	1 (2%)	2 (4%)
Alternaria	6 (13%)	7 (15%)

Cladosporium	2 (4%)	2 (4%)
Seasonal Asthma controlled with albuterol	17 (36)	15 (33)

FEV1: Forced Expiratory Volume in 1 second; FVC: Forced Vital Capacity; ENT: Ear, Nose and Throat; SPT: Skin Prick Test.

TABLE II. Effect of Intradermal Immunotherapy on Primary and Secondary Outcomes (Intention-to-Treat)

	Control (n=47)	Intradermal Immunotherapy (n=46)	Difference (95% CI)	P value
Primary Outcome	**Median (IQR)**			
CSMS during entire season	487 (365–717)	502 (333–841)	14 (-172.5 to 215.1)	.80
Secondary Outcomes				
Symptom score during entire season	264 (156–398)	335 (183–503)	59 (-1.3 to 110.9)	.24
Medication Score during entire season	263 (129–482)	242 (116–405)	-19 (-153.0 to 100.2)	.44
CSMS Score during peak season	365 (278–508)	356 (232–521)	-8 (-75.8 to 66.3)	.90
Nasal symptom score during entire season	121 (81–200)	174 (120–207)	35 (4.0 to 67.5)	.03
Mouth symptom score during entire season	14 (5–45)	34 (8–90)	10 (3.8 to 24)	.05
Eye symptom score during entire season	78 (52–180)	79 (41–153)	-7 (-18.5 to 2.9)	.54
Lung symptom score during entire season	12 (0–34)	17 (3–32)	4 (-1 to 15)	.17
Nasal Allergic Symptoms measured by VAS	122 (54–184)	156 (104–275)	53 (-11.6 to 125.2)	.05
Eye Allergic Symptoms measured by VAS	144 (41–176)	84 (32–197)	-3 (-46.0 to 35.8)	.40
Global Evaluation of Symptom Scores	3 (1–4)	3 (2–4)	0 (0 to 1)	.48
Symptom Free Days	41 (23–61)	35 (19–53)	-6 (-17 to 3)	.15
No. days prednisone used during entire season	0 (0–0)	0 (0–0)	0 (0 to 0)	.36
Medication Free Days	76 (65–94)	81 (65–93)	4 (-11 to 21)	.22
Mini-RQLQ	18 (10–25)	16 (13–23)	-0.3 (-4.2 to 3.7)	.89
EQ-5D-5L	88 (81–94)	87 (83–94)	9 (-24.8 to 43.6)	.59

Median difference between groups calculated by stratified Hodges-Lehmann.
P values based on stratified Mann-Whitney U test (Van Elteren's test) adjusted for stratification factors
P values for mini-RQLQ and EQ-5D-5L based on linear mixed model adjusted for stratification factors

Entire grass pollen season: 13 May-3 August 2013; Peak season: 12 June-26 July 2013.
CSMS: combined symptom & medication score, VAS: Visual-analog scale
Mini-RQLQ: mini-Rhinoconjunctivitis Quality-of-Life Questionnaire, EQ-5D-5L: EuroQoL instrument

TABLE III. Effect of Intradermal Immunotherapy on Primary and Secondary Outcomes (Per-Protocol Sensitivity Analysis)

	Control (n=39)	Intradermal Immunotherapy (n=45)	Difference (95% CI)	P value
Primary Outcome		**Median (IQR)**		
CSMS during entire season	453 (279–685)	517 (344–841)	82 (-121.8 to 280.1)	.23
Secondary Outcomes (median (IQR)				
Symptom score during entire season	241 (150–398)	340 (189–503)	76 (25.9 to 133.5)	.09
Medication Score during entire season	254 (113–358)	255 (119–405)	21 (-125.0 to 157.0)	.83
CSMS Score during peak season	342 (242–476)	363 (242–546)	18 (-73.2 to 127.5)	.51
Nasal symptom score during entire season	119 (80–205)	173 (123–207)	40 (13.3 to 71.5)	.02
Mouth symptom score during entire season	14 (4–43)	38 (8–90)	14 (4.9 to 32.0)	.02
Eye symptom score during entire season	72 (48–145)	80 (41–153)	0 (-16.0 to 17.6)	.85
Lung symptom score during entire season	11 (0–21)	17 (3–32)	9 (1.0 to 17.0)	.05
Nasal Allergic Symptoms measured by VAS	118 (50–154)	162 (105–275)	68 (8.3 to 134.6)	.008
Eye Allergic Symptoms measured by VAS	114 (42–159)	90 (32–197)	1 (-52.8 to 62.0)	.49
Global Evaluation of Symptom Scores	3 (1–3)	3 (2–4)	1 (0.0 to 1.0)	.25
Symptom Free Days	44 (25–67)	34 (19–47)	-12 (-22.0 to -2.0)	.04
No. days prednisone used during entire season	0 (0–0)	0 (0–0)	0 (0 to 0)	.33
Medication Free Days	78 (66–98)	80 (65–92)	-1 (-20.0 to 17.0)	.87
Mini RQLQ	17 (10–22)	16 (13–23)	- 2.0 (-5.89 to 1.88)	.31
EQ-5D-5L	88 (84–94)	88 (83–94)	3 (-28.4 to 35.2)	.83

Data for primary outcome and all symptom scores represent Area Under Curve values

Median difference between groups calculated by stratified Hodges-Lehmann.

P values based on stratified Mann-Whitney U test (Van Elteren's test) adjusted for stratification factors

P values for mini-RQLQ and EQ-5D-5L based on linear mixed model adjusted for stratification

factors

Entire grass pollen season: 13 May-3 August 2013; Peak season: 12 June-26 July 2013.
CSMS: combined symptom & medication score, VAS: Visual-analog
scale
Mini-RQLQ: mini-Rhinoconjunctivitis Quality-of-Life Questionnaire, EQ-5D-5L: EuroQoL
instrument

Table IV. Effect of Intradermal Immunotherapy on Daily and Visual-Analog Scale Organ Symptom Scores (Intention-to-Treat, Post-hoc analysis)

	Control (n=47)	Intradermal Immunotherapy (n=46)	Difference (95% CI)	P value
Daily Organ Symptom Scores	**Median (IQR)**			
Nose				
Sneezing	55 (35.0-71.0)	76 (43.3-103.0)	21 (7.0 to 34.0)	.01
Blockage	36 (12.5-61.0)	41 (14.0-74.5)	6 (-2.5 to 13.5)	.33
Running	46 (22.5-65.4)	51 (30.0-81.5)	10 (-3.0 to 22.8)	.17
Mouth				
Itching	8 (1.0-25.0)	19 (4.0-52.3)	4 (1.8 to 6.8)	.06
Drying	3 (0.0-15.0)	7 (0.0-40.0)	3 (0.0 to 9.6)	.18
Eyes				
Itching	44 (26.0-72.5)	48 (21.0-68.0)	-1 (-5.0 to 2.0)	.99
Redness/sore	14 (7.0-45.0)	17 (4.0-42.0)	-1 (-6.0 to 3.0)	.55
Streaming	14 (2.0-24.0)	11 (2.0-19.0)	0 (-4.0 to 3.0)	.69
Swelling	5 (0.0-14.0)	2 (0.0-9.0)	-2 (-4.0 to 0.0)	.03
Lungs				
Breathlessness	0 (0.0-8.1)	0 (0.0-4.0)	0 (0.0 to 2.0)	.27
Cough	1 (0.0-12.1)	8 (1.0-23.3)	2 (0.0 to 6.0)	.02
Wheezing	0 (0.0-8.0)	3 (0.0-7.0)	0 (0.0 to 2.0)	.25
Tightness	0 (0.0-4.0)	2 (0.0-4.0)	0 (0.0 to 2.0)	.08
VAS Organ Symptom Scores				
Nose				
Blockage	118 (39.1-178.8)	152 (71.4-238.7)	39 (1.6 to 82.8)	.12
Running	117 (62.0-162.7)	169 (96.0-265.6)	58 (-8.2 to 124.5)	.006
Itching	81 (41.9-141.6)	138 (93.2-281.7)	64 (-16.3 to 165.4)	.003

Sneezing	125 (46.1-182.4)	187 (133.1-295.3)	77 (-1.6 to 150.9)	.006
Eyes				
Itching	135 (41.9-217.8)	120 (53.7-248.3)	4 (-35.3 to 46.1)	.97
Watering	71 (33.6-119.4)	69 (21.0-129.5)	1 (-40.5 to 55.5)	.79

Data shown represent Area Under Curve values

Median difference between groups calculated by stratified Hodges-Lehmann.

