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High dose pollen intralymphatic immunotherapy: two RDBPC trials question the benefit of dose increase

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Laila Hellkvist contributed to the study design and recruited, treated and took samples from the patients in Stockholm, analyzed the clinical data, contributed to interpretation of the immunological results and prepared the draft manuscript.

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Dan Weinfeld recruited and treated the patients in Borås, interpreted the serology data regarding IgE and IgG4 and revised the manuscript.

Åslög Dahl analyzed the relationship between the pollen levels and CSMS.

Agneta Karlsson coordinated the enrollment, treatment, and follow-up of patients in Stockholm and Lund, monitored the adherence to the symptom diary and put together the clinical data file.

Marit Westman and Karin Lundkvist helped to recruit and treat patients in Stockholm and revised the manuscript.

Ola Winqvist contributed to the study design and interpretation of the immunological results.

Susanna Kumlien Georén contributed to the study design, planning and interpretation of the flow cytometry analysis and revised the manuscript.

Ulla Westin recruited and treated the patients in Lund and revised the manuscript.

Lars Olaf Cardell planned the project, contributed to interpretation of the clinical and immunological results and revised the manuscript.

Short title

Intralymphatic immunotherapy in two up-dosing trials

Declarations of interest

The studies were funded by grants from the Swedish Research Council and the Asthma and Allergy Association. The first author has received grants from Hesselman's Research Foundation and ACTA Otolaryngologica.

Registration in public trials registry

ILIT after SCIT-10 000 was registered at ClinicalTrials.gov with ID NCT02679105. ILIT de novo- 3000 was registered at ClinicalTrials.gov with ID NCT02975479.

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Abbreviations

AR	Allergic rhinitis
AIT	Allergy immunotherapy
AUC	Area under the curve
CSMS	Combined symptoms and medication score
CM	Central memory cell
DC	Dendritic cell
EM	Effector memory cell
GAM	Generalized Additive Model
ILIT	Intralymphatic immunotherapy
MFI	Median fluorescence intensity
MS	Medication score
NPT	Nasal provocation test
PBL	Peripheral blood
RR	Relative risk

RQLQ	Rhinoconjunctivitis quality of life questionnaire
RDBPC	Randomized double-blind placebo-controlled
SCIT	Subcutaneous immunotherapy
SPT	Skin prick test
SS	Symptom score
Th cell	T helper cell
Treg cell	T regulatory cell
VAS	Visual analogue scale
QoL	Quality of life

Abstract

Background

The same dosing schedule, 1000 SQ-U times three, with one-month intervals, have been evaluated in most trials of intralymphatic immunotherapy (ILIT) for the treatment of allergic rhinitis (AR). The present studies evaluated if a dose escalation in ILIT can enhance the clinical and immunological effects, without compromising safety.

Methods

Two randomized double-blind placebo-controlled trials of ILIT for grass pollen induced AR were performed. The first included 29 patients that had recently ended 3 years of SCIT and the second contained 39 not previously vaccinated patients. An up-dosage of 1000-3000-10 000 (5000 + 5000 with 30 minutes apart) SQ-U with one month in between was evaluated.

Results

Doses up to 10 000 SQ-U was safe after recent SCIT. The combined symptom-medication scores (CSMS) were reduced by 31% and the grass specific IgG4 levels in blood were doubled. In ILIT de novo, the two first patients that received active treatment developed serious adverse reactions at 5000 SQ-U. A modified up-dosing schedule; 1000-3000-3000 SQ-U appeared to be safe but failed to improve the CSMS. Flow cytometry analyses showed increased activation of lymph node derived dendritic but not T-cells. Quality of life and nasal provocation response did not improve in any study.

Conclusion

ILIT in high doses after SCIT appears to further reduce grass pollen induced seasonal symptoms and may be considered as an add-on treatment for patients that do not reach full symptom control after SCIT. Up-dosing schedules de novo with three monthly injections that exceeds 3 000 SQ-U should be avoided.

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Introduction

Allergy immunotherapy (AIT) is the only treatment for allergic rhinitis (AR) that is both symptom ameliorating and changes the course of the disease, although rarely eliminates the symptoms totally (1). It is usually given as subcutaneous immunotherapy (SCIT) with repeated injections at hospital or as sublingual immunotherapy (SLIT) with daily administrations at home (1, 2). Both routes involve treatment during at least three years and problems with side effects (3) and adherence (4) limit the use.

More than ten years ago, intralymphatic injections were proposed as a new route for AIT, based on a trial of three low dose (1000 SQ-U) grass allergen injections given in lymph nodes (5), and the concept of intralymphatic immunotherapy (ILIT) was born. In ILIT, superficial lymph nodes in the groin are visualized with ultrasound guidance and injected with allergen, aiming at the lymph node cortex. During the last years, several studies have evaluated the same or equivalent doses as in the original study. The extracts used have included a recombinant cat dander allergen (6), house dust mite allergen (7), cedar pollens (8, 9), grass pollen (10-13), and in our own research group combinations of grass and/or birch pollens (14-17). Different up-titration schedules have been applied with grass allergen (18) and a combination of house dust mite, dog and cat allergens (19, 20). Most trials, but not all (10), have indicated improvement of allergen triggered symptoms and have had few and mild side effects.

The efficacy of AIT usually corresponds to a high allergen dose (21). What often limits the level of the maintenance doses is the risk of allergic side reactions (22). Based on the good safety profile of previous ILIT studies, a dose-escalation seemed to be the next step to develop ILIT. The two studies presented in this article are the first randomized double-blind placebo-controlled (RDBPC) trials that explore ILIT with ALK Alutard 5-grasses® and ALK Diluent as placebo, in up-dosing schedules. The overall aim was to investigate if the dose increase 1000- 3000- 10 000 (divided into 5000 + 5000 with 30 minutes apart) SQ-U could be used safely and to evaluate the potentially additional clinical improvement. Firstly, in "ILIT after SCIT- 10 000", we investigated whether the schedule was safe among patients that had recently been treated with SCIT and presumably had a tolerance to the allergen. In the second study, "ILIT de novo- 3000", the up-dosing schedule was attempted in patients without preceding SCIT. However, the first two patients had anaphylactic reactions at 5000 SQ-U. Therefore, the up-dosing protocol 1000- 3000- 3000 SQ-U was used. To track immunological changes following the treatment, we measured IgE and IgG₄ antibody levels as well as the activation of T-cells in blood and dendritic cells (DCs) in the lymph nodes. See Figure 1 and the supplementary section for a description of the study outline and the methods used.

Results

Patients

The baseline characteristics were equal in the active and placebo group in ILIT after SCIT-10 000, except for more patients with high symptom scores (SS) being randomized to the active group. In ILIT de novo-3000, the demographics were also balanced except for higher overall estimation of disease severity on visual analogue scale (VAS) in the placebo group. (See Table 1.)

After screening and treatment, 12 placebo and 13 active patients remained for analysis in ILIT after SCIT-10 000. In ILIT de novo-3000, 19 placebo and 16 active patients completed the protocol. See the supplementary information and Figure E1 for the flow of patients.

Safety

The adverse events in ILIT after SCIT-10 000 were mostly mild. Small reactions at the injection site were common after the up-dosing injections, but also occurred after placebo injections. One active patient had rhinorrhea during 2 days after the first injection. One placebo patient suffered from pain, swelling in the groin, fever and general muscle pain 4 days after the second injection, perhaps representing a mild, self-limited infection.

In ILIT de novo-3000, the first dose with 1000 SQ-U did not elicit any moderate or severe adverse events. After 3000 SQ-U one active patient reported tightness over the chest and head, itching in the palms, soles and at the neck without erythema, 6-12 hours after the injection. This patient was withdrawn from the study. Three other active patients reported isolated mild skin itching distant from the injection site, without erythema, after the second injection and stayed in the study. In addition, one active patient reported heavy breathing after the second injection. It was at this time point considered unspecific and the patient proceeded with the up-dosing protocol, see below.

Four randomized patients continued to 5000 SQ-U, aiming at 10 000 SQ-U. Two patients, later confirmed having received active treatment, developed anaphylactic reactions at 5000 SQ-U. Patient number 1 had intense rhinitis and urticarial hives 15 minutes after the 5000 SQ-U injection. He received anaphylaxis treatment with intramuscular adrenaline, oral corticosteroids and antihistamines. The reaction resolved promptly, and he could be discharged from the hospital after 2 hours. Patient number 2 experienced an odd sensation immediately after the injection, dizziness and weakness after 5 minutes followed by abdominal pain, nausea, itching chest erythema, flush, facial angioedema and hypotension. Unfortunately, S-Tryptase was not measured. The patient received immediate anaphylaxis treatment as above with the addition of intravenous corticosteroids, nasal oxygen and intravenous fluids, was stabilized after 30 minutes and could be discharged from the hospital the same day. This patient had reported a late reaction with heavy breathing, mild nasal obstruction, increased heart rate and mild itching at the injection site already after 3000 SQ-U.

Consequently, the dose-escalation was interrupted. The protocol 1000-3000-3000 SQ-U could be performed without any serious adverse events. Two active patients had a large (>10 cm) erythema at the injections site at the last injection, both were correlated with signs of leakage of the allergen outside of the lymph node visualized with ultrasound. See Table 2 for details.

Pollen counts

According to the local pollen counts during the study periods in 2015-2017, the grass pollen levels were 17-21% lower in 2016 than in 2015 and 25-46% lower in 2017 than in 2016. See Figure 2 and Table E1.

Primary outcome measure

In ILIT after SCIT- 10 000, the median combined SMS scores (CSMS) were reduced by 31% in the active group at the pollen season after treatment compared to the season before treatment. The CSMS in the placebo group did not change (see Figure 3 and Table 3.) When comparing the active group with the placebo group there was no difference in CSMS after treatment; the active group (n=13) had median

CSMS 76.3 (IQR 22.7-109.9) after treatment, and the placebo group (n=12) had median CSMS 46.2 (IQR 30.1-71.2), p=0.31. Excluding one patient that had incomplete SMS registration at the baseline season did not change the result.

In ILIT de novo- 3000, both the placebo and active groups improved the year after treatment. The active group reduced the mean CSMS with 24% and the placebo group reduced the mean scores with 28% (see Figure 3 and Table 3). Three patients had received a different up-dosing (1000-1000-3000 or 1000-3000-5000 SQ-U) but were not outliers. There was no difference between the active group versus the placebo group after treatment, the active group had mean CSMS 68.9 (SD 28.9 [95% CI 53.5-84.3]), n=16 and the placebo group had mean CSMS 74.8 (SD 34.7 [95% CI 57.5-92.0]), n= 18, p=0.60.

Secondary outcomes

SMS at the total study period

In ILIT after SCIT-10 000, the daily symptom scores (SS) did not change when comparing the scores before versus after treatment. In contrast, the medication use was significantly lowered in the active group the year after treatment, measured as a 52% reduction of the median medication scores (MS). The placebo group did not change. (See Figure 3 and Table 3.) When comparing the active versus the placebo group, the SS was unbalanced at baseline with 41% lower scores in the placebo group than in the active group (p=0.02, see Table 1). After treatment, the gap between the groups had narrowed and there was no significant difference. The placebo group (n=11) had median SS 21.8 (IQR 13.1-32.6) and the active group (n=13) had median SS 43.7 (IQR 13.4-53.2), p=0.31. The MS was balanced at baseline and the result of the between group comparison had not changed after treatment. The placebo group (n=11) had median MS 28.0 (IQR 6.5-33.5) after treatment, and the active group (n=13) had median MS 27.5 (IQR 3.0-63.3) after treatment, p=0.91.

In ILIT de novo- 3000, the SS and MS were lower in both the active and placebo group the year after treatment. The SS was reduced by 25% in active group and 20% in the placebo group. MS was reduced by 32% in active group and 36% in the placebo group. (See Figure 3 and Table 3.) Analysis between the active

(n=16) and placebo group (n=18) after treatment did not reveal any differences in SS ($p=0.49$) or MS ($p=0.64$). (See Table 3.)

SMS at the peak pollen season

In ILIT after SCIT- 10 000, the median MS in the active group was reduced with 26% at the peak pollen season after treatment compared to before treatment. The placebo group did not improve. There was no change in SS or CSMS within any of the groups and no difference between the active (n=13) or placebo group (n=11) in the SS ($p=0.19$), MS ($p=0.97$) or CSMS ($p=0.58$).

In ILIT de novo- 3000, the placebo group reduced the CSMS and the MS after treatment, while the active group did not improve. There was no difference between the active (n=15) and placebo group (n=18) after treatment in SS ($p=0.92$), MS ($p=0.81$) or CSMS ($p=0.84$). (See Table E2).

Serology

In ILIT after SCIT- 10 000, the allergen specific IgG₄ levels decreased by 41% in the placebo group during the course of the study. In the active group the IgG₄ levels were doubled 4 weeks after treatment with an increase from median level 3.4 to 8.3 mg/L but had returned to baseline levels 8 months after treatment. We could not find any correlation between the IgG₄ induction and CSMS.

In ILIT de novo- 3000 there was also a statistically significant, but modest, increase in allergen specific IgG₄ levels. 4 weeks after active treatment the levels had increased by about 42% from median 0.24 to 0.34 mg/L, $p=0.0001$. 8 months after treatment the levels had declined towards baseline; median specific IgG₄ was 0.25 mg/L, $p=0.03$. The placebo group did not change.

The allergen specific IgE antibodies increased transiently in the active group 4 weeks after treatment in both studies. At the follow up 8 months after treatment the levels had normalized in ILIT after SCIT- 10 000 but were still elevated compared to the baseline level in ILIT de novo- 3000. The placebo group did not change. (See Figure 4 and Table E3.)