P values based on stratified Mann-Whitney U test (Van Elteren's test) adjusted for baseline stratification factors

Fig. 1

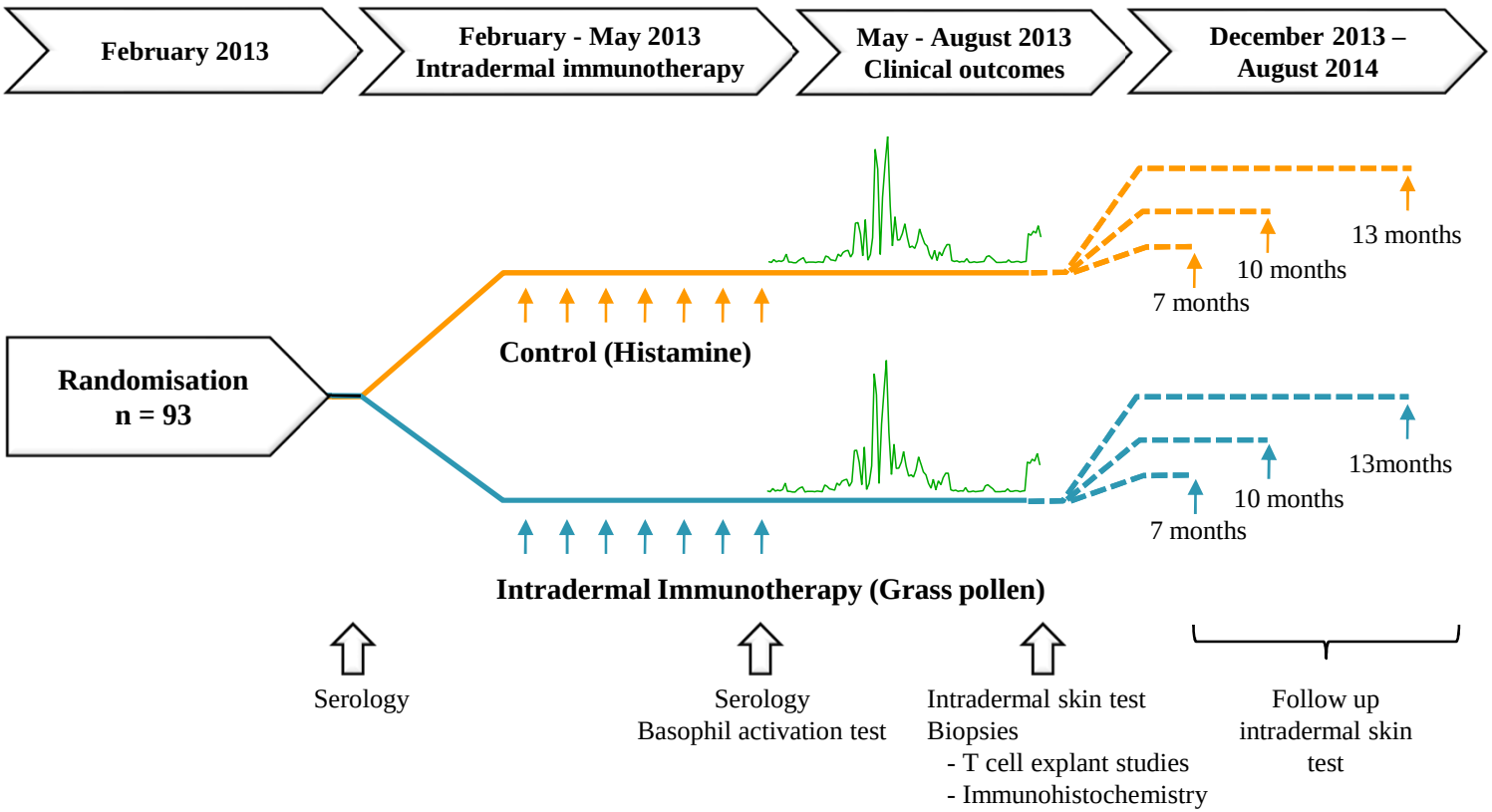
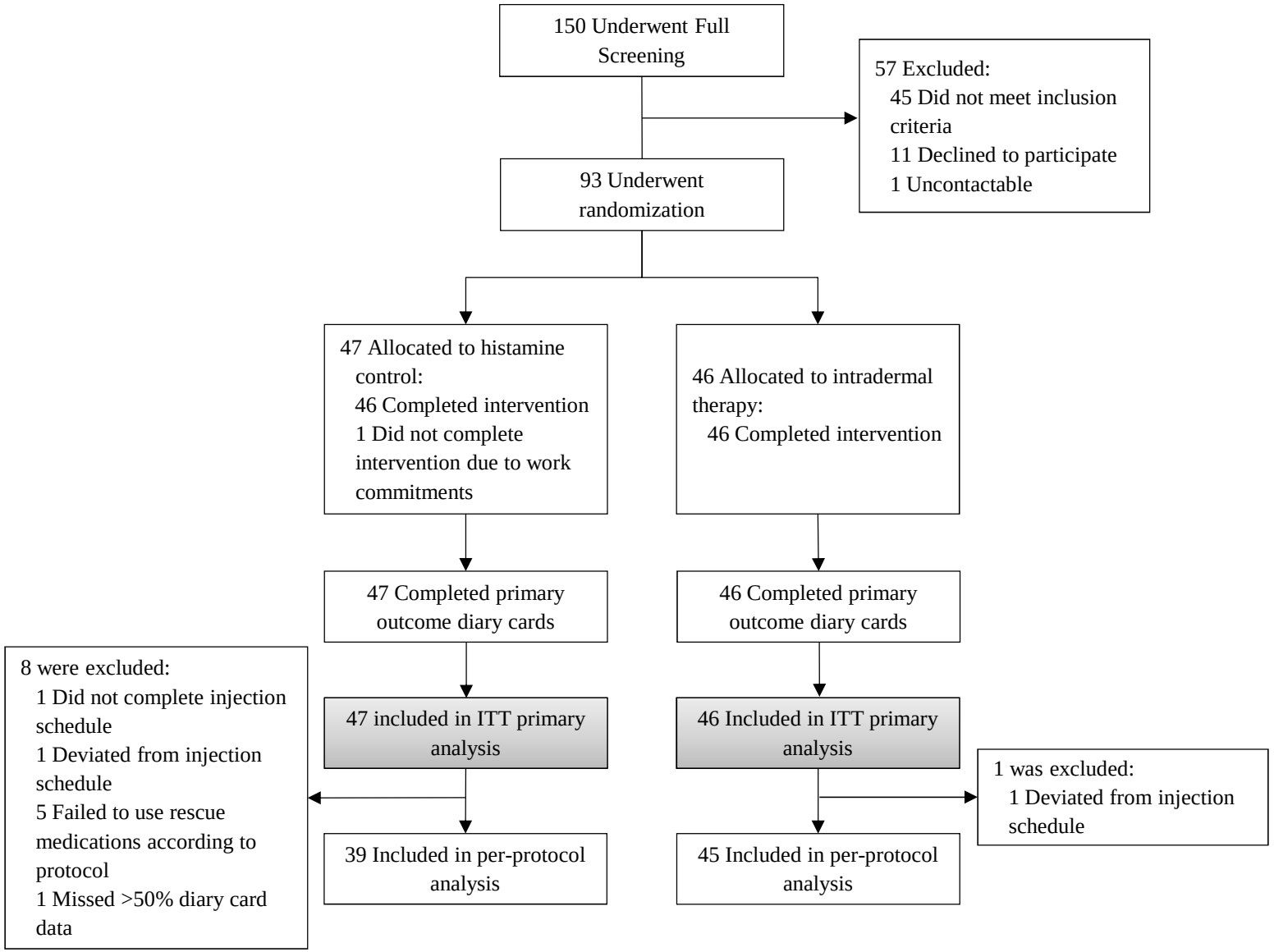


Fig. 2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1 Uncontactable
93 Underwent randomization
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46 Completed intervention
1 Did not complete intervention due to work commitments
46 Allocated to intradermal therapy:
46 Completed intervention
47 Completed primary outcome diary cards
46 Completed primary outcome diary cards
8 were excluded:
1 Did not complete injection schedule
1 Deviated from injection schedule
5 Failed to use rescue medications according to protocol
1 Missed >50% diary card data
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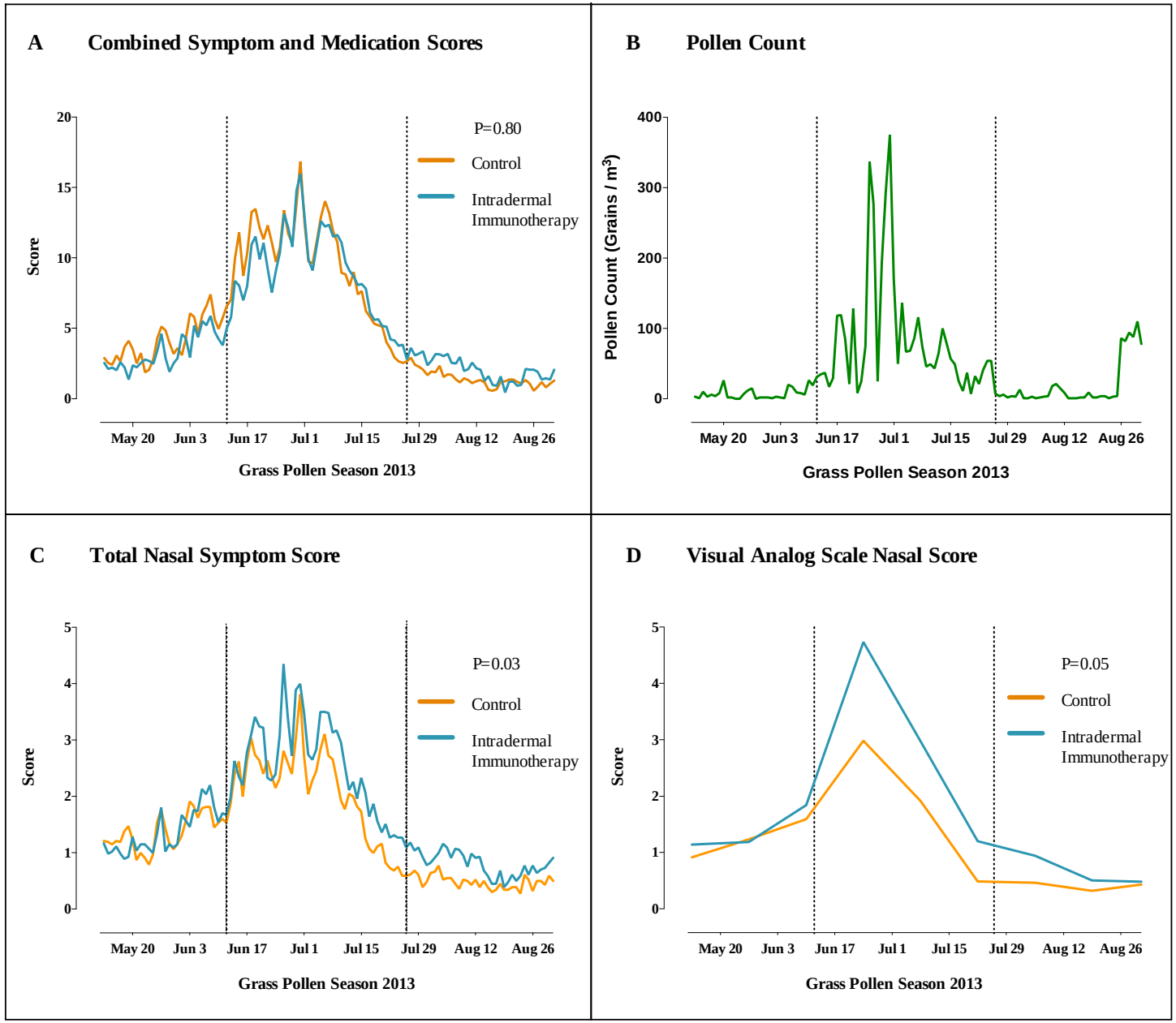
1 Deviated from injection schedule
39 Included in per-protocol analysis
45 Included in per-protocol analysis

Fig. 3



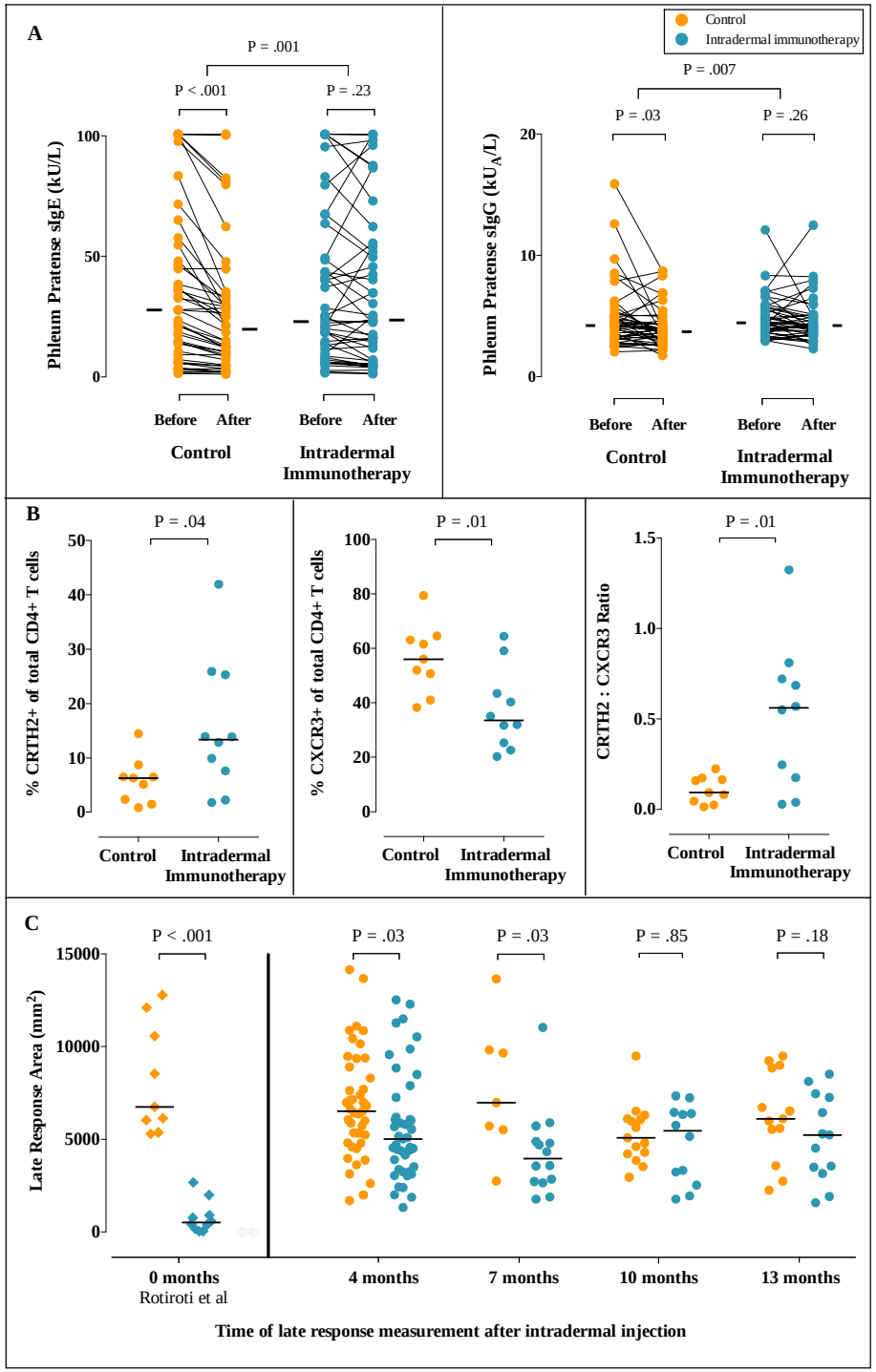


TABLE E1. Verification of participant blinding

Patient Guess Trial Arm	Trial Arm	
	Control (n=43)	**Intradermal Immunotherapy (n=44)**
Intradermal Immunotherapy (n=44)	22	22
Control (n=43)	21	22

At the end of the pollen season participants verified blinding by guessing if they had received active or control treatment

TABLE E2. Effect of Intradermal Immunotherapy on Primary and Secondary Outcomes (Intention-to-Treat); Missing data imputed

	Control (n=47)	Intradermal Immunotherapy (n=46)	Difference (95% CI)	P value
Primary Outcome		**Median (IQR)**		
CSMS during entire season	509 (365–738)	502 (333–841)	8 (-174.7 to 210.9)	.91
Secondary Outcomes				
Symptom score during entire season	264 (156–434)	335 (183–525)	61 (-7.8 to 123.2)	.22
Medication Score during entire season	263 (129–482)	242 (116–405)	-24 (-173.1 to 107.5)	.39
CSMS Score during peak season	370 (292–573)	363 (232–570)	-11 (-95.8 to 77.5)	.80
Nasal symptom score during entire season	131 (80–200)	178 (120–218)	33 (0.3 to 68.5)	.03
Mouth symptom score during entire season	14 (6–45)	39 (8– 90)	11 (3.1 to 26.1)	.05
Eye symptom score during entire season	78 (52–180)	79 (41–158)	-7 (-20.0 to 3.0)	.51
Lung symptom score during entire season	12 (0– 40)	20 (3– 32)	4 (-1.0 to 15.3)	.17
Nasal Allergic Symptoms measured by VAS	124 (66–166)	162 (107–275)	59 (-3.7 to 133.2)	.02
Eye Allergic Symptoms measured by VAS	112(42–169)	97 (37–197)	2 (-45.6 to 49.0)	.56
Global Evaluation of Symptom Scores	3 (1–3)	3 (2–4)	0 (0 to 1)	.43
Symptom Free Days	41 (23–61)	35 (19–53)	-6 (-17 to 3)	.15
No. days prednisone used during entire season	0 (0–0)	0 (0–0)	0 (0 to 0)	.36
Medication Free Days	76 (56–94)	81 (65–93)	4 (-11.0 to 21.0)	.22
Mini-RQLQ	18 (10–25)	16 (13–23)	-0.3 (-4.2 to 3.7)	.89
EQ-5D-5L	88 (81–94)	87 (83–94)	9 (-24.8 to 43.6)	.59

Data for primary outcome and all symptom scores represent Area Under Curve values

Median difference between groups calculated by stratified Hodges-Lehmann.

P values based on stratified Mann-Whitney U test (Van Elteren's test) adjusted for stratification factors

P values for mini-RQLQ and EQ-5D-5L based on linear mixed model adjusted for stratification factors

Entire grass pollen season: 13 May-3 August 2013; Peak season: 12 June-26 July 2013.

CSMS: combined symptom & medication score, VAS: Visual-analog scale

Mini-RQLQ: mini-Rhinoconjunctivitis Quality-of-Life Questionnaire, EQ-5D-5L: EuroQoL instrument

TABLE E3. Frequency of Adverse Events reported from first intradermal allergen immunotherapy or control injection until end of pollen season

	Control (n=47)				Intradermal Immunotherapy (n=46)			
	No. Participants with ≥1 AE	% Participants	No. Events	Event Rate (%)	No. Participants with ≥1 AE	% Participants	No. Events	Event Rate (%)
Any AEs	42	89	145		40	87	148	
Serious Adverse Events	2	4.3	2	1.4	1	2.2	1	0.7
Tonsillitis	0	0	0	0	1	2.2	1	0.7
Overnight stay for Polysomnography	1	2.1	1	0.7	0	0	0	0
Extraction of infected dental plate	1	2.1	1	0.7	0	0	0	0
Relation of AE to treatment								
Definite/Probable	6	13	14	9.7	3	6.5	15	10
Possible	0	0	0	0	0	0	0	0
Remote	34	72	70	48	30	65	68	46
None	34	72	61	42	32	70	65	44
AE withdrawals	0	0	0	0	0	0	0	0
Systemic Adverse Reactions	6	13	13	9.0	3	6.5	15	10
Generalised Pruritus	4	8.5	9	6.2	2	4.3	8	5.4
IgE-mediated lymphangitis	0	0	0	0	1	2.2	7	4.7
Light-headedness	2	4.3	2	1.4	0	0	0	0
Facial flushing/feeling hot	2	4.3	3	2.1	0	0	0	0
Systemic Adverse Reactions*								
Grade 1	6	13	12	8.3	3	6.5	15	10
Grade 2	0	0	0	0	0	0	0	0
Grade 3	0	0	0	0	0	0	0	0

Grade 4	0	0	0	0	0	0	0	0

Statistical comparison was by Fisher's Exact test for ≤5 events and Chi^2 test for >5 events.