Activation of dendritic cells and CD4 T-cells

In patients treated with active ILIT, DCs in the lymph node showed increased activation after treatment, with upregulated expression of T-cell co-stimulatory molecules. Mean MFI for CD80 increased from 345.4 (SD 128.2 [95% CI 186.2-504.6]) to 1050 (SD 652.2 [95% CI 548.4-1515]), $p=0.028$, Mean MFI for CD86 increased from 3927 (SD 2171 [95% CI 1231-6623]) to 6710 (SD 1806 [95% CI 5322-8098]), $p=0.09$. DCs in active ILIT increased the expression of CD141. Mean MFI in placebo after treatment was 3420 (SD 1796 [95% CI 2214-4627]) compared to active ILIT 6257 (SD 1990 [95% CI 4727-7786]), $p=0.002$. Flow cytometry histogram for a representative staining is presented in Figure E5. No changes in DCs from lymph node samples were detected in the placebo-treated patients. DCs in PBL did not change in any of the groups. For the statistical analysis, a 2-way ANOVA with Fishers LSD test was used (see Fig. 5).

In PBL, there were no significant changes of the amount of CCR5⁺ CM T-cells or CD25⁺ CD4⁺ EM T-cells. No significant difference in the amount of allergen activated CD4⁺ T-cells could be detected between the placebo and active group after treatment. (See Figure E2 and supplementary information.)

VAS, Quality of Life, Nasal Provocation Test

The global estimation of improvement on VAS did not show any difference after treatment in any of the studies. The Quality of Life (QoL) was unchanged in ILIT after SCIT- 10 000 but improved in the placebo group and not in the active group in ILIT de novo- 3000. The Nasal Provocation Test (NPT) did not change in any of the studies. (See Figure E3 and supplementary information.)

Relationship between pollen levels and CSMS

The patients treated in Malmö in ILIT after SCIT- 10 000 (12 active and 7 placebo) were included in a subgroup analysis regarding responsiveness to allergen exposure measured by the local pollen counts. With a generalized additive model, we attempted to analyze if the relationship between the risk for

worsening of symptoms and increased pollen concentration changed during the study. Before treatment, at an increase with 10 grain/m³ the RR for aggravated CSMS was 1.017 [SE 1.010-1.024] p= 0.029 in the active group. After treatment, the relationship between increased pollen concentration and worsening of the symptoms did not reach statistical significance. However, in the placebo group the pattern was the same. (See Table E4 and Figure E4.)

Discussion

In the present two studies, doses up to 10 000 SQ-U was safe after recent SCIT, but up-dosing to 5000 SQ-U caused serious adverse reactions in previously non-AIT treated patients. Grass-specific IgG₄-levels were boosted in both studies but the CSMS was improved only in ILIT after SCIT- 10 000. ILIT de novo- 3000 showed increased activation of DCs in the lymph nodes but no induction of Treg cells in blood.

The dose-escalation 1000-3000-5000 SQ-U with one-month intervals in de novo-patients seems hazardous and should probably be avoided. No technical problems at the injections can explain the result. It is possible that the allergen was drained fast to the hilus of the lymph node and further to the thoracic duct and systemically through the venous system. One of the patients that experienced an anaphylactic reaction had reported heavy breathing after the previous ILIT-injection, which in retrospect can be understood as a risk factor (19). However, the other patient in the present study with anaphylactic reaction had not reported any previous symptoms making up-dosing contraindicated.

At the planning stage of the trials, a previous up-dosing ILIT study had used ALK Center-AL® Phleum pratense in doses up to 250 PNU (18). We estimated that 5000-10 000 SQ-U Alutard® could be in the same range, but it is hard to translate allergen doses between different extracts. The dose escalation after SCIT could probably be carried out since the patients had a remaining tolerance to the allergen.

In ILIT after SCIT- 10 000, the median CSMS was 31% lower in the active group at the pollen season after treatment compared to the season before. In general, it is recommended to use between groups comparisons in AIT-trials to measure the relative treatment effect in relation to the placebo effect and avoid the confounding factor of different pollen counts during two seasons of comparison. When baseline registration of symptoms is available, within group comparisons of before versus after treatment is also valid (23). Our limited sample size, with unbalanced group at baseline, made it difficult to achieve a

positive result in the comparison of the active versus placebo group after treatment. The pollen levels were 17-21% lower the season after treatment compared to baseline. Still, the placebo group did not report less symptoms. This supports a true improvement in the active group.

In ILIT after SCIT- 10 000, the GAM regression analysis showed higher CSMS at increasing grass pollen concentrations before treatment. This result was similar to what have been found in other grass pollen studies in Sweden (24), France and Switzerland (25). However, our results could not be reproduced after treatment, probably due to few days with high pollen counts and a limited sample size.

In ILIT de novo- 3000, none of the parameters CSMS, MS, SS, QoL or NPT were improved. The pollen counts were 25-46% lower the year after treatment compared to baseline, which might explain the intra-season results (26). Alternatively, the treatment effect might be rather short and perhaps not lasting for some patients that completed the ILIT treatment up to 6 months before the start of the grass pollen season. The study may also have been underpowered for detecting changes in secondary outcomes. However, not even a trend for improvement could be seen. In addition, at the peak pollen season the placebo but not the active group had improved CSMS, MS and QoL. This speaks against a favorable effect of up-dosing ILIT in de-novo patients.

Immunologically, serum IgG₄ increased markedly in ILIT after SCIT and to a smaller extent in ILIT de novo-3000. It seems that the SCIT treated patients were already primed towards inducible IgG₄ production and responded after booster ILIT therapy with an expected recall response from the memory B cell population. Another observation is that the IgE-levels increased early after active ILIT, as in other AIT. The weak IgG₄ response in ILIT de novo- 3000 reduced the IgG₄/IgE ratio, which could indicate an incomplete immunological skew (27, 28). It is possible that that the failure to improve the symptoms in ILIT de novo-3000 is linked to the weak IgG₄ induction.

In the presented studies we used allergen adsorbed to aluminum, widely used in AIT to enhance the availability of the allergen for antigen presentation (29). Aluminium containing extracts have also been used in ILIT animal studies (30) and in veterinary trials (31, 32), as well as in the first human ILIT study from 2008 (5) and in ILIT up-dosing (18). In the presented study, when increasing the doses from 1000 to 3000 and 5000 SQ-U, also the aluminium content was increased from 0.033 to 0.099 and 0.165 mg respectively. This might not be beneficial, since aluminium also have been described skewing the immune balance towards more activation of T helper (Th) type 2 cells (33). The advantage of intralymphatic

administration is that the allergen is delivered directly to a high density of immunologically active cells. Hence, aluminium enhanced antigen presentation might not be necessary in ILIT. Even though no aluminium related adverse events have been noted in ILIT, potential undesired accumulation in the lymph nodes can not be excluded. Evaluation of ILIT with aluminum-free extracts and more specific Th1 skewing adjuvants would be of great interest.

In ILIT de novo, we could indeed see signs of Th2 type of activation. Lymph node derived DCs increased the expression of CD141, previously described on DC:s promoting differentiation to Th2 (34). Our previous low dose ILIT study (16) showed activation of Th1 and T regulatory (Treg) cells in PBL. This could not be repeated in the present study. Activation of T cells is dependent on concentration of stimulants, direction of stimuli and timing. Thus, dosing and dose intervals seem to be important to find the therapeutic window. This hypothesis is supported by a previous ILIT trial with lack of symptom improvement when the time intervals between the injections were shortened, which increased the allergen dose per time unit (10, 35). The mode of action in ILIT may include a combination of multiple mechanisms as in conventional AIT, such as Treg expansion, Th1 skewing and the induction of allergen specific IgG₄ responses. Although under debate, with regards to the origin of IgE memory B cells(36, 37), an additional mechanism for long term effect in ILIT may be a fine-tuned apoptosis of high affinity IgE memory B cells due to the abundance of allergen in the lymph nodes upon ILIT-injections. Thus, it is not surprising that allergen dose, adjuvant composition and time intervals needs to be titrated to find the optimal tolerance inducing window.

This is not a consensus experience for other forms of AIT. But according to the literature, one previous birch SLIT study have shown that a dose increase did not confer more symptom relief (38) and a grass SLIT study even showed a lower treatment effect in a high-dose group (39). In the present study of ILIT in de novo patients, the lack of clinical improvement and favorable immunological changes after 3000 SQ-U suggests that the optimal dose for ILIT with injections four weeks apart is 1000 SQ-U or below.

ILIT after SCIT- 10 000 shows that three intralymphatic injections up to 10 000 SQ-U might give additional symptom relief. Since SCIT rarely eliminates the symptoms completely, pre-seasonal ILIT after SCIT might play a role in the AIT toolbox in the future as a cost-effective supplementary treatment.

Future treatments of airborne allergies probably involve the expansion of sublingual at-home administration and the development of hypoallergenic allergoids or peptide vaccines (40, 41). Especially SLIT has very much improved the availability of AIT during the last decades. However, SLIT is not suitable

for all patients and health care systems due to the adherence problems, oral side effects and costs. It is very appealing to be able to offer the patients a short, safe and inexpensive treatment, as an alternative to SCIT and SLIT. If our knowledge about the mechanisms in ILIT can be increased, and the dose, time interval and technique of ILIT could be further optimized, ILIT may play an important role in personalized AIT treatment in the future.

Conclusion

De novo treatment with ILIT in patients with grass pollen induced allergic rhinitis should not exceed 1000 SQ-U, due to risk for severe side effects and limited improvement gain. An up-dosing schedule to 10 000 SQ-U after previous SCIT seems safe, appears to improve the seasonal symptoms and might be used to boost a previously given treatment.

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Table 1. The demographics and characteristics at baseline of the patients that were included in the data analysis.

	ILIT after SCIT- 10 000			ILIT de novo- 3000		
	Placebo group	Active group	P-value†	Placebo group	Active group	P-value†
Number of patients	12	13		19	16	
Gender			0.38			0.50
Female, no. (%)	4 (33%)	2 (15%)		6 (32%)	7 (44%)	
Male, no. (%)	8 (67%)	11 (85%)		13 (68%)	9 (56%)	
Study site			0.12			1.0
Stockholm, no. (%)	3 (25%)	1 (8%)		12 (63%)	10 (63%)	
Lund, no. (%)	7 (58%)	12 (92%)		7 (37%)	6 (37%)	
Borås, no. (%)	2 (17%)	0		0	0	
Seasonal asthma, no. (%)	3 (25%)	4 (31%)	1.0	4 (21%)	4 (25%)	1.0
Sensitization to birch pollen with positive SPT, no. (%)	11 (92%)	13 (100%)	1.0	12(63%)	8 (50%)	0.51
Age, mean (SD [range])	34 (8[22-49])	34 (6[24-43])	0.87	33(10 [20-53]	35 (8 [20-47]	0.17
Nasal provocation test, median (SD [range])	104 (55 [13-208])	118 (78 [35 - 333])	0.52	185 (51 [102-285])	170 (61 [53-290])	0.75
Timothy specific IgE (kU/L), median (SD [range])	18.5 (33.1 [1.8-100.0])	11.9 (11.8 [1.9-35.7])	0.23	11.0 (14.8 [0.9-54.0])	5.2 (14.5 [0.4-48.8])	0.28
RQLQ, median (SD [range])	1.8 (0.7 [0.5-2.4])	2.4 (1.3 [0.6-5.1])	0.08	2.8 (1.0 [1.1-4.6])	2.2 (1.0 [0.7-4.1])	0.18
Overall allergy severity estimated on VAS, mean (SD [range])	4.2 (2.5 [1.0-8.8])	4.8(2.4[1.2-8.9])	0.48	7.8 (1.6 [2.9-9.3])	6.4 (1.3 [3.6-8])	0.006
CSMS expressed as AUC, mean (SD [range]), median	62 (41 [10-157]), median: 62	98 (49 [17-167] median: 110	0.06	104 (43 [37-182])	90 (31 [43-146])	0.53
SS expressed as AUC, mean (SD [range]), median	25 (14 [5-45]), median: 27	45 (22 [5-85]), median: 46	0.02	51 (24 [17-92])	49 (17 [27-80])	>0.99
MS expressed as AUC, mean (SD [range]), median	38 (36 [0-127]), median: 33	56 (36 [3-101]), median: 58	0.22	53 (28 [11-95])	45 (28 [1-92])	0.44