*Classified using the World Allergy Organization grading system for systemic reactions to subcutaneous immunotherapy, Cox L et al. JACI 125:569-574, e567.

Table E4. Microarray gene expression profiles of activated CD4+ T cells derived from skin biopsy explants

Gene	P value	Fold-Difference
Intradermal Immunotherapy down versus Control group		
LOC100133042	.02	-1.80
CEP55	.03	-1.78
GFOD1	.00	-1.77
HIST2H2AB	.04	-1.62
H2AFZ	.02	-1.61
LOC730534	.01	-1.57
HSD17B4	.02	-1.57
HIST1H2AD	.03	-1.56
HDAC1	.01	-1.55
CCL3L1	.03	-1.53
CALR	.02	-1.52
CDCA5	.01	-1.52
PRDX5	.01	-1.51
FEN1	.02	-1.50
Intradermal Immunotherapy up versus Control group		
EPS15	.02	1.51
MYB	.01	1.52
GK	.03	1.53
RNASET2	.03	1.55
LOC729383	.02	1.56
GPR171	.00	1.59
LOC729387	.04	1.60
SLC11A2	.02	1.60
HS.508682	.04	1.68
IL5	.03	1.71
GBP5	.05	1.79
TNFSF8	.01	1.79
TNIP3	.03	1.87
CENTA1	.05	2.11

Data analyzed by 3 way-ANOVA model.

Fig. E1

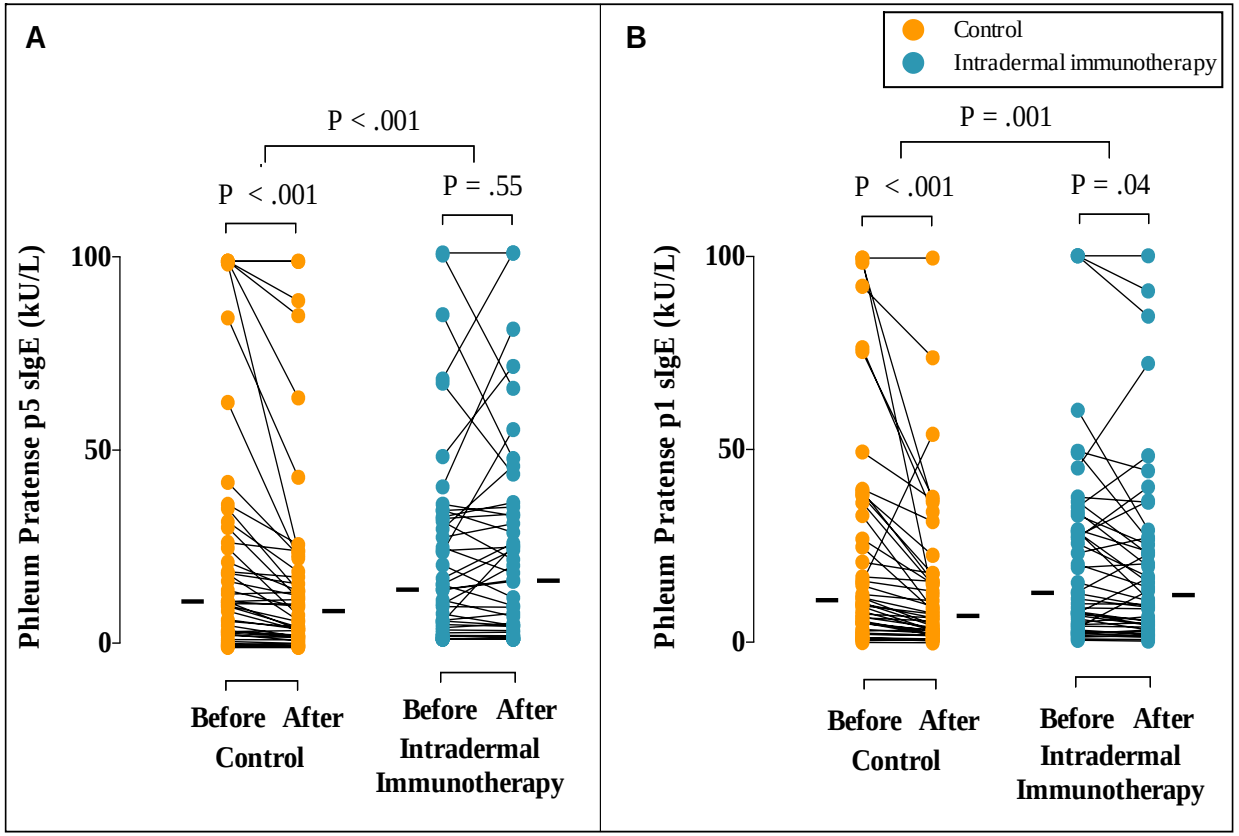
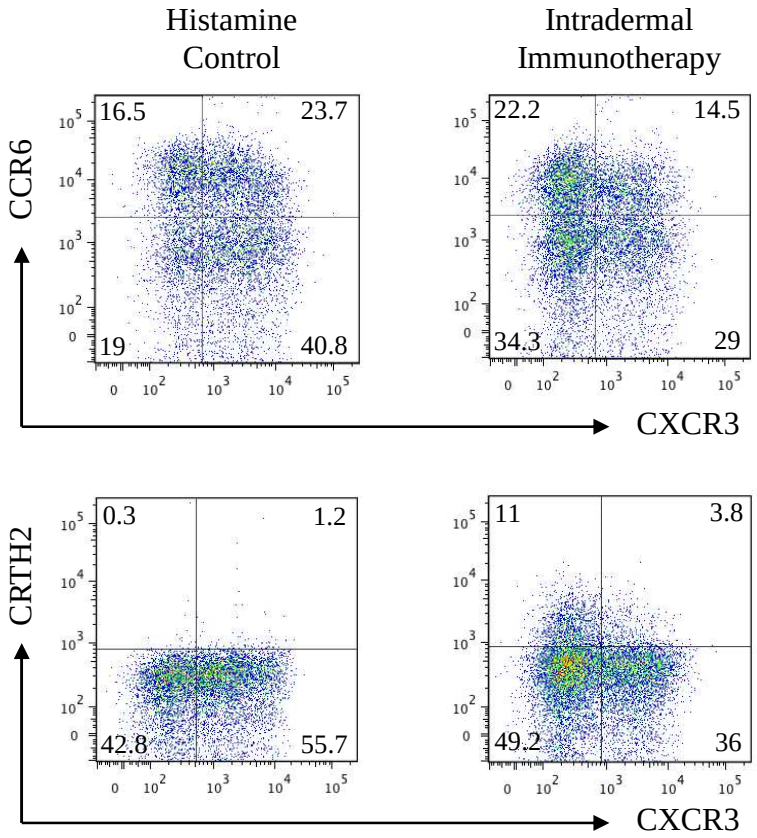


Fig. E2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Control
Intradermal
Immunotherapy
CCR6
CXCR3
16.5
23.7
19
40.8
22.2
14.5
34.3
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CRTH2
CXCR3
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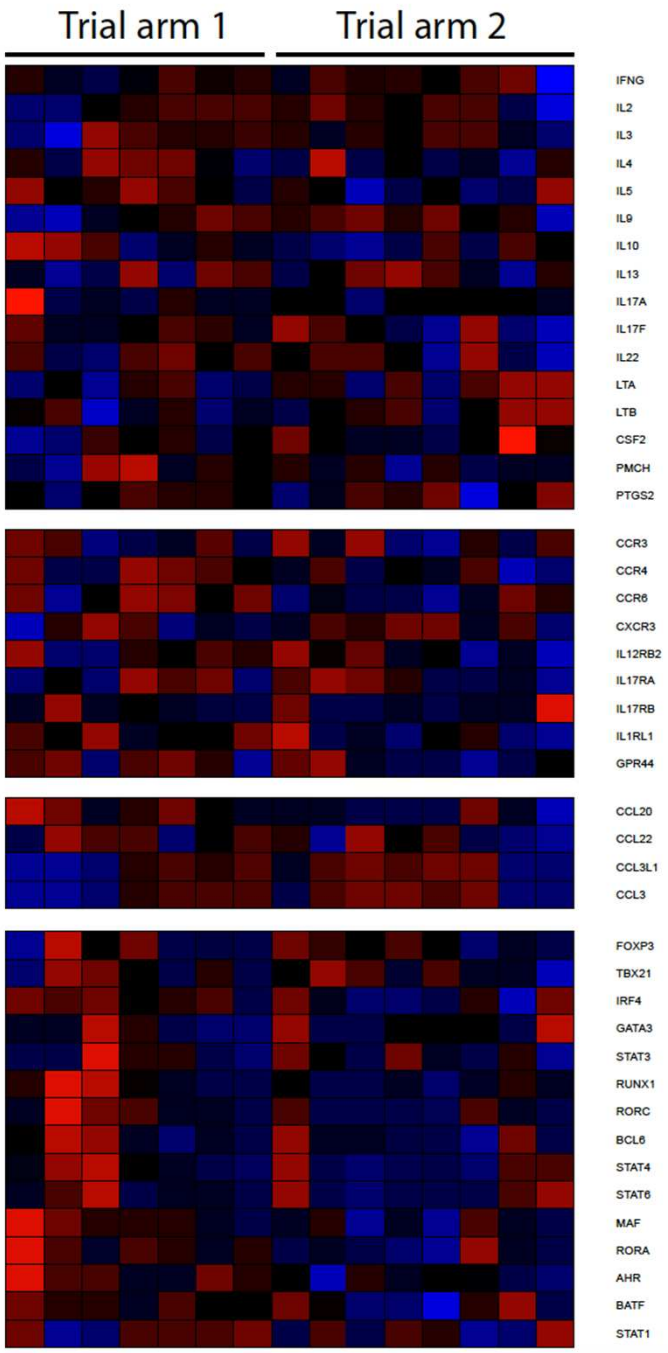
1.2
42.8
55.7
11
3.8
49.2
36

Fig. E3


Trial arm 1
Trial arm 2
IFNG
IL2
IL3
IL4
IL5
IL9
IL10
IL13
IL17A
IL17F
IL22
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LTB
CSF2
PMCH
PTGS2
CCR3
CCR4
CCR6
CXCR3
IL12RB2
IL17RA
IL17RB
IL1RL1
GPR44
CCL20
CCL22
CCL3L1
CCL3
FOXP3
TBX21
IRF4
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Fig. E4

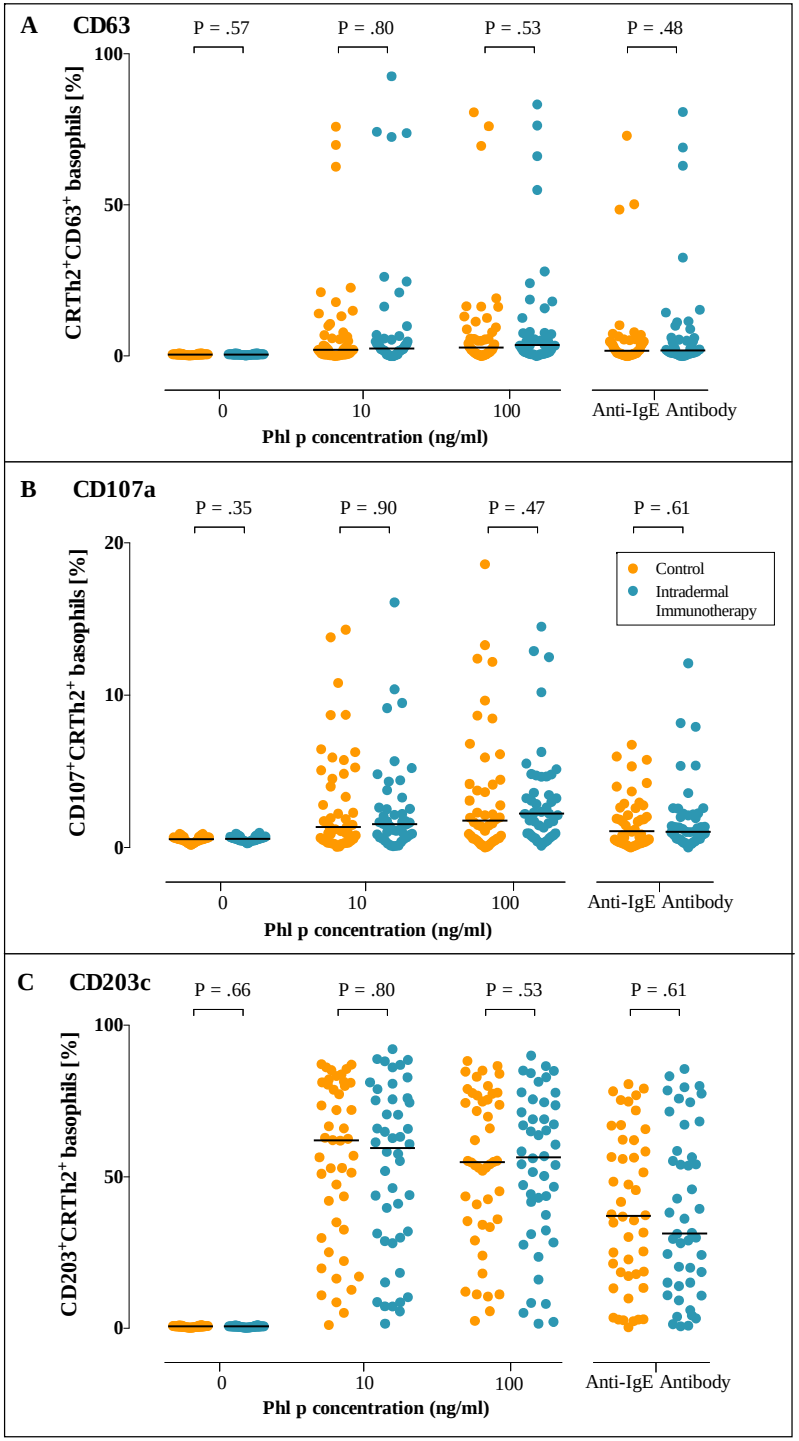


Fig. E5

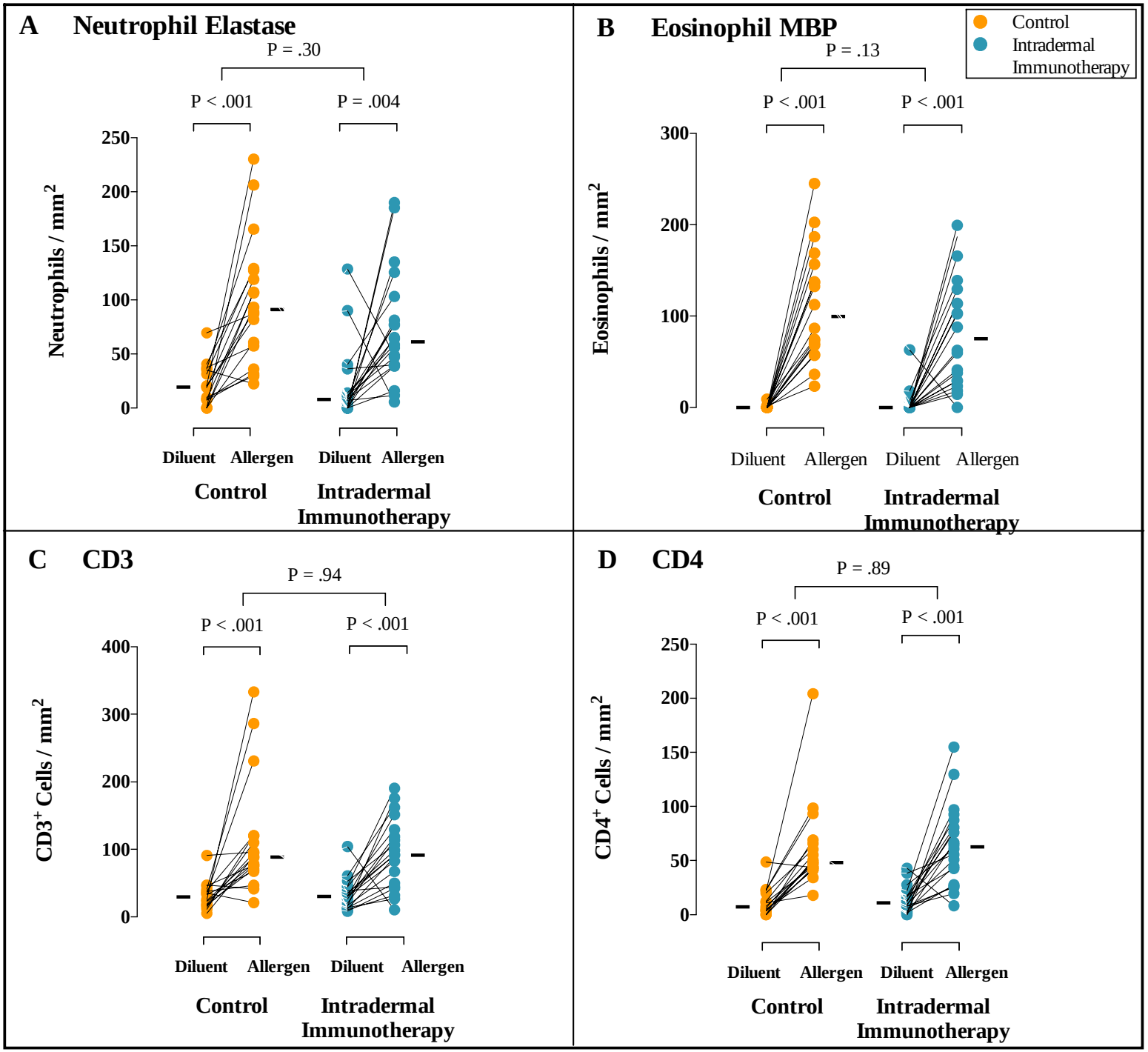
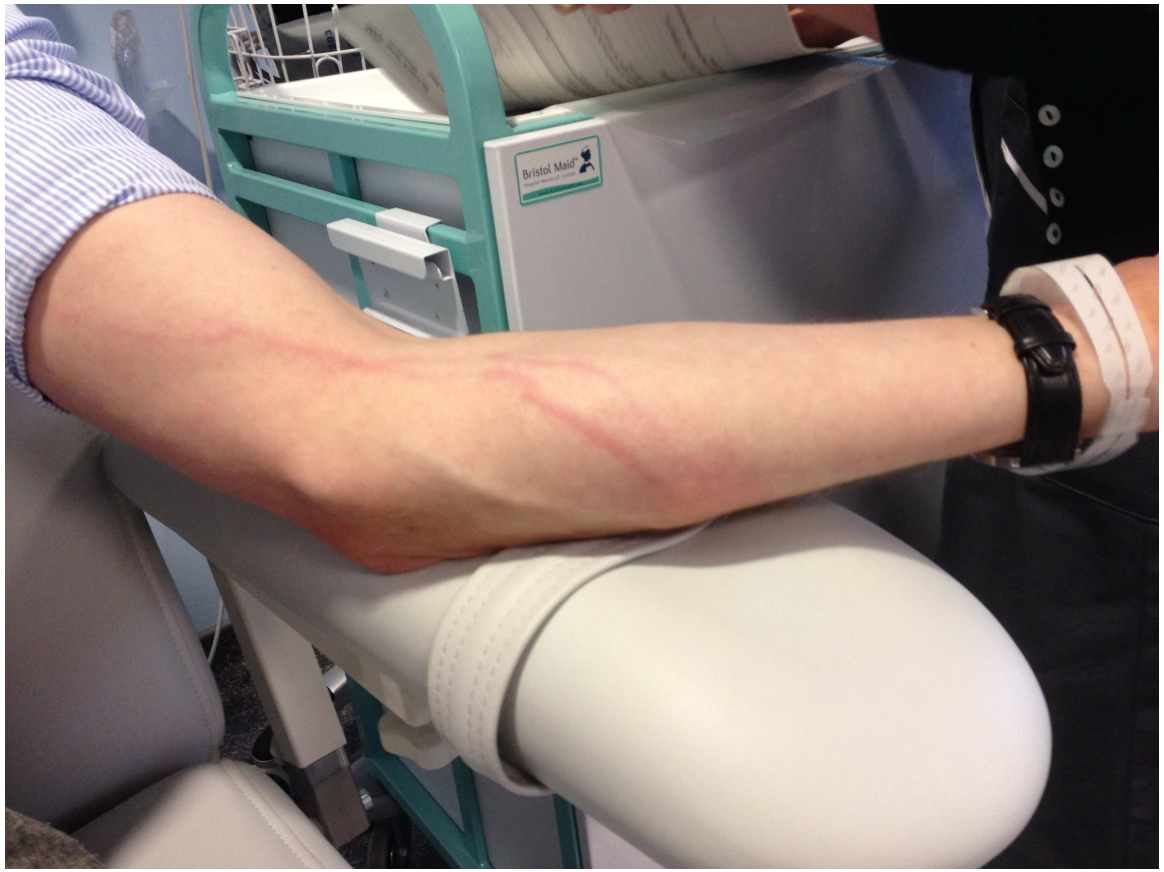