†Mann Whitney test

Table 2. Adverse events

Treatment	ILIT after SCIT- 10 000						ILIT de novo- 3000					
	Events						Events					
	(No. patients)						(No. patients)					
	% of injections						% of injections					
Number of injection	1 st	2 nd	3 rd	1 st	2 nd	3 rd	1 st	2 nd	3 rd	1 st	2 nd	3 rd
Dose SQ-U	1000	3000	10 000				1000	3000	3000			
Anaphylaxis	0 (13) 0%	0 (13) 0%	0 (13) 0%	0 (12) 0%	0 (12) 0%	0 (12) 0%	0 (20) 0%	0 (18) 0%	0 (13) 0%	0 (20) 0%	0 (19) 0%	0 (19) 0%
Redness or itching ≤ 1 cm at injection site	5 (13) 38%	6 (13) 46%	7 (13) 54%	2 (12) 17%	0 (12) 0%	0 (12) 0%	9 (20+) 45%	3 (18) 17%	6 (13) 46%	5 (20) 25%	5 (19) 26%	4 (19) 21%
Redness >1cm ≤10 cm at injection site	1 (13) 8%	0 (13) 0%	2 (13) 15%	0 (12) 0%	1 (12) 8%	0 (12) 0%	2 (20+) 10%	4 (18) 22%	3 (13) 23%	0 (20) 0%	0 (19) 0%	0 (19) 0%
Redness > 10 cm ≤20 cm at injection site	0 (13) 0%	0 (13) 0%	0 (13) 0%	0 (12) 0%	0 (12) 0%	0 (12) 0%	0 (20+) 0%	0 (18) 0%	2 (13) 15%	0 (20) 0%	0 (19) 0%	0 (19) 0%
Sneezing, rhinorrhea, itchy eyes/nose/ throat	2 (13) 15%	1 (13) 8%	1 (13) 8%	2 (12) 17%	2 (12) 17%	2 (12) 17%	3 (20+) 15%	4 (18) 22%	0 (13) 0%	4 (20) 20%	5 (19) 26%	4 (19) 21%
Heavy breathing or tightness over the chest	0 (13) 0%	0 (13) 0%	0 (13) 0%	0 (12) 0%	0 (12) 0%	0 (12) 0%	1 (20+) 5%	2 (18) 11%	0 (13) 0%	0 (20) 0%	1 (19) 5%	1 (19) 5%
Mild abdominal symptoms or nausea	0 (13) 0%	0 (13) 0%	0 (13) 0%	0 (12) 0%	0 (12) 0%	0 (12) 0%	2 (20+) 10%	0 (18) 0%	0 (13) 0%	1 (20) 5%	0 (19) 0%	1 (19) 5%
Fatigue	0 (13) 0%	1 (13) 8%	1 (13) 8%	2 (12) 17%	3 (12) 25%	2 (12) 17%	1 (20+) 5%	0 (18) 0%	1 (13) 8%	5 (20) 25%	1 (19) 5%	2 (19) 11%
Headache	0 (13)	0 (13)	0 (13)	0	0	0	0	1 (18)	0 (13)	0 (20)	2 (19)	0

	0%	0%	0%	(12)	(12)	(12)	(20†)	6%	0%	0%	11%	(19)
				0%	0%	0%	0%					0%
Fever/muscle pain	0 (13)	0 (13)	0 (13)	0	1	0	1	1 (18)	0 (13)	1 (20)	0 (19)	0
	0%	0%	0%	(12)	(12)	(12)	(20†)	6%	0%	5%	0%	(19)
				0%	8%	0%	5%					0%
Skin itching distant from injection site	0 (13)	0 (13)	1 (13)	0	0	0	0	4 (18)	0 (13)	1 (20)	2 (19)	0
	0%	0%	8%	(12)	(12)	(12)	(20†)	22%	0%	5%	11%	(19)
				0%	0%	0%	0%					0%
Palpitations or dizziness	0 (13)	0 (13)	0 (13)	0	0	0	0	1 (18)	0 (13)	0 (20)	0 (19)	1
	0%	0%	0%	(12)	(12)	(12)	(20†)	6%	0%	0%	0%	(19)
				0%	0%	0%	0%					5%
Reactivation of herpes zoster	0 (13)	0 (13)	0 (13)	0	0	1	0	0 (18)	0 (13)	0	0	0
	0%	0%	0%	(12)	(12)	(12)	(20†)	0%	0%	(20)	(19)	(19)
				0%	0%	8%	0%			0%	0%	0%
Follow up after treatment	4 weeks	8 months		4 weeks	8 months		4 weeks	8 months		4 weeks	8 months	
Low Lymphocytes in blood	0 (13)	0 (13)		0 (12)	1 (12‡)		0 (19)	1 (19 ‡)		0 (20)	0 (20)	
	0%	0%		0%	8%		0%	5%		0%	0%	
Low Neutrophils in blood	1 (13§)	0 (13)		0 (12)	0 (12)		0 (19)	1 (19 §)		0 (20)	1 (20 §)	
	8%	0%		0%	0%		0%	5%		0%	5%	

† One patient received 1000 SQ-U two times. ‡ Lymphocytes in blood $1.0\text{--}1.02 \times 10^9/\text{L}$. § Neutrophils in blood $1.6 \times 10^9/\text{L}$.

Table 3. CSMS, SS and MS for the entire study periods

ILIT after SCIT- 10 000

		CSMS	SS	MS
		mean (SD [95%CI]), median (IQR)	mean (SD [95%CI]), median (IQR)	mean (SD [95%CI]), median (IQR)
Active N= 13	Before treatment	97.8 (48.8 [68.3-127.3]), 110.1 (53.11–132.8)	43.5 (21.3 [30.6-56.5]), 45.6 (26.3–58.0)	54.3 (34.7 [33.4-75.3]), 57.5 (20.5–85.0)
	After treatment	72.3 (44.8 [45.3-99]), 76.3 (22.7–109.9)	36.7 (23.6 [22.5-51.0]), 43.7 (13.4–53.2)	36.0 (33.4 [15.8-56.1]), 27.5 (3.0–63.3)
	P-value before vs. after treatment †	0.005	0.15	0.003
Placebo N=11	Before treatment	61.5 (40.7 [34.2-88.9]), 61.9 (24.9-83.7)	24.4 (13.5 [15.3-33.5]), 27.4 (10.1-36.2)	37.4 (34.8 [13.8-60.5]), 33.0 (15.0–41.5)
	After treatment,	55.7 (45.1 [25.5-86.1]), 45.1 (29.9-65.7)	24.8 (15.1 [14.7-35.0]), 21.8 (13.1–32.6)	31.0 (33.3 [8.8-53.4]), 28.0 (6.5–33.5)
	P-value before vs. after treatment †	0.24	0.90	0.068

ILIT de novo- 3000

		CSMS	SS	MS
		mean (SD [95%CI])	mean (SD [95%CI])	mean (SD [95%CI])
Active N=16	Before treatment	90.4 (30.9 [74.0–107.0])	49.1 (16.5 [40.3–57.9])	44.5 (27.8 [29.7-59.3])
	After treatment,	68.9 (28.9 [53.5–84.3])	36.7 (17.6 [27.4–46.1])	30.3 (20.3 [19.5–41.2])
	P-value before vs. after treatment ‡	0.003	0.004	0.005
Placebo N=18	Before treatment	104.2 (42.7 [82.9-125.5])	51.2 (23.7 [39.4-63.0])	53.0 (27.7 [39.2-66.8])
	After treatment	74.8 (34.7 [57.5-92.0])	40.9 (16.8 [32.5-49.2])	33.9 (24.3 [21.8-46.0])
	P-value before vs. after treatment†	0.003	0.02	0.005

† Wilcoxon matched pairs signed rank test. ‡ Paired t-test. IQR interquartile range.

Figure 1. The overall study timeline in ILIT after SCIT- 10 000 and ILIT de novo- 3000. The time frame from the last ILIT-injection to the first follow-up was 4-6 weeks, to the start of the grass pollen season 3-6 months and to the second follow-up 6-9 months. SCIT= Subcutaneous Immunotherapy. SPT= Skin Prick Test. NPT= Nasal Provocation Test. CSMS= Combined Symptoms and Medication Scores. RQLQ= Rhinitis Quality of Life Questionnaire.

Figure 2. The mean daily CSMS and the pollen levels before and after treatment at the different study sites. A. The baseline season of ILIT after SCIT-10 000. **B.** The season after treatment in ILIT after SCIT- 10 000. **C.** The baseline season of ILIT de novo- 3000. **D.** The season after treatment in ILIT de novo- 3000.

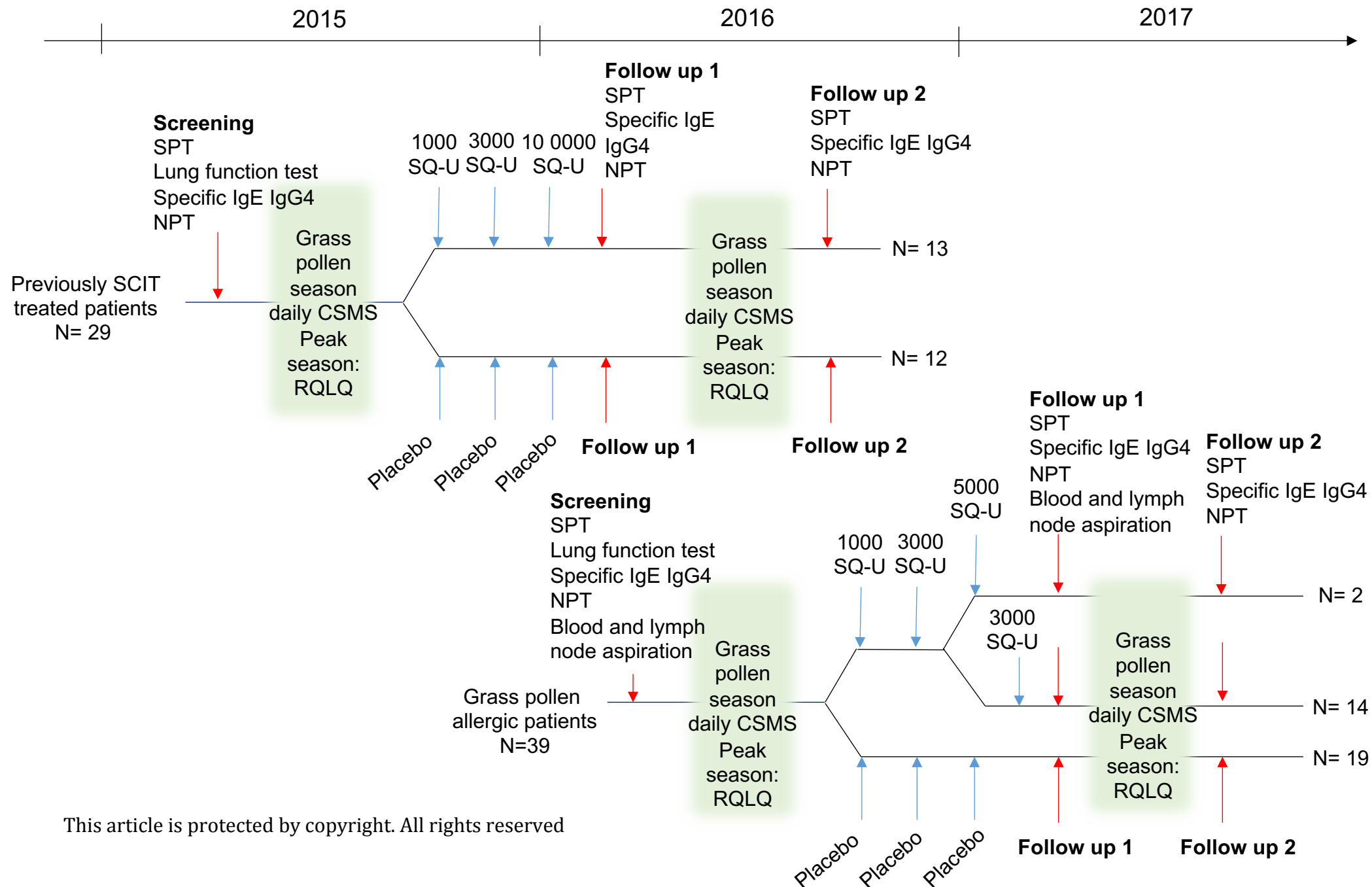
Figure 3. The symptoms and medication diary. A. The CSMS in ILIT after SCIT- 10 000 was reduced in the active ($p= 0.005$) but not in the placebo group. The SS did not improve significantly after treatment. The MS improved in the active group ($p=0.003$) but not in the placebo group. **B.** In ILIT de novo- 3000 the

CSMS, SS and MS improved in both the active and placebo group after treatment. * $p < 0.05$, ** $p < 0.01$, n.s.=not significant.

Figure 4. Serologic changes after ILIT. Timothy specific IgG₄ levels in A. ILIT after SCIT- 10 000 and B. ILIT de novo- 3000. Timothy specific IgE levels in C. ILIT after SCIT- 10 000 and D. ILIT de novo- 3000. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s.= not significant.

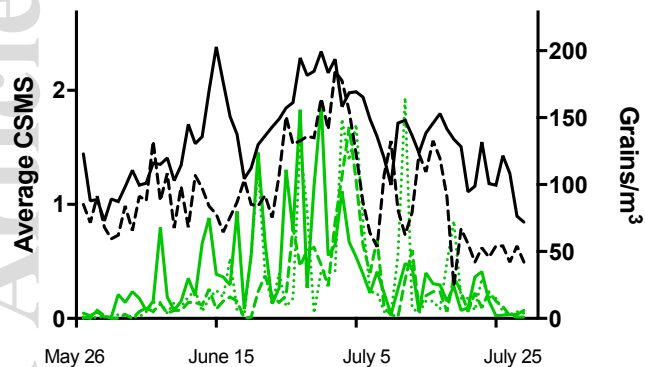
Figure 5. The expression of CD80 and CD141 increases on lymph node DCs after active ILIT.

Fig. A, B and C. The level of expression on DCs in lymph nodes. **Fig. D, E and F.** The level of expression on DCs in blood. **Fig A-F.** The scatter plot represents the median levels of expression revealed by flow cytometry. * $P < 0.05$, ** $P < 0.01$, Horizontal lines represent the mean value and SD.



A

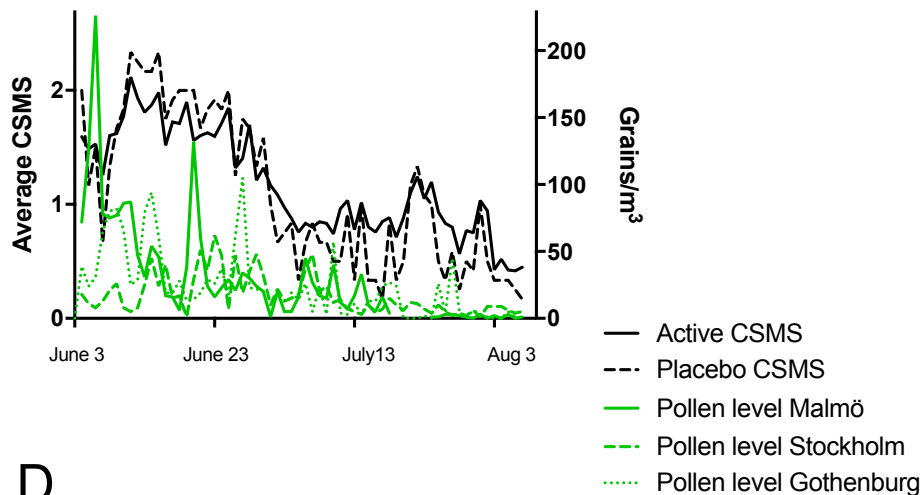
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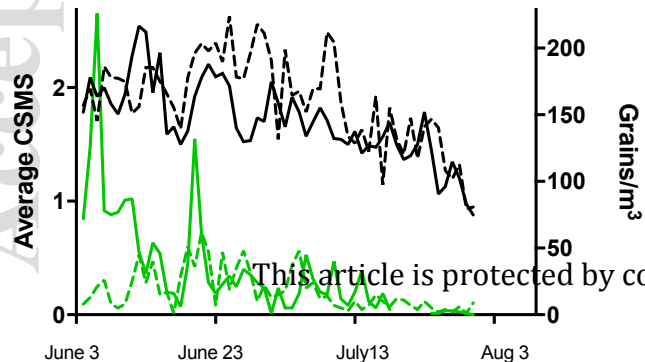
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2016 ILIT after SCIT- 10 000



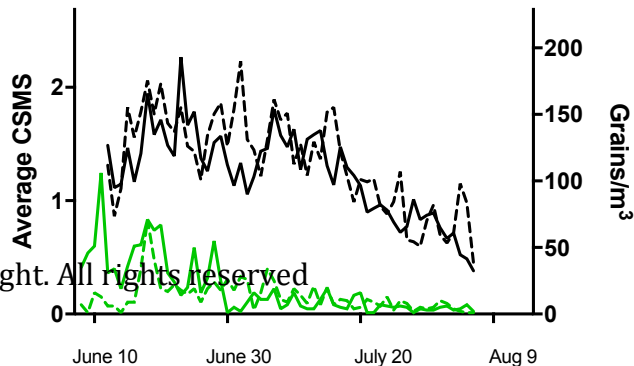
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2016 ILIT de novo- 3000



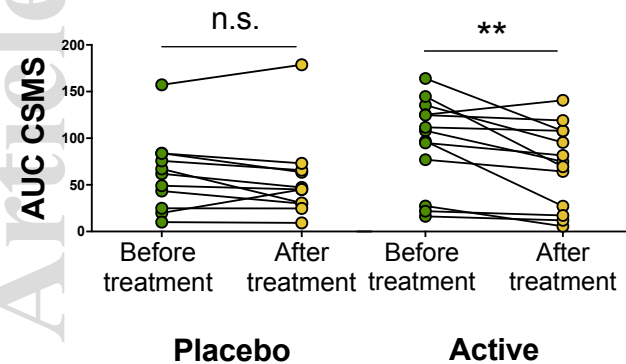
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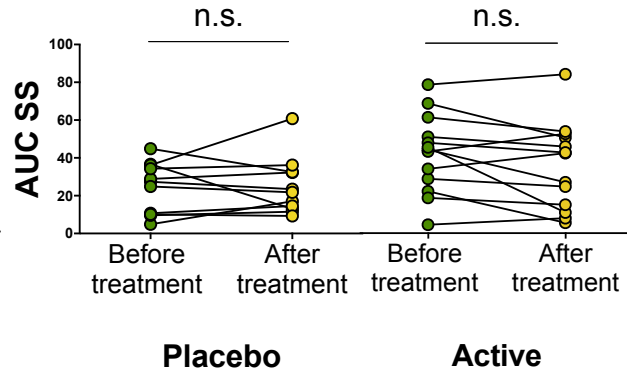


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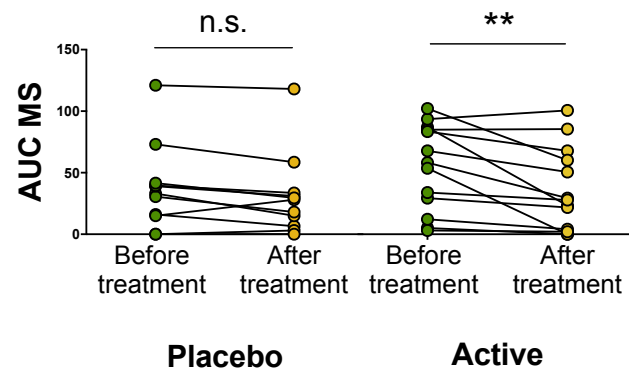
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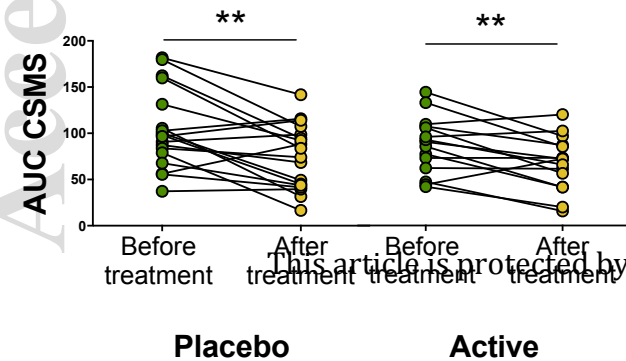
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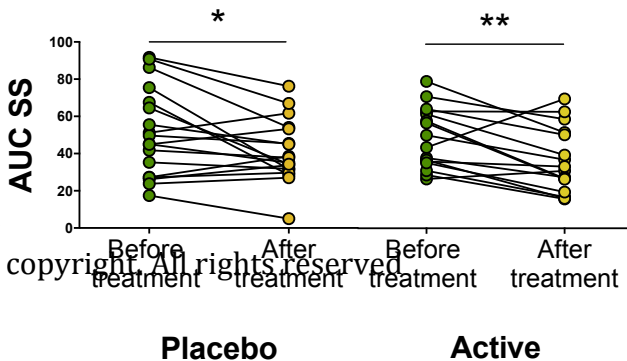
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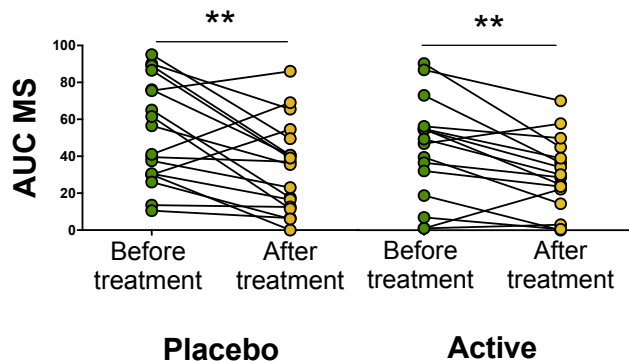
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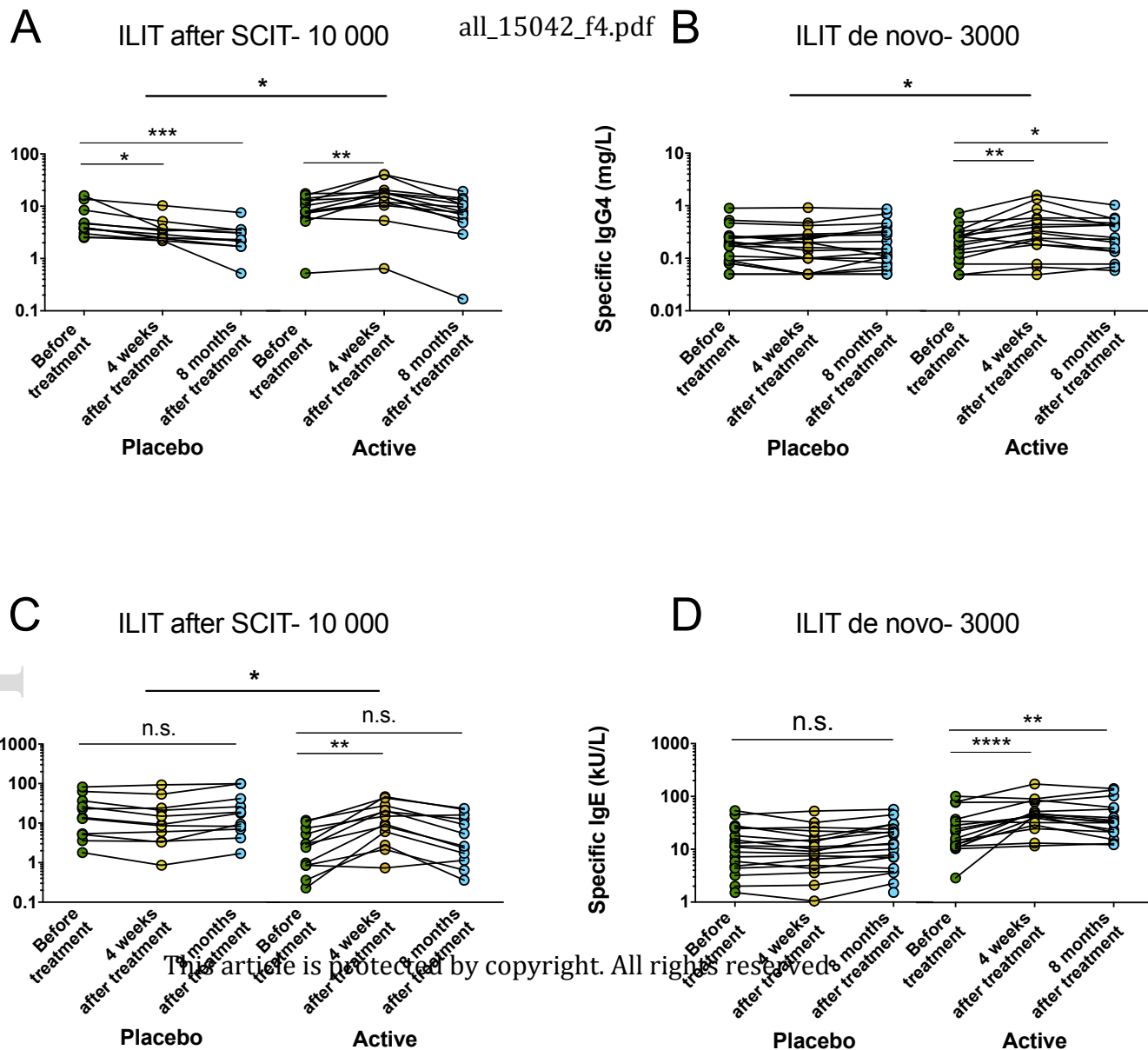


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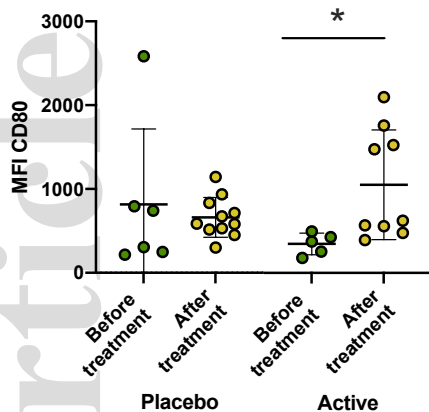