Fig. E6



Online Repository

Supplementary Methods

Participants

Full inclusion criteria were adults aged 18 to 65 years with a history of moderate-severe symptoms of grass pollen allergic rhinitis according to ARIA classification[1] in May, June, or July, for a minimum of 2 years interfering with daily activities or sleep and remaining troublesome despite treatment with medication. Participants were required to have a positive skin prick test response (wheal diameter at least 3 mm) to *Phleum pratense* together with a positive specific IgE (at least IgE class two) against *Phleum pratense*. Women of childbearing age were included if willing to use an effective form of contraception for the duration of intradermal injections. Participants were able to consent and comply with study procedures.

Exclusion criteria were as follows: pre-bronchodilator FEV_1 less than 70% of predicted value at screening; seasonal grass pollen-induced asthma requiring regular treatment with albuterol or inhaled corticosteroids (those with mild seasonal grass pollen-induced asthma, controlled with occasional albuterol only, were included); significant symptomatic seasonal allergic rhinitis and/or asthma due to tree pollen or weed pollen near or overlapping the grass pollen season (patients with mild intermittent symptoms requiring only occasional antihistamines were included); significant perennial rhinitis (patients with mild intermittent symptoms requiring only occasional antihistamines were included); an emergency room visit for asthma in the previous 12 months; chronic obstructive pulmonary disease; recurrent acute sinusitis; chronic sinusitis; previous grass pollen immunotherapy within the previous 5 years; previous life-threatening anaphylaxis or angioedema; history of intolerance of grass pollen immunotherapy or rescue medications; a positive serum or urine pregnancy test within 72 hours of enrolment; lactating females; use of any investigational or immunosuppressive drug within 30 days of screening; use of leukotriene receptor antagonists, beta-blockers, calcium channel blockers, tricyclic antidepressants, monoamine oxidase inhibitors or anti-IgE monoclonal antibody; a medical condition that the investigator deemed incompatible with participation in the trial; infection of the upper respiratory tract, sinuses or middle ear at randomization; insufficient understanding of the trial protocol. Current smokers or subjects with greater than or equal to 5 pack years were also ineligible.

Power Calculations

Sample size calculations for the primary outcome (combined symptom and medication score) were performed based on raw data from a previous clinical trial of subcutaneous grass pollen immunotherapy.[11] The power calculation was conservatively based on the detection of a clinical effect size 80% of that reported in that

trial. Using this method and a two-sided non-parametric test based on a Monte Carlo approach, group sample sizes of 35 and 35 achieved 90% power to detect such a difference in AUC of the combined symptom and medication scores at a significance level of .05. To make allowance for the unknown distribution of the primary outcome and based on the lower bound for the asymptotic relative efficiency of the Mann-Whitney U test, the sample size was increased by a further 15% to 40 in each arm. Further accounting for a post-randomization dropout rate of up to 10% consistent with previous trials of grass pollen immunotherapy, a total sample size of 90 (45 each arm) was estimated as required.

Skin Biopsy Randomization

In August 2013, the King's Clinical Trial Unit randomly selected participants to be approached to undergo skin biopsies. The first 40 participants who consented then underwent biopsy. Also in August 2013, all participants were randomized a second time to one of three groups for repeat intradermal allergen injections at seven, ten or thirteen months after the final intradermal immunotherapy or control injection, to assess if low dose intradermal allergen immunotherapy was associated with prolonged suppression skin responses.

Masking

All physicians, researchers, research nurses, outcome assessors and patients were blinded to treatment allocation until primary and secondary analyses were complete. Active and control study medication vials appeared identical. Only the King's Clinical Trial Unit randomization provider and the manufacturing pharmacy had access to blinding information. Unmasking could be performed for emergencies only. To verify blinding, participants guessed whether they had received the active or control intervention post-pollen season.

Procedures

Each active intradermal allergen injection contained 10 Biological Units (BU) (33.3 SQ-U) of *Phleum pratense* soluble grass pollen extract (Aquagen SQ^{TM} Timothy, ALK Abello, Reading UK) in a 20 µl volume (i.e. 500 BU/ml (1666.7 SQ-U/ml)). Individual vials for each participant and each visit were pre-prepared and pre-labeled by Guy's Hospital Pharmacy under GMP conditions. In brief, Aquagen SQ^{TM} Timothy grass pollen extract was reconstituted in manufacturer-supplied diluent to the maximum recommended concentration (30'000 BU/ml (100'000 SQ-U/ml) i.e. 60-times final working strength; shelf life 6 months at 2-8^{o}C after reconstitution) and 0.15 ml aliquoted into glass study vials. At each visit for intradermal injection the investigator added 8.85 ml of clinical grade 0·9% normal saline at ambient temperature to the vial corresponding to that participant's visit to achieve a 60-fold dilution. Twenty microliters was then aspirated from this vial and administered directly. The

allergen required dilution on the day of administration, as the recommended shelf life of Aquagen SQ™ Timothy Grass Pollen extract at 500 BU/ml (1666.7 SQ-U/ml) is 14 days. The control intervention was histamine only, administered at a concentration of 100 μg/ml for the 1st and 2nd injections. To help preserve blinding, histamine concentrations were reduced to 30 μg/ml for the 3rd and 4th injections, and 10 μg/ml for 5th, 6th and 7th injections. To match the grass pollen extract dilution and preserve blinding, histamine was also aliquoted into study vials at 60-times final working strength in 0.15 ml volumes, for dilution with 8.85 ml of clinical grade 0.9% normal saline immediately prior to injection. Active and control study medications appeared identical.

The injection site was alternated between left and right arms at each visit. Intradermal injections were administered in a 20 μl volume using a 29 gauge insulin syringe (Becton Dickinson Micro-Fine™). In the event of an injection being administered too deeply (i.e. into subcutaneous tissue) to elicit an immediate injection 'bleb' and subsequent characteristic wheal, the injection was repeated 1 cm from the original site. Following an intradermal injection participants were able to take an antihistamine to reduce the local itching and swelling if they so wished.

Study Outcomes

The primary outcome was a combined symptom and medication score during the grass pollen season (13th May-31st August 2013, 111 days), as recommended by World Allergy Organisation (WAO) guidelines for allergic rhinitis immunotherapy trials.[10] Participants scored symptoms from 0 to 3 in the nose (sneezing, blockage, and running), eye (itching, redness, tears, and swelling), mouth and throat (itching and dryness), and chest (breathlessness, cough, wheezing, and tightness). Daily rescue medication was scored as follows: desloratadine, 5mg, up to 1 tablet daily (6 points daily); olopatadine eye drops, 1 mg/ml, up to 1 drop per eye twice daily (1·5 points per drop, maximum 6 points daily); fluticasone nasal spray, 50 μg per spray, up to 2 sprays per nostril once daily (2 point per spray, maximum 8 points daily); and prednisone, 5 mg per tablet, up to 6 tablets daily (2 points per tablet, maximum 12 points daily). Symptom and medication scores were expressed as the Area Under Curve (AUC) for the entire grass pollen season. Scores for symptoms (maximum 39 points daily) and medications (maximum 32 points daily) were normalized before combining as recommended by the WAO.[10]

Safety

Adverse Events

Adverse events and side effects were recorded from the first treatment injection throughout the study regardless of severity or relation to study participation. As a precaution against systemic allergic reactions, all participants were observed after the first injection for one hour, and if there was no systemic reaction, for 30 minutes after subsequent injections. In the event of experiencing a Grade 1 reaction, the observation period for that individual remained at 1 hour after subsequent injections.

The following Adverse Events were anticipated and not reported:

1) Symptoms due to aeroallergen exposure i.e. nasal blockage, rhinorrhea, itching or sneezing; Itching, watering redness or swelling of eyes; itching or dryness of mouth/throat; breathless, cough, wheeze and chest tightness.
2) Transient discomfort from intradermal injections.
3) Appearance of an itchy edematous wheal with surrounding erythema after intradermal injection.
4) Appearance of swelling (edema) within hours of intradermal injection.
5) Temporary discomfort, bleeding, bruising, swelling at the needle site following venesection.
6) Mild localised itching arising from skin prick testing during screening.