F MS ILIT de novo- 3000

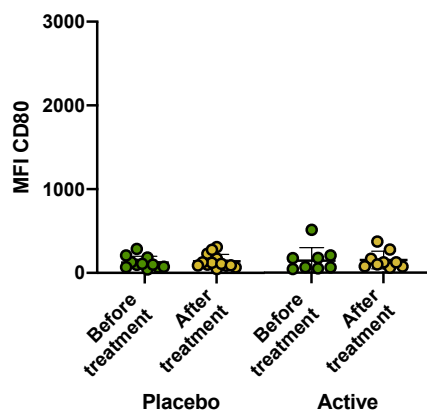




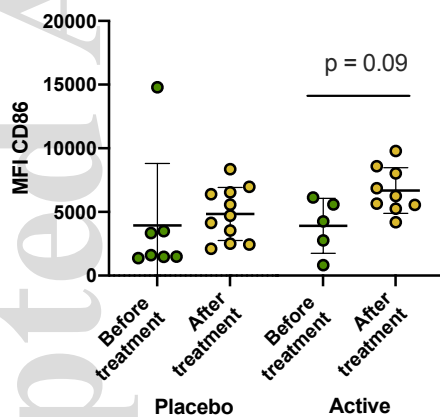
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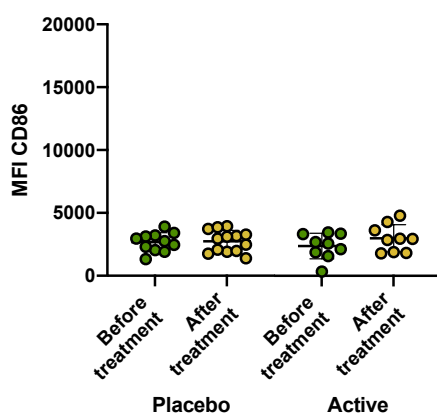
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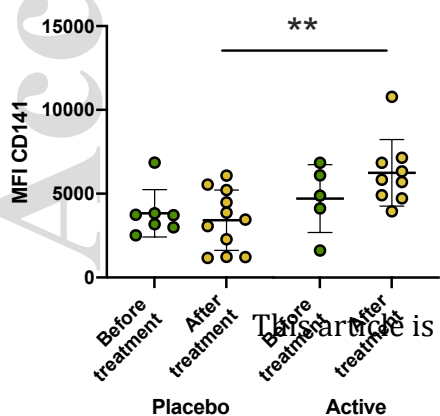
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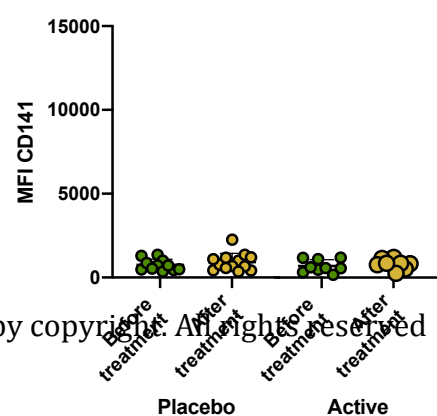
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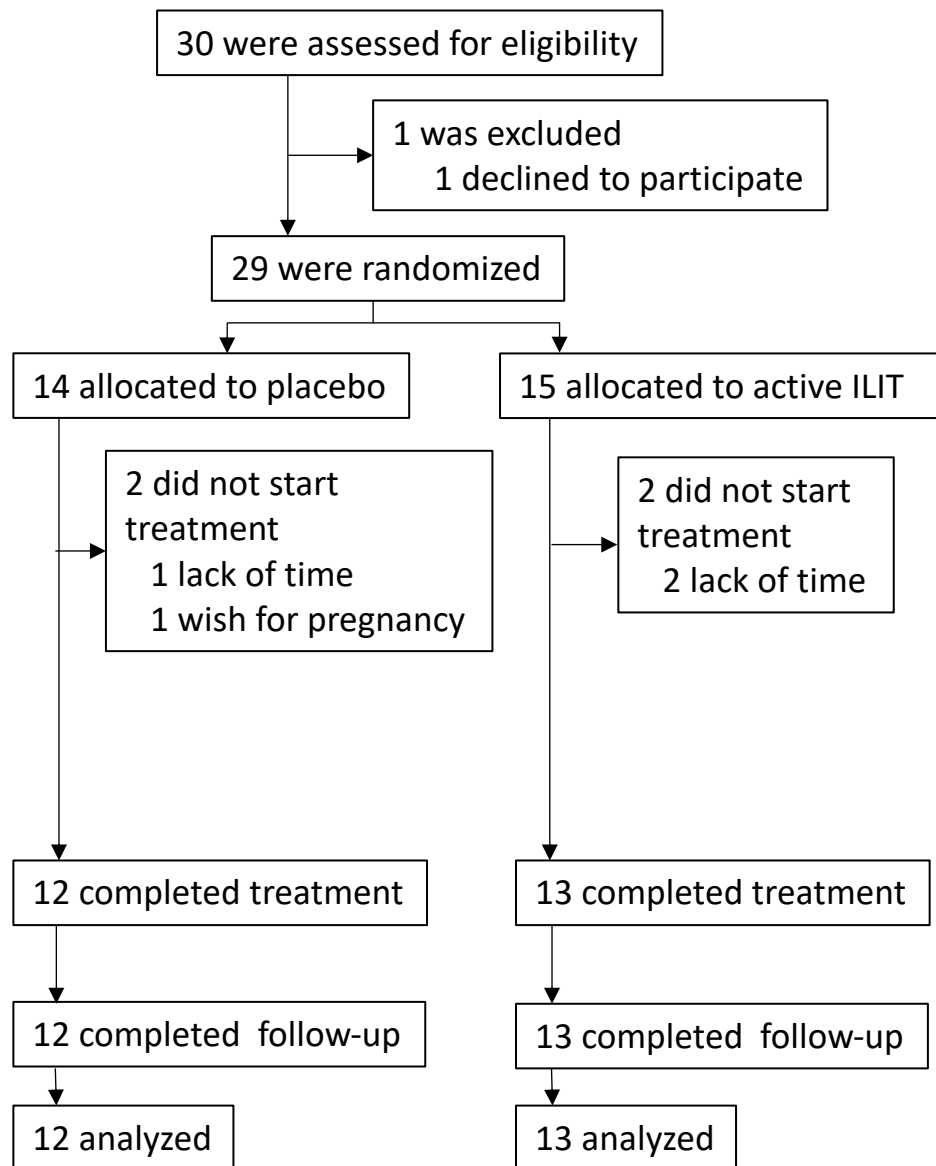
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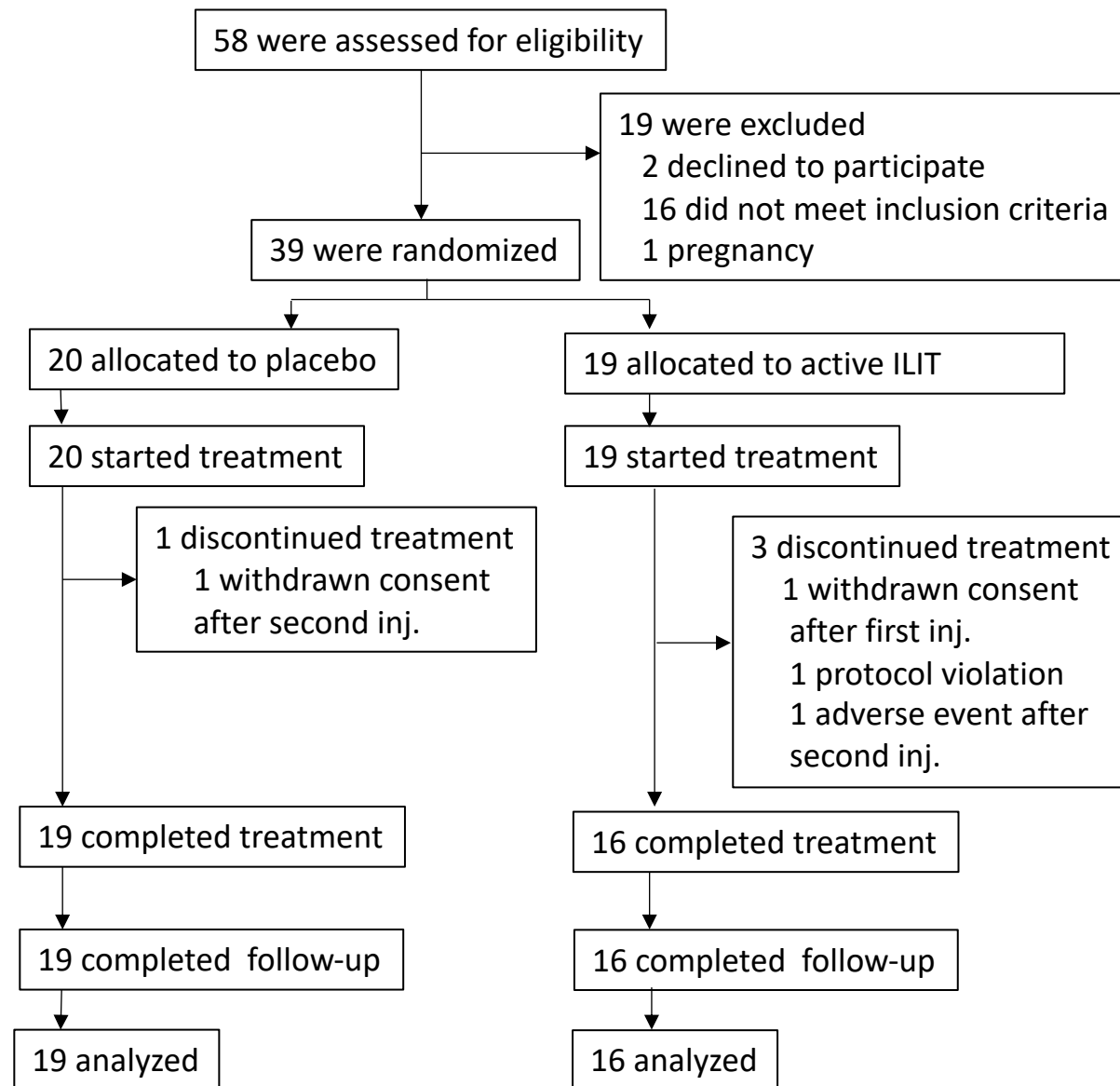
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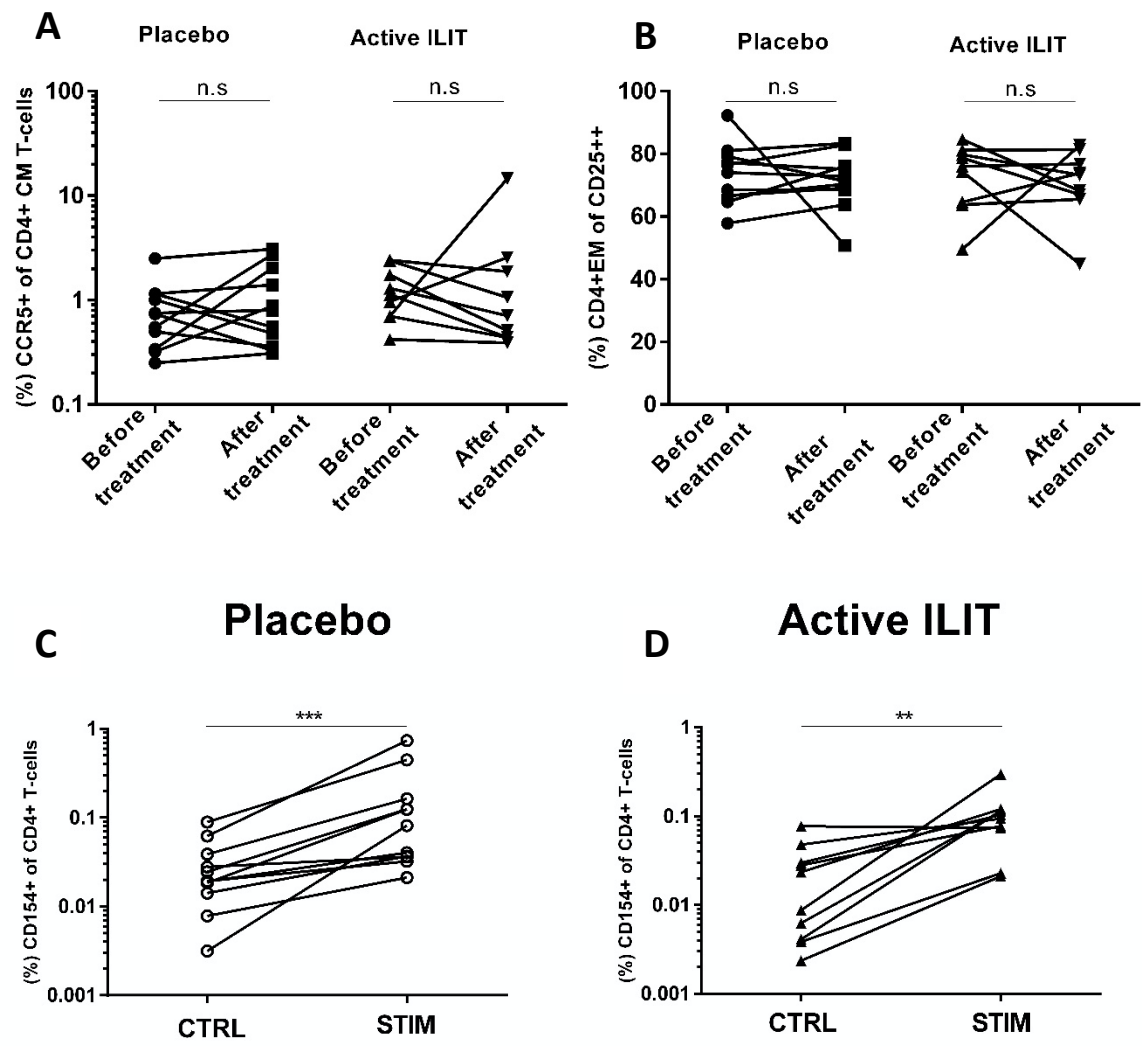


ILIT after SCIT- 10 000



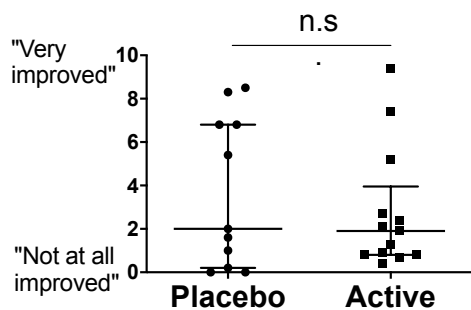
ILIT de novo- 3000



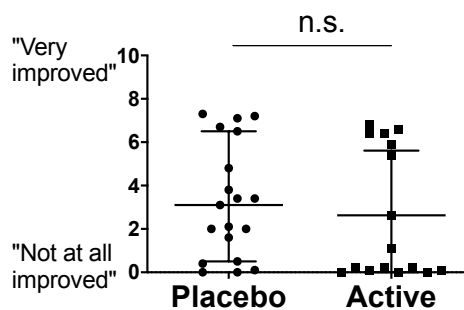


A. Improvement on VAS

ILIT after SCIT- 10 000

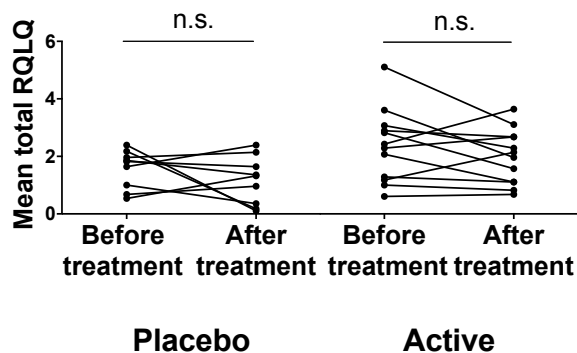


ILIT de novo- 3000

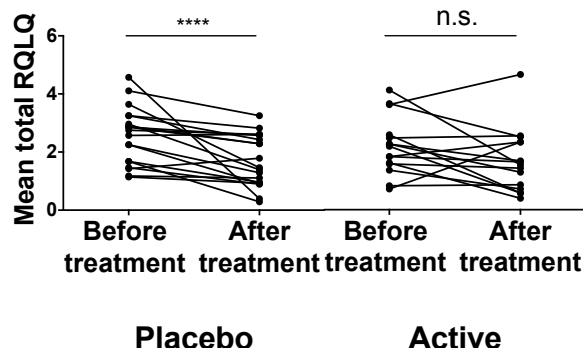


B. Quality of life at grass pollen season on RQLQ

ILIT after SCIT- 10 000

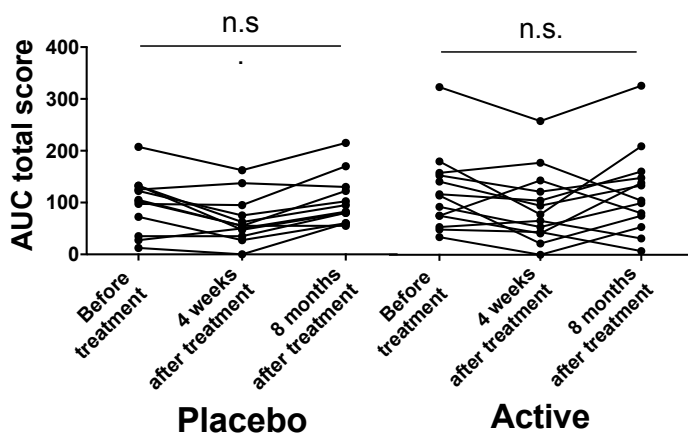


ILIT de novo- 3000

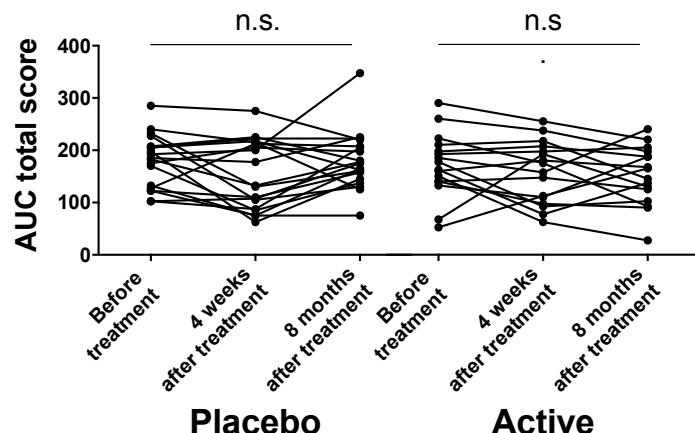


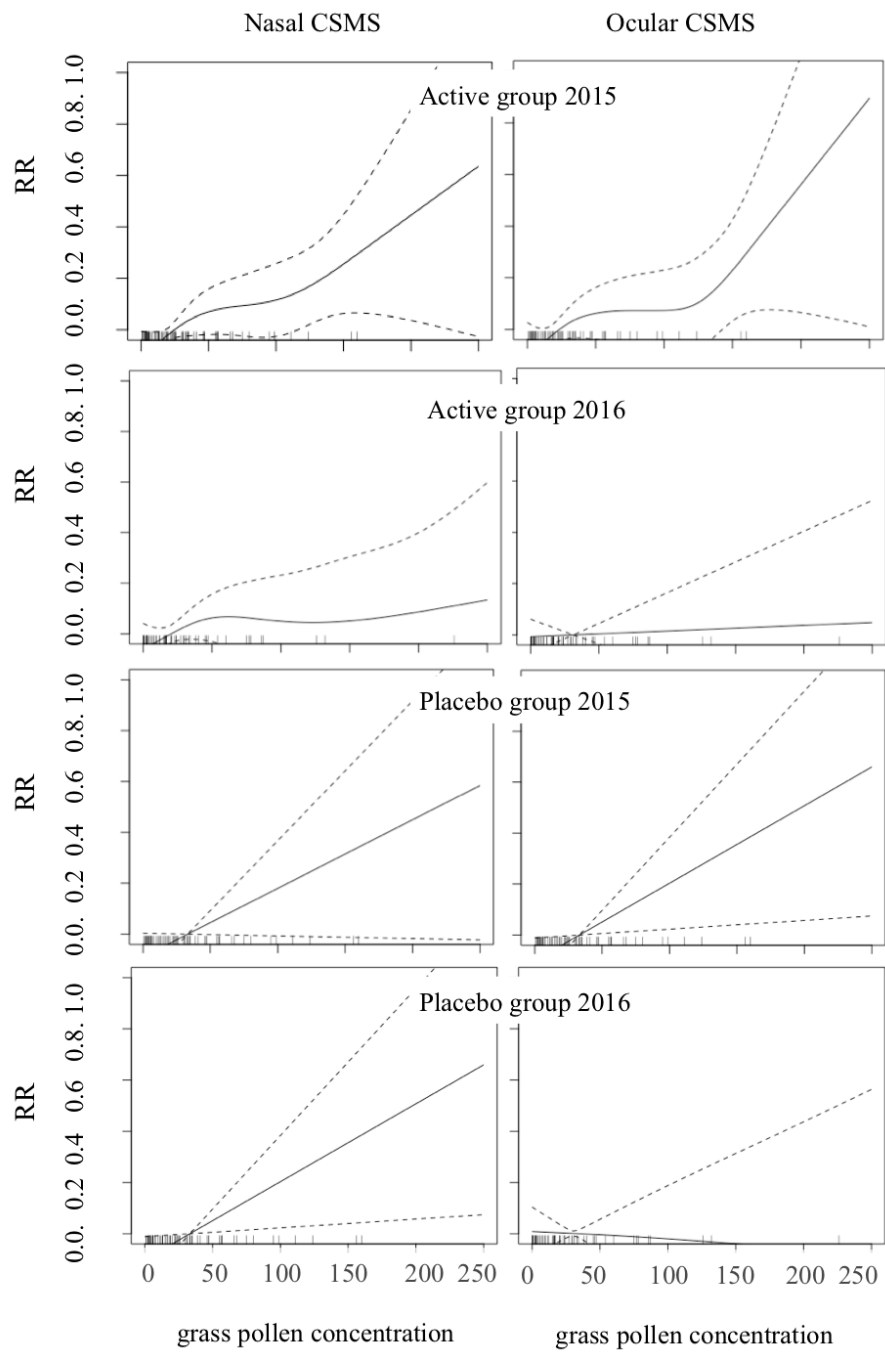
C. Nasal provocation test

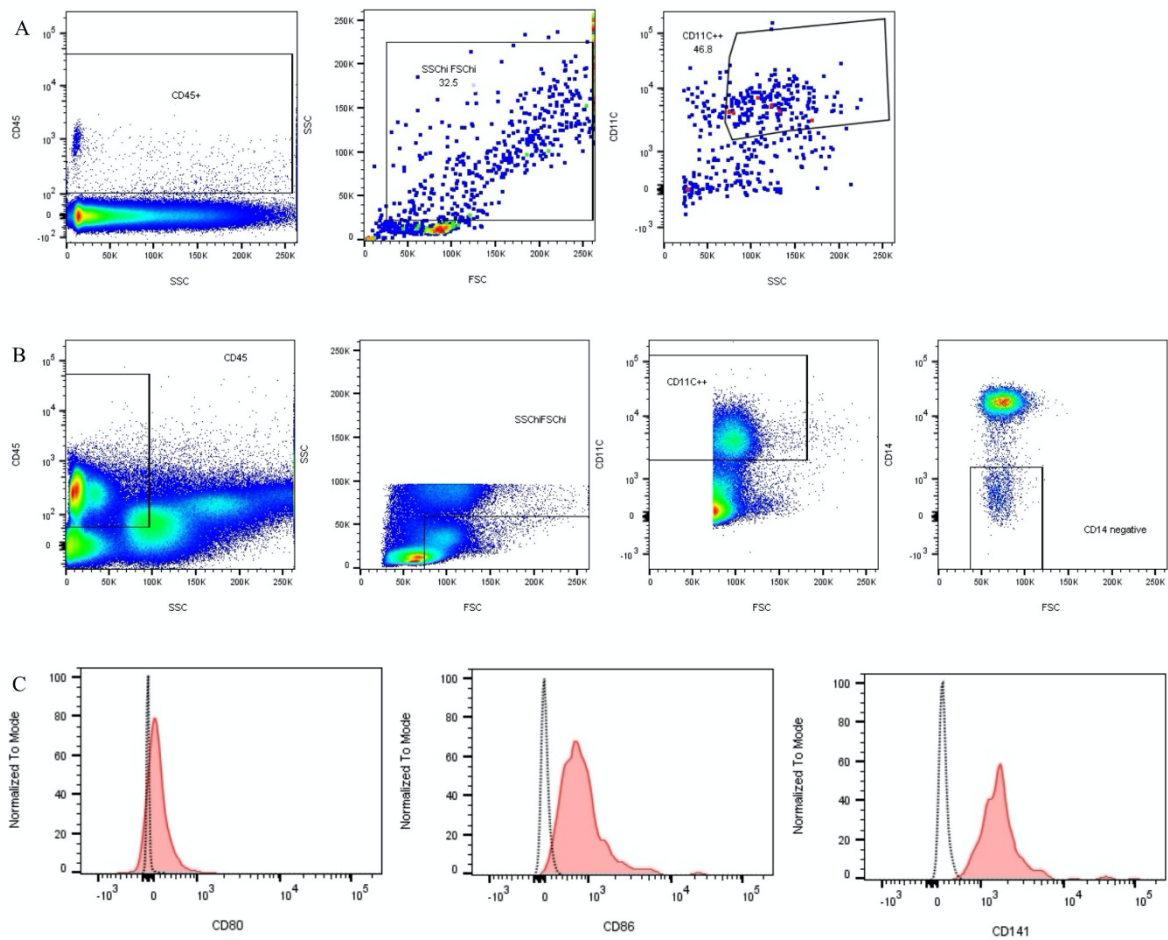
ILIT after SCIT- 10 000

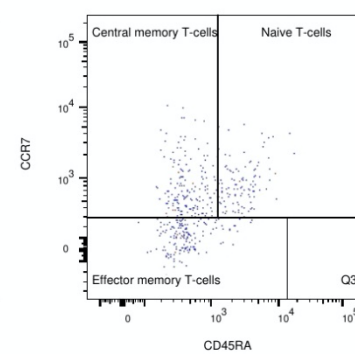
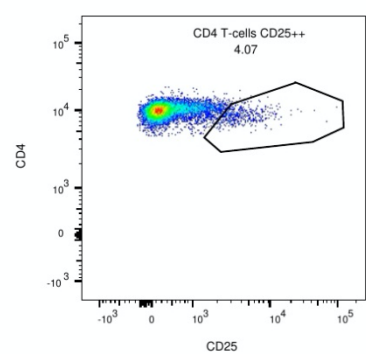
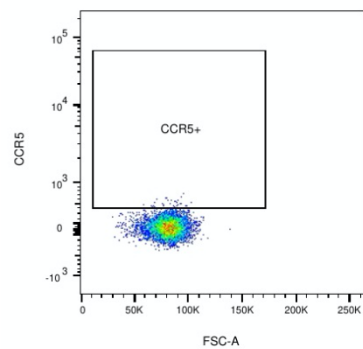
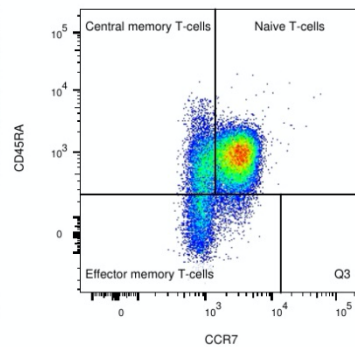
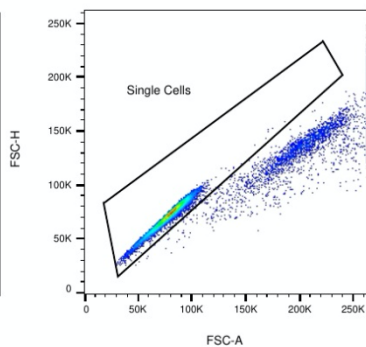
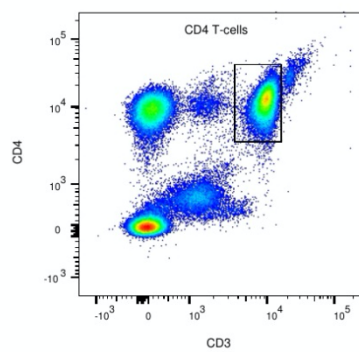


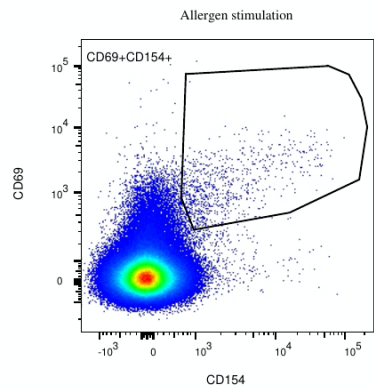
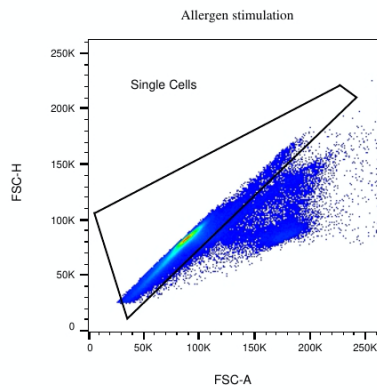
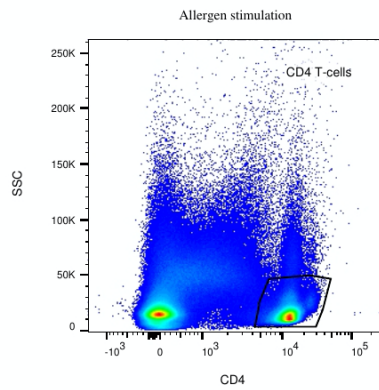
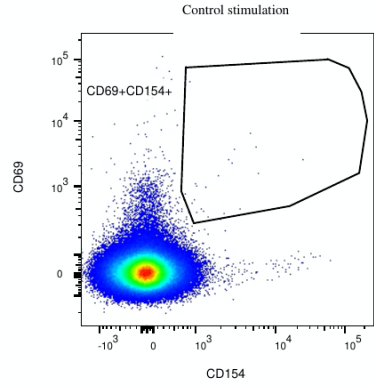
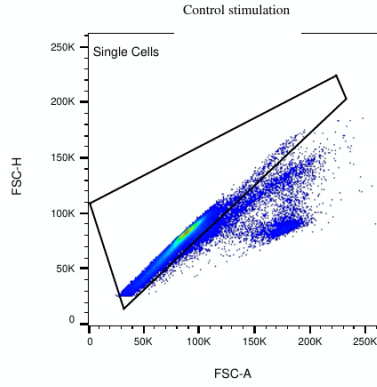
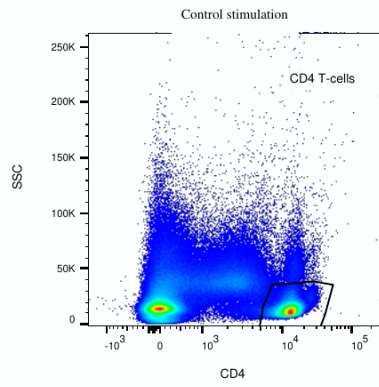
ILIT de novo- 3000











1Supplementary information

2

3Methods

4

5Study design

6

7Two separate studies were performed, "ILIT after SCIT- 10 000" and "ILIT de novo- 3000".