Withdrawal criteria and stopping rules

The pre-specified criteria for discontinuation of the study therapy (active or control) were as follows:

1) Inability or failure to attend for intervention within 3 weeks of previous allergen/histamine administration.
2) Inability or failure to receive 7 or 8 injections within the dates specified.
3) Two Grade 2 systemic reactions, or a single systemic reaction of Grade 3 or above after administration of study therapy. Systemic reactions were graded according to the World Allergy Organization criteria[3]:
    a. Grade 1: symptoms of one organ system (cutaneous, upper respiratory tract, conjunctival, gastrointestinal, other).
    b. Grade 2: symptoms of more than one organ system present or asthma symptoms/signs (cough, wheezing, shortness of breath but <40% drop in peak expiratory flow [PEF] or FEV1).
    c. Grade 3: asthma symptoms/signs (with $\geq$ 40% drop in PEF or FEV1), upper respiratory tract (laryngeal, uvula, tongue) edema with or without stridor.
    d. Grade 4: respiratory failure or hypotension with or without loss of consciousness.

4) An adverse event that, in the judgment of the principal investigator or the medical monitor, presented an unacceptable consequence or risk to the participant.
5) An illness or infection not associated with the condition under study and that required treatment not consistent with protocol requirements; or, if a participant developed an intercurrent illness that in the judgment of the principal investigator in any way justified discontinuation.
6) An inability or unwillingness to comply with the study protocol, with the protocol deviations being sufficient to jeopardize the participant's well-being or the integrity of the study.
7) Pregnancy occurring during study participation.

Pre-defined study stopping rules included the occurrence of five grade 3 reactions or a single grade 4 reaction.

Intradermal skin challenge testing

All patients underwent intradermal skin challenge testing four months after the final intradermal allergen immunotherapy or control injection (September 2013). Participants were then randomized to undergo a repeat follow up test at either seven, ten or 13 months later to assess persistence of late response suppression by comparing late phase response sizes in those who had received active intradermal immunotherapy or the control intervention. The procedure for the intradermal skin challenge testing and the dose of allergen used was identical to that for an active intradermal allergen immunotherapy injection. In brief, grass pollen extract (10 BU, equivalent to 33.3 SQ-U, of Aquagen SQ™ Timothy, ALK Abelló, Reading, UK) in a 20 µl volume of allergen diluent was injected intradermally into the extensor aspect of each forearm. A negative control injection of 20 µl diluent was injected into the contralateral forearm. Participants were asked to refrain from taking antihistamines or oral steroids for a minimum of five days and two weeks beforehand, respectively. Early phase responses were measured 15 minutes after the intradermal injection. The wheal outline was traced and transferred into the patient record. Late phase responses were measured after 24 hours by palpation of the outline of edema. The area of the late response was also traced and transferred to the patient record. A single clinician performed all measurements under double-blind conditions. The early and late phase response areas were calculated from scaled scanned images of the tracings with NIS Elements v4·2 software (Nikon Instruments). Early and late phase response areas were then compared in the intradermal immunotherapy and control arms at each time point.

Skin biopsy

Forty participants (20 in each trial arm) were randomized to undergo 3 mm skin punch biopsies immediately after measurement of late phase responses (i.e. 24 hours after challenge) four months after completing their final treatment injections, in September 2013. Biopsies were collected from both allergen challenged and diluent control sites. Local anaesthesia was achieved with lidocaine hydrochloride 10 mg/mL with adrenaline 1 in 200 000 (5 micrograms/mL). In the first 20 subjects, biopsies were divided with a scalpel into two pieces and one half piece was fixed in 4% paraformaldehyde (Sigma-Aldrich, Poole, UK) for 2 hours. In the rest of the subjects, entire biopsies were processed for immunohistochemistry by fixation in 4% paraformaldehyde at room temperature for 4 hours, After washing twice in 15% sucrose, biopsies were mounted in OCT embedding medium (Bayer UK Ltd., Basingstoke, United Kingdom) and stored at -80^{o}C pending analysis. The remaining unfixed half-biopsy pieces were cultured directly for T cell analysis.

Analysis of T cells cultured from skin biopsies

Skin biopsy tissue was finely dissected and resuspended in complete medium (RPMI supplemented with 10% fetal calf serum, Penicillin (100 U/ml), Streptomycin (100 µg/ml) and L-glutamine (2 mM) (all from Life Technologies, Warrington, UK). Tissues were then cultured at 37°C in a humidified atmosphere containing 5% CO_2 in the presence of IL-2 (50 U/ml). After 3-4 days, cells were passed through a 0.2 µm cell strainer to obtain single cell suspensions and restimulated with immobilized anti-CD3/CD28 antibodies for a further 3 days, followed by expansion for 4 days in the presence of IL-2.

Expanded T cells were stained with the viability dye eFluor®780 (eBioscience, Vienna, Austria) prior to surface staining with anti-CD4 PerCP-Cy5.5 (BioLegend, London, UK), anti-CD8 BV510 (BD Biosciences, Oxford, UK), anti-CRTH2 PE (BioLegend), anti-CXCR3 BV421 (BioLegend), anti-CCR6 PE-Cy7 ((BD Biosciences) and anti-IL-25 receptor AF647 (kind gift of Dr Andrew McKenzie). Samples were resuspended (FACSFlow™, BD Biosciences) for flow cytometric analysis (FACSCalibur™, BD Biosciences). Data were analysed using FlowJo™ v7.6 software (Tree Star, Inc., Oregon, USA).

For microarray studies, cells were activated for 4 hours with ionomycin (500 ng/ml) and phorbol 12-myristate 13-acetate (PMA) (5 ng/ml) (both Sigma Aldrich). RNA was isolated from cell pellets using the miRNeasy™ mini kit and RNeasy MinElute™ cleanup kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. cDNA synthesis and amplification was performed with the Ovation PicoSL™ WTA system V2 kit (NuGEN, Leek, Netherlands) as per the manufacturer's instructions. Purity and yield was then analyzed using

the Bioanalyzer platform (Agilent, Stockport, UK) and NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Loughborough, UK) respectively, before amplified cDNA was biotin-labeled with the NuGEN Encore™ BiotinIL module according to the manufacturer's instructions. Biotin-labeled cDNA was hybridized to an Illumina Human HT-12 v4 Expression BeadChip™ before scanning with the iScan™ system (Illumina, Essex, UK) untilising GenomeStudio™ software. Data analysis was performed with the Partek Genomics Suite™ software (Partek Incorporated, Missouri, USA). [1]

Immunohistochemistry

Immunohistochemical staining of skin biopsies was performed using the modified alkaline phosphatase anti-alkaline phosphatase (APAAP) method to stain for eosinophils, neutrophils, CD4+ T cells, and CD3+ T cells.[4,5] In brief, 8-10 μm thickness tissue sections were air dried overnight on poly-L-lysine coated slides. For immunostaining, slides were incubated at room temperature in a humidified chamber with the primary mouse mAb (neutrophil elastase, Dako, Ely, UK; eosinophil major basic protein, Abcam, Cambridge, UK; CD3 and CD4, both Dako) suspended in 5% human serum/PBS for predetermined optimzed incubation times. Sections were then washed in PBS and incubated with rabbit anti-mouse Ig (Dako) for 30 minutes, then washed again. Slides were then incubated with a third layer of soluble complexes of AP and mouse anti-APAAP (Serotec, Kidlington, UK) for 30 minutes, washed and developed with Fast Red (Sigma-Aldrich, Poole, UK) for a further 20 minutes. Sections were washed extensively in PBS before counter-staining with Harris' hematoxylin (BDH, Poole, UK) and mounted in glycerol gel. For negative controls, each primary antibody was substituted with the appropriate isotype-matched irrelevant mAb. Slides were counted blind in random order by two observers. Allergen and diluent biopsy sections were evaluated from each subject. The total number of positive cells was expressed as the number of cells per square millimeter of biopsy. Inter-observer variability was 7%, assessed on repeat counts of 19 slides. The difference between the two counts was plotted against the mean of the two counts; all but one of the differences fell within two standard deviations of the mean difference, indicating satisfactory agreement between observers.

[1] The following link has been created to allow review of record GSE72324 while it remains in private status: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=evsfsmqyxdgffod&acc=GSE72324

Serum antibody measurements

Sera were analyzed for concentrations of *Phleum pratense-specific* IgG, IgG4 and IgE, and IgE specific to the major allergens Phl p 5 and Phl p 1 using a commercial assay system according to the manufacturer's instructions (ImmunoCAP^{TM}, ThermoFisher Scientific, Horsham UK).

Basophil Activation Test

Basophil activation tests were performed in 92 participants following administration of the final intradermal allergen immunotherapy or control injection (May 2013). Whole blood was collected and tested within 2 hours of sampling under blinded conditions by a single investigator (AG). Heparinized whole blood was immunostained with anti-human CD3 PE-Cy7 (BD Biosciences), CD294 PE (Miltenyi Biotec, Woking, UK), CD203c PerCP-Cy5·5 (BioLegend), CD303 APC (Miltenyi Biotec), CD107a Brilliant Violet 421 (BioLegend), CD63 FITC (BioLegend) and isotype controls. Basophils were then stimulated with anti-human IgE (1000 ng/ml, positive control; Abcam) or *Phleum Pratense* extract (ALK Abelló) at 10 ng/ml and 100 ng/ml for 15 minutes at 37^{o}C. Samples were then lyzed (BD FACS Lysing Solution, BD Biosciences), washed and resuspended (CellFix^{TM}, BD Biosciences) for flow cytometric analysis (FACSCalibur™, BD Biosciences). Data were analyzed using FlowJo^{TM} v7.6 software (Tree Star, Inc., Oregon, USA), gating on CD3^{neg}CD303^{neg}CD294^{pos} basophils. Basophil activation was expression as percentage CD63^{pos}, CD203c^{pos} or CD107a^{pos} basophils of the entire basophil population.