8Both were national, multicenter, 1:1 randomized, double-blind, placebo-controlled (RDBPC),

9parallel-group studies in patients with moderate to severe AR. The studies were conducted

10at the outpatient offices of the Ear-, Nose- and Throat departments at Karolinska University

11Hospital in Stockholm and Skånes University Hospital in Lund, Sweden. ILIT after SCIT- 10 000

12also included patients at the Asthma and Allergy Clinic Outpatient Unit at South Alvsborgs

13Central Hospital, Borås, Sweden. In ILIT after SCIT- 10 000, previous SCIT-patients were

14screened before the pollen season in 2015, registered baseline symptoms and medication

15during the pollen season, received randomized treatment during the autumn and winter,

16were followed up 4 weeks after treatment, registered seasonal symptoms and medication at

17the consecutive pollen season in 2016 and had the last follow up visit after the second pollen

18season. ILIT de novo- 3000 started one year later, had the same disposition and included

19blood samples and lymph node aspirates before treatment and 1 month after treatment, for

20exploratory immunological investigations. (See Figure 1 in the main text for the study

21outline). The studies were approved by the Ethics Committee in Stockholm and the Swedish

22Medical Products Agency, registered at ClinicalTrials.gov with ID NCT02679105 and

23NCT02975479 and conducted according to good clinical practice guidelines. All patients

24provided written informed consent before participation and an independent data monitoring

25consult reviewed the adherence to the clinical report forms.

26

27

28

29Patients

30

31The participants in ILIT after SCIT- 10 000 were recruited from the AIT clinics at the study

32sites. Patients that had recently (within 20 months) completed a full SCIT protocol with

33maintenance doses of 100 000 SQ-U for 3-4 years, with improvement but not full symptom

34relief, were eligible. For ILIT de novo- 3000, patients with moderate to severe AR, without

35previously AIT, were recruited from newspaper advertisements and from referrals. Inclusion

36criteria for both studies were men and women in age 18-55, a positive skin prick test with a

37wheal diameter of ≥ 3 mm for grass and timothy specific IgE level ≥ 0.35 kU/mL. Mild

38seasonal or exercise induced asthma was permitted, with symptom relief after short acting

39 β_2 -agonist inhalation, $FEV_1 > 70\%$ of predicted value at spirometry and reversibility of $< 12\%$

40on post bronchodilator test out of the pollen season. Exclusion criteria were the same for

41the two studies; perennial or uncontrolled asthma, other pulmonary disease, known

42autoimmune or collagen disease, chronic infection or other significant disease, severe atopic

43dermatitis, use of beta blockers or angiotensin converting enzyme inhibitors as

44antihypertensive medications, symptomatic sensitization to house dust mite or furry animals

45with daily exposure, chronic upper airways disease, pregnancy, wish for pregnancy, nursing,

46 inability to register symptoms electronically, obesity with BMI >30 or withdrawn informed
47 consent. In ILIT after SCIT, the patients were carefully informed about the difference
48 between the completed conventional SCIT-treatment and the clinical experimental RDBPC
49 study with voluntary participation. In ILIT de novo- 3000, previous AIT was prohibited. The
50 recalled rhinoconjunctivitis total symptom score, in ILIT de novo- 3000, had to be ≥ 8 (the
51 sum of rhinorrhea, nasal obstruction, sneezing, nasal itching, itchy/red eyes and watery eyes,
52 each scored 0-3) at the last pollen season, with symptomatic treatment.

53

54 Allocation to treatment and blinding

55

56 In both studies, the patients were randomized 1:1 to active or placebo treatment. The
57 allocation to the treatment arms was aided by a computer randomization program, without
58 stratification. A nurse not connected to the studies prepared sealed emergency envelopes
59 according to the randomization lists. The envelopes were not opened until the studies were
60 completed. The preparation and labelling of the medical product for each patient was also
61 performed by an independent nurse. The allergen extract for injection 1 and 2 (10 000 SQ-U/
62 mL) had the same smell and look as placebo. The extract used for injection 3 (100 000 SQ-U/
63 mL) had a cloudy appearance and was therefore diluted 1:1 with ALK Diluent® to the
64 concentration 50 000 SQ-U/mL. The nurse that prepared the allergen covered the barrel of
65 the 1.0 mL syringe with the label tape in a way that only the lower needle hub was free
66 (visible for aspiration before injection). By this procedure the active product could not be
67 distinguished from placebo. The randomization lists were stored in a locker. All participants,
68 physicians that administered the interventions and study staff that evaluated the outcomes
69 remained blinded throughout the studies.

70

71 Ultrasound guided intralymphatic injections

72

73 The same node, as far as possible with aid from pictures saved on the ultrasound machine,
74 received the allergen at all three injections. Spinal needles with size 25 Gauge were used for
75 the injections. When needed, aspiration was performed to rule out risk for intravascular
76 injection. During the injection, correct placement inside the lymph node could be verified as
77 a transient swelling of the node seen with the ultrasonographic imaging. All injections were
78 performed with aseptic technique. Peak expiratory flow, local reactions at the injections site
79 and systemic reactions were surveilled for 30 minutes after each injection. The patients were
80 requested to inform the study staff of any adverse events during the subsequent 24 hours.

81

82

83

84 Treatment protocol

85

86 The patients were premedicated at home, 1-7 hours before ILIT, with one tablet of the oral
87 second or third generation antihistamine they used during pollen season (Loratadine,
88 Cetirizine or Ebastine 10 mg, Desloratadine 5 mg, or Fexofenadine 120 mg). ALK Alutard 5-
89 grasses® SQ, grass allergen adsorbed to aluminumhydroxide, was used for active treatment.

90ALK Diluent® with human albumin was used as placebo. Both products contained phenol,
91sodium chloride and sodium bicarbonate. By this, the two treatment arms were similar
92regarding smell and look and as similar as possible except for the active ingredient to allow
93interpretation of the treatment effect due to aluminum adsorbed allergen. According to the
94protocol the doses were 0.1 mL of 10 000 SQ-U/mL (1000 SQ-U) at treatment 1, 0.3 mL of
9510 000 SQ-U/mL (3000 SQ-U as a slow injection) at treatment 2 and 0.1 mL + 0.1 mL of
9650 000 SQ-U/mL (5000 + 5000 SQ-U with 30 minutes in between) at treatment 3. The
97treatment interval aimed at 4 weeks between the injections but was extended to a
98maximum of 7 weeks if needed. The last ILIT-injection was given in the late winter time for
99some patients, but not if the patient had symptoms from alder, hazel or birch pollen
100exposure. The time frame between the last ILIT-injection and the start of the grass pollen
101season was 3-6 months.

102

103All patients in ILIT after SCIT- 10 000 could follow this protocol.

104

105In ILIT de novo- 3000, the first two active patients that received 5000 SQ-U had anaphylactic
106reactions. Therefore, the investigators (the authors) modified the protocol and the
107remaining patients in ILIT de novo- 3000 received 3000 SQ-U again (or placebo) at the third
108treatment. By this, the up dosing schedule was 1000- 3000- 3000 SQ-U. The adverse events
109and protocol changes were reported immediately to the Medical Products Agency, the Ethics
110Committee and ILIT interested researchers and the registration at Clinicaltrials.gov was
111updated. In addition, one active patient could not proceed to injection 2 within the time
112frame of 7 weeks due to an upper airway infection. For safety reasons, this patient received
113a modified up-dosing; 1000- 1000- 3000 SQ-U with 8 weeks in between injection number 1
114and 2. To summarize, the active group in ILIT de novo- 3000 received an up-dosing schedule
115with doses 1000-3000-3000 SQ-U, except for two patients that received 1000-3000-5000 SQ-
116U and one patient 1000-1000-3000 SQ-U.

117

118

119Pollen data

120

121Pollen data were registered with Burkard 7-day Volumetric Spore Traps situated at roof tops
122at Dep. of Meteorology, Frescati, Stockholm University (15 m above ground level),
123Sahlgrenska University Hospital ("Östra"; 40 m above ground level), and Skåne University
124Hospital, Malmö, (40 m above ground level) and were analyzed at Swedish Natural History
125Museum and Gothenburg University, respectively. There was no pollen trap in Borås, where
126some of the patients resided in ILIT after SCIT- 10 000 and daily concentration data from the
127closest trap in Gothenburg are not valid due to the distance between the cities (ca 80 km).
128But the start and the general course of the pollen season was assumed to be similar. In the
129two other areas, the participants lived within a radius of 50 kilometers from the pollen trap.
130

131The pollen season was defined as the period between the dates when pollen was registered
132at three consecutive dates at the first and the last time, respectively. The peak pollen season
133for the study period is the 14 consecutive days with highest sum of pollen level.

134

135

136

137Outcome measures

138

139*Symptoms and medication scores*

140The primary outcome measure was the daily combined symptoms and medication score
141(CSMS), that was the sum of the medication score (MS) and symptom score (SS); maximum
142CSMS score was 6. It was measured with EAACI:s recommended scoring system (E1) and
143compared before versus after treatment. The patients scored rhinorrhea, nasal obstruction,
144itchy nose, sneezing, itchy/red eyes, watery eyes (0=no symptoms, 1=mild symptoms, easy
145to tolerate, 2=moderate symptoms, troublesome but tolerable, 3=severe symptoms, hard to
146tolerate). The daily SS was the average value of the symptoms, maximum score 3. The
147patients were instructed to use rescue medication in a stepwise, as-needed, manner. When
148symptoms occurred, the patients first used oral and/or topical antihistamines (=1 point),
149followed by intranasal steroids (2 points, with/without antihistamines). If the patients still
150had troublesome symptoms, they were offered oral corticosteroids (=3 points, with/without
151other medications). The maximum daily MS score was 3.

152

153The symptoms and medication questions were administered daily by e-mail, or in a non-
154public iPhone app that was developed in a separate research project (IntegrIT at Karolinska
155Institutet). The patients were requested to complete the questionnaires within 24 hours and
156received reminders if compliance failed. Before the study, the start of the registration period
157was decided to be just before the anticipated peak grass pollen period, in order to avoid
158confounding scores due to birch pollen exposure. When the patients temporarily were in
159climate zones outside of the relevant pollen areas the registrations were paused (e.g.,
160Mediterranean area, Asia). Occasional days when patients reported symptoms due to
161exposure to furry animals, were also decided beforehand to be excluded. In our small study,
162this was to optimize the chances for correct measurement of grass pollen related symptoms
163and to avoid registrations with no, or wrong, allergen exposure. The maximum level of
164missing replies was decided before the studies to be 50% of the days.

165

166CSMS was expressed as the area under the curve (AUC) for each patient during the total
167registration period and during the peak pollen season (the 14 consecutive days with highest
168local pollen counts) at the baseline year and the year after treatment.

169SS and MS expressed as AUC were evaluated as secondary outcomes during the total
170registration period and the peak pollen season.

171

172Missing data were imputed as the last observation carried forward when there was missing
173reply at the very first or last day of the registration period. Missing data that did not occur at
174the first or last days were not imputed, instead the method with using the AUC on the
175available data was considered a suitable approximation method.

176

177

178

179*Nasal provocation test*

180A nasal provocation test (NPT) was conducted outside of the pollen season before the
181treatments, 4 weeks after the treatments and 8 months after treatments. It was rescheduled
182if any contraindications were fulfilled, such as recent upper airways infection or intake of
183prohibited concomitant medications. The test was performed based on recommendations

184for standardization (E2). After room temperature acclimation, 0.1 mL of water-soluble grass
185allergen, ALK Aquagen SQ[®] timothy, 10 000 SQ-U/mL (1000 SQ-U), was sprayed into each
186nostril. A trained nurse asked the study subjects to score the allergic symptoms on a Likert
187scale (0=no symptoms, 1=mild symptoms, 2=moderate symptoms and 3=severe symptoms).
188The symptoms evaluated were nasal obstruction, rhinorrhea, itchy nose, itchy eyes, watery
189eyes, itchy palate/ears and number of sneezes. The scores were registered before challenge,
190after 5 minutes, 15 minutes and 30 minutes. The result was expressed as AUC for each
191patient at each examination. We attempted to perform a rhinomanometry as an objective
192measurement of the nasal obstruction, but due to technical problems this parameter was
193abandoned.

194

195VAS

196After the second pollen season the participants assessed the overall symptom improvement
197on a visual analogue scale (VAS) ranging from 0 ("no improvement") to 10 ("total symptom
198relief").

199

200Quality of life

201The quality of life (QoL) at the pollen seasons was assessed with the Juniper
202Rhinoconjunctivitis Quality of Life (RQLQ(S)) questionnaire, validated for allergic
203rhinoconjunctivitis (E3). The forms were completed on one occasion at the heights of the
204grass pollen season before treatment, and at the pollen season after treatment. The score
205was calculated as the average of 28 questions, each ranging 0-6 (resulting in maximum RQLQ
206score 6 points) and the minimal clinically important improvement is 0.5 point.

207

208Skin prick test

209A skin prick test with ALK Soluprick[®] SQ was performed before treatment, 4 weeks after
210treatment and 8 months after treatment. It was a standard panel with 10 common airborne
211allergens with the concentration corresponding to 10 Histamine Equivalent in prick testing, a
212positive control with histamine chloride in concentration 10 mg/mL, and a negative control
213with saline buffer. A wheal reaction of ≥ 3 mm in diameter (horizontal mm+ vertical mm/2)
214was deemed positive.

215

216Serology

217Timothy specific IgE and IgG4 antibodies were measured at the screening visit before the
218baseline pollen season, 4 weeks after treatment and 8 months after treatment. Venous
219blood was collected in tubes containing EDTA (Vacuette 454209) for differential leukocyte
220blood counts. Allergen specific IgE and IgG₄ levels were measured with ImmunoCAP (Thermo
221Fisher Scientific, Uppsala, Sweden), according to the manufacturer's instructions.

222

223Lymph node aspirations

224In ILIT de novo- 3000 at the study site in Stockholm, lymph node aspirations and additional
225blood samplings were performed before treatment and 4 weeks after treatment. With
226ultrasonographic guidance the lymph node in the groin that received ILIT, was punctured
227with a 22 Gauge spinal needle and lymph node material was aspirated.

228

229Flow cytometry

230The amount of material on each lymph node aspirate was small, which limited the assays.

231 In this study we only focused on DCs. The expression of CD80, CD86 and CD141 in DCs was
232 determined with flow cytometry. For the detection of DCs we used expression of CD45+,
233 SSC++ and CD11C+. In analysis of blood we also included CD14 to exclude monocytes. As a
234 continuation of our previous ILIT studies (E4), we analyzed changes in the amount of central
235 memory (CM) and effector memory (EM) T-cells. An assay with allergen activated CD4 T-cells
236 was also included.

237

238 When sampling blood, vacutainer tubes (BD Biosciences, San Jose, Calif) containing sodium
239 Heparin were used. Lymph node aspirates were suspended in sterile Tissue Storage solution
240 (Miltenyi Biotec, Bergisch Gladbach, GE). Monoclonal antibodies and reagents used were
241 purchased from BD. The included antibodies were used in three panels. CD11C (clone B-ly6),
242 CD14, (clone MφP9), CD16 (clone 3G8), CD45 (clone 2D1), CD80 (clone L307.4), CD86 (clone
243 FUN-1), CD141 (clone 1A4) and HLA-DR (clone G46-6) were used for detection and analysis
244 of dendritic cells. CD3 (clone UCHT1), CD4 (clone RPA-T4), CD25 (clone 2A3), CD45RA (clone
245 HI100), CCR5 (clone 2D7) and CCR7 (clone 3D12) were used for detection and analysis of CD4
246 T-cells. CD4 (clone RPA-T4), CD69 (clone FN50) and CD154 (clone TRAP-1) were used for
247 detection and analysis of allergen activated CD4 T-cells. Other reagents used were BD Comp-
248 beads anti-mouse, BD Comp-beads anti-rat and Cytometer set-up and tracking beads.
249 All antibodies were titrated for optimal performance before use, dilutions ranging from 1:5
250 to 1:50.

251

252 After staining of blood and FNA with appropriate antibodies, red blood cells were lysed with
253 ammonium chloride solution and fixed with 1% formaldehyde. For discrimination of doublet
254 from analysis, forward scatter area and forward scatter height were used. Background versus
255 positive staining was ascertained by using internal controls and fluorescence minus one
256 (FMO) controls. Cells were analyzed on an LSR fortessa (BD Biosciences, San Jose, Calif), and
257 data were processed with FlowJo software© version 10.6.1 (Tree Star, Inc., Ashland, USA).
258 Compensation was performed with single-stained beads comp-beads. The flow cytometer
259 was validated before every run with BD cytometer set-up and tracking beads.

260 In addition, at the blood sampling after treatment, a T-cell allergen stimulation assay was
261 performed. Human peripheral blood mononuclear cells (PBMC) were isolated and stimulated
262 with allergen before the flow cytometry analysis, in order to study allergen specific CD4+ T-
263 cells. Within 12 hours from sampling, PBMC were isolated from blood by density
264 centrifugation using Ficoll Paque Plus (GE Healthcare, Buckinghamshire, UK) and SepMate™
265 tubes (STEMCELL Technologies, Vancouver, CA). The PBMC suspension were washed three
266 times in PBS (Gibco, Life Technologies, Uppsala, Sweden) then resuspended at 7.5×10^6
267 cells /ml in stimulation medium ((TexMACS Medium, Miltenyi Biotec, Bergisch Gladbach,
268 GE), 5% autologous serum and 1% Penicillin-Streptomycin (Gibco, Life Technologies,
269 Uppsala, Sweden)). PBMC were incubated with anti-CD40 1µg/ml (Miltenyi Biotec, Bergisch
270 Gladbach), for 20 minutes to block future interaction between CD40 and CD40 ligand. PBMC
271 were stimulated with 4000 SQ-U of Timothy Aquagen (ALK, Hørsholm, DK) in a total volume
272 of 200µl for 12 hours at 37°C and 5% humidified CO₂. The allergen stimulation was run in
273 triplicates, as a negative control stimulation medium without added allergen was used.

274

275 *Time-series analysis of daily CSMS and pollen levels*

276 In a time-series analysis we used the generalized additive model (GAM) to evaluate the
277 relative risk (RR) for allergy symptoms in relation to the pollen levels. A curve indicating the

278pollen dose-response relationship between the symptoms and pollen concentration was
279modelled through the introduction of a spline function and displayed the increment in risk
280for symptoms with an increment of 10 pollen grains/m³. Apart from the symptoms in focus,
281we included “time (ordinal day number)” and “maximum temperature” as possibly
282confounding variables. The model was tested with the function “gam” in the package
283“mgcv”, with a logit link for discrete response, with the software R (Version R 3.3.3 GUI 1.69
284Mavericks build, Vienna, Austria). We present the RR values and 95% confidence intervals for
285the significant relationships found. The equation used was CSMS ~ pollen concentration +
286s(time, k = 4, fx = F) + s(Tmax, k = 4, fx = F). In the equation s= spline function, k=degrees of
287freedom and fx=F indicates that a penalized regression spline is included.

288

289Statistical analysis

290

291All patients were included in an intention-to-treat safety analysis. Efficacy outcomes were
292assessed as modified intention-to-treat analyses without patients that did not complete the
293intervention or had missing outcome data. Paired observations of SS, MS, CSMS, RQLQ and
294T-cell activation that were normally distributed were analyzed with paired t-test or when not
295normally distributed Wilcoxon matched-pairs signed rank test. Comparisons between the
296active and placebo groups of VAS, CSMS, SS, MS, RQLQ, IgE, IgG4, NPT, SPT and T-cell
297activation were analyzed with Mann-Whitney test to compare rank if not normally
298distributed, and with unpaired t-tests when passing normality tests. Repeated measures of
299IgE, IgG4, NPT and SPT were analyzed with Friedman’s test followed by Dunn’s multiple
300comparison post-hoc test for non- parametric data, or repeated measurements one-way
301ANOVA with Dunnett’s multiple comparisons test for parametric data. DC activation in lymph
302nodes and blood was analyzed with a 2-way ANOVA with Fishers LSD test.
303Normally distributed data were presented as mean value (SD [95% CI]) and not normally
304distributed data with medians (interquartile ranges). Pearson correlation test was used to
305explore correlations between clinical improvement and immunological alterations. A p-value
306of 0.05 or less was considered significant. The analyses were performed with GraphPad
307Prism 6.01 software (GraphPad Software, La Jolla, Calif). In addition, a generalized additive
308model (GAM) was used in a time-series analysis with R-software, to evaluate the relative risk
309(RR) for allergy symptoms in relation to the pollen levels, see above.

310

311We performed a power calculation based on our previous low-dose ILIT study where we
312found a 28 % improvement in nasal provocation test (4). To our knowledge there is no
313previous published ILIT study that have used the CSMS as the outcome measure. Since the
314effect of allergy immunotherapy is considered to be dose dependent, in these dose
315escalation studies we calculated for a 50% improvement of the CSMS in the active group, a
316standard deviation of 50%, aimed at a power of 0.80 and used a type 1 error rate α level of
3170.05. The calculated sample size was 16 in each group for ILIT after SCIT- 10 000. After the
318baseline CSMS registration in ILIT after SCIT- 10 000, a new power calculation was performed
319for ILIT de novo- 3000. If we expected 40% improvement of CSMS in the active group and a
320standard deviation of 45%, we assumed that d_{active} was 100, d_{placebo} 55 and SD 45. Aiming at a
321power of 0.80 and using a type 1 error rate α level of 0.05, the calculated sample size was 20
322in each group in ILIT de novo- 3000.

323

324

325

326Results

327

328Patients

329

330In ILIT after SCIT-10 000, 31 patients were telephone interviewed, 30 patients were
331screened, and 29 patients were included in the study. None of the patients were found to be
332a non-responder to SCIT. The narrow inclusion criteria of recent completed SCIT limited the
333enrollment. 4 patients dropped out before randomized treatment. All patients that started
334the treatment completed the protocol and follow up. 1 placebo treated patient did not
335complete the symptom diary, that was the primary outcome measure, sufficiently the year
336before treatment, due to an unforeseen travel for 3 weeks that included the peak pollen
337season. This patient was excluded from the analyses of CSMS, SS, MS and RQLQ and VAS.
338The same patient also did not complete the diary sufficiently at the peak pollen period the
339year after treatment, partly due to travel. The patient was not included in the analysis of
340SMS of the peak days but had acceptable compliance of the total registration period and
341remained in the comparison of the SMS of the active versus placebo group after treatment.
342One active patient had grass allergen specific IgE level >100 kU/mL at all three blood sample
343occasions and the specific level could not be determined at the hospital lab. This patient was
344excluded from the analysis of grass specific IgE since we couldn't measure the changes. 1
345placebo patient had a missing value of grass specific IgG4 4 weeks after treatment and was
346excluded from the analysis of this parameter. One active and two placebo patients
347completed the QoL questionnaires out of the season time frame at the baseline year. They
348were excluded from the comparison of QoL before versus after treatment but remained in
349the between group comparisons of active versus placebo after treatment. (Figure E1 shows
350the flow of patients.)

351

352In ILIT de novo- 3000, 58 patients were screened and 39 were included and randomized. The
353predetermined inclusion criteria with a moderate to high minimal level of disease severity
354and a limited time frame for screening hampered the enrollment of patients. One placebo
355patient withdrew consent after the first injection due to previously known and exacerbated
356stress symptoms. One active patient was revealed having a chronic skin infection, that the
357patient had not reported to the study staff previously. That patient therefore fulfilled a
358contraindication and was withdrawn from the study after two injections. One active patient
359got the first injection without complications but then withdrew consent due to fear of
360adverse events. One active patient had a suspect late adverse reaction with lower airways
361symptoms after the second injection and was withdrawn from the study. One active patient
362could not proceed to injection 2 within the time frame of 7 weeks due to an upper airway
363virus infection. This patient instead "started over" with the protocol after the recovery and
364made a modified up-dosing; 1000- 1000- 3000 SQ-U with 8 weeks in between injection
365number 1 and 2. This patient, and the two patients that received 5000 SQ-U as the last
366treatment, were analyzed together with the rest of the active group. One placebo patient
367completed only 48% of the registration days at the pollen season after treatment and was
368excluded from the analysis of the symptoms and medication scores. One active patient was
369away for travel at the heights of the grass pollen season after treatment and did not
370complete the QoL questionnaires and was not included in the peak pollen analysis of CSMS,
371SS or MS. (Figure E1 shows the flow of patients.)

372

373

374Pollen data

375

376In ILIT after SCIT- 10 000, the 2015 baseline study period included both the pre- and
377postpeak periods of the pollen season. In 2016 in Malmö, the peak days occurred at the very
378start of the study period, and thus, mainly the postpeak period was included. But in this
379year, the prepeak period was rather short. Before the start of the study period, 300 grains
380were registered. During both years, the study period included the peak dates.

381In ILIT de novo- 3000, the peak grass pollen season started early in Malmö both study years.
382Therefore, the registration started in the beginning of the peak pollen season, but included
383the peak pollen day, both years. In Stockholm the courses of the grass pollen seasons were
384as expected and the registration periods included both the prepeak season and the postpeak
385seasons. (See Table E1 for pollen counts during the registration periods.)

386

387

388Daily registrations

389

390The patients received daily electronic questionnaires for 64 days at the grass pollen season
391in ILIT after SCIT- 10 000, and for 56 days in ILIT