Supplementary References

E1. Zuberbier T, Bachert C, Bousquet PJ, et al. GA(2) LEN/EAACI pocket guide for allergen-specific immunotherapy for allergic rhinitis and asthma. *Allergy* 2010; **65**(12): 1525-30.

E2. Canonica GW, Baena-Cagnani CE, Bousquet J, et al. Recommendations for standardization of clinical trials with Allergen Specific Immunotherapy for respiratory allergy. A statement of a World Allergy Organisation (WAO) taskforce. *Allergy* 2007; **62**(3): 317-24.

E3. Cox L, Larenas-Linnemann D, Lockey RF, Passalacqua G. Speaking the same language: The World Allergy Organisation Subcutaneous Immunotherapy Systemic Reaction Grading System. *J Allergy Clin Immunol* 2010; **125**(3): 569-74, 74.e1-74.e7.

E4. Frew AJ, Kay AB. The relationship between infiltrating CD4+ lymphocytes, activated eosinophils, and the magnitude of the allergen-induced late phase cutaneous reaction in man. *J Immunol* 1988; **141**(12): 4158-64.

E5. Gaga M, Frew AJ, Varney VA, Kay AB. Eosinophil activation and T lymphocyte infiltration in allergen-induced late phase skin reactions and classical delayed-type hypersensitivity. *J Immunol* 1991; **147**(3): 816-22.

E6. Varney VA, Gaga M, Frew AJ, Aber VR, Kay AB, Durham SR. Usefulness of immunotherapy in patients with severe summer hay fever uncontrolled by antiallergic drugs. *BMJ* 1991; **302**(6771): 265-9.

Supplementary Figure Legends

Fig E1. Effects of Intradermal Immunotherapy on Phl p 5- and Phl p 1-specific IgE

Levels of IgE specific for major allergens Phl p 5 and Phl p 1 before and after completion of seven intradermal allergen or histamine control injections. P values for pre- and post-treatment comparisons based on the Wilcoxon signed-rank test. P values for between group comparisons are based on ANCOVA.

Fig E2. Flow cytometric analysis of CD4+ T cells from skin biopsy explants

Representative flow cytometry plots illustrating surface staining for CCR6, CXCR3 and CRTH2, gated on skin biopsy-derived CD4+ T cells, in a participant who received histamine control (left) and a participant who received grass pollen intradermal injections (right).

Fig E3. Heatmap showing expression of selected genes associated with Th1/Th2 phenotypes and allergic inflammatory responses.

Fig E4. Basophil activation tests

Percentage of basophils staining positive for activation markers CD63 (A), CD107a (B) and CD203c (C). Whole blood was stimulated under the conditions shown. P values are based on the Mann-Whitney U test.

Fig E5. Immunohistochemistry analysis of skin biopsies

Comparison of allergen-induced inflammatory cell numbers in skin biopsies from intradermal immunotherapy and control arm participants. Data shown indicate numbers of neutrophils (A), eosinophils (B), $CD3^+$ cells (C) and CD4+ cells (D) in skin biopsies taken after diluent and *Phleum Pratense* intradermal skin challenges in September 2013. Cells were stained using the APAAP method. Solid bars represent median values. P values comparing diluent and allergen-challenged biopsies are based on the Wilcoxon signed-rank test. P values for between group comparisons are based on ANCOVA.

Fig E6. Lymphangitis in a participant who received active intradermal immunotherapy. Photograph taken 40 minutes after intradermal injection.

Supplementary Tables

TABLE E1. Verification of participant blinding

	Trial Arm	
Patient Guess Trial Arm	**Intradermal Immunotherapy (n=44)**	**Control (n=43)**
Intradermal Immunotherapy (n=44)	22	22
Control (n=43)	22	21

287 At the end of the pollen season participants verified blinding by guessing if they had received active or control treatment

TABLE E2. Effect of Intradermal Immunotherapy on Primary and Secondary Outcomes (Intention-to-Treat); Missing data imputed

	Intradermal Immunotherapy (n=46)	Control (n=47)	Difference (95% CI)	P value
Primary Outcome	Median (IQR)			
CSMS during entire season	502 (333–841)	509 (365–738)	8 (-174.7 to 210.9)	.91
Secondary Outcomes				
Symptom score during entire season	335 (183–525)	264 (156–434)	61 (-7.8 to 123.2)	.22
Medication Score during entire season	242 (116–405)	263 (129–482)	-24 (-173.1 to 107.5)	.39
CSMS Score during peak season	363 (232–570)	370 (292–573)	-11 (-95.8 to 77.5)	.80
Nasal symptom score during entire season	178 (120–218)	131 (80–200)	33 (0.3 to 68.5)	.03
Mouth symptom score during entire season	39 (8– 90)	14 (6–45)	11 (3.1 to 26.1)	.05
Eye symptom score during entire season	79 (41–158)	78 (52–180)	-7 (-20.0 to 3.0)	.51
Lung symptom score during entire season	20 (3– 32)	12 (0– 40)	4 (-1.0 to 15.3)	.17
Nasal Allergic Symptoms measured by VAS	162 (107–275)	124 (66–166)	59 (-3.7 to 133.2)	.02
Eye Allergic Symptoms measured by VAS	97 (37–197)	112(42–169)	2 (-45.6 to 49.0)	.56
Global Evaluation of Symptom Scores	3 (2–4)	3 (1–3)	0 (0 to 1)	.43
Symptom Free Days	35 (19–53)	41 (23–61)	-6 (-17 to 3)	.15
No. days prednisone used during entire season	0 (0–0)	0 (0–0)	0 (0 to 0)	.36
Medication Free Days	81 (65–93)	76 (56–94)	4 (-11.0 to 21.0)	.22
Mini-RQLQ	16 (13–23)	18 (10–25)	-0.3 (-4.2 to 3.7)	.89
EQ-5D-5L	87 (83–94)	88 (81–94)	9 (-24.8 to 43.6)	.59

Data for primary outcome and all symptom scores represent Area Under Curve values

Median difference between groups calculated by stratified Hodges-Lehmann.

P values based on stratified Mann-Whitney U test (Van Elteren's test) adjusted for stratification factors

P values for mini-RQLQ and EQ-5D-5L based on linear mixed model adjusted for stratification factors

Entire grass pollen season: 13 May-3 August 2013; Peak season: 12 June-26 July 2013.

CSMS: combined symptom & medication score, VAS: Visual-analog scale

Mini-RQLQ: mini-Rhinoconjunctivitis Quality-of-Life Questionnaire, EQ-5D-5L: EuroQoL instrument

TABLE E3. Frequency of Adverse Events reported from first intradermal allergen immunotherapy or control injection until end of pollen season

	Intradermal Immunotherapy (n=46)				Control (n=47)			
	No. Participants with ≥1 AE	**% Participants**	**No. Events**	**Event Rate (%)**	**No. Participants with ≥1 AE**	**% Participants**	**No. Events**	**Event Rate (%)**
Any AEs	40	87	148		42	89	145	
Serious Adverse Events	1	2.2	1	0.7	2	4.3	2	1.4
Tonsillitis	1	2.2	1	0.7	0	0	0	0
Overnight stay for Polysomnography	0	0	0	0	1	2.1	1	0.7
Extraction of infected dental plate	0	0	0	0	1	2.1	1	0.7
Relation of AE to treatment								
Definite/Probable	3	6.5	15	10	6	13	14	9.7
Possible	0	0	0	0	0	0	0	0
Remote	30	65	68	46	34	72	70	48
None	32	70	65	44	34	72	61	42
AE withdrawals	0	0	0	0	0	0	0	0
Systemic Adverse Reactions	3	6.5	15	10	6	13	13	9.0
Generalised Pruritus	2	4.3	8	5.4	4	8.5	9	6.2
IgE-mediated lymphangitis	1	2.2	7	4.7	0	0	0	0
Light-headedness	0	0	0	0	2	4.3	2	1.4
Facial flushing/feeling hot	0	0	0	0	2	4.3	3	2.1
Systemic Adverse Reactions*								
Grade 1	3	6.5	15	10	6	13	12	8.3
Grade 2	0	0	0	0	0	0	0	0
Grade 3	0	0	0	0	0	0	0	0
Grade 4	0	0	0	0	0	0	0	0

Statistical comparison was by Fisher's Exact test for ≤5 events and Chi^2 test for >5 events.

*Classified using the World Allergy Organization grading system for systemic reactions to subcutaneous immunotherapy, Cox L et al. JACI 125:569-574, e567.

Table E4. Microarray gene expression profiles of activated CD4+ T cells derived from skin biopsy explants

Gene	P value	Fold-Difference
Intradermal Immunotherapy down versus Control group		
LOC100133042	.02	-1.80
CEP55	.03	-1.78
GFOD1	.00	-1.77
HIST2H2AB	.04	-1.62
H2AFZ	.02	-1.61
LOC730534	.01	-1.57
HSD17B4	.02	-1.57
HIST1H2AD	.03	-1.56
HDAC1	.01	-1.55
CCL3L1	.03	-1.53
CALR	.02	-1.52
CDCA5	.01	-1.52
PRDX5	.01	-1.51
FEN1	.02	-1.50
Intradermal Immunotherapy up versus Control group		
EPS15	.02	1.51
MYB	.01	1.52
GK	.03	1.53
RNASET2	.03	1.55
LOC729383	.02	1.56
GPR171	.00	1.59
LOC729387	.04	1.60
SLC11A2	.02	1.60
HS.508682	.04	1.68
IL5	.03	1.71
GBP5	.05	1.79
TNFSF8	.01	1.79
TNIP3	.03	1.87
CENTA1	.05	2.11

290 Data analyzed by 3 way-ANOVA model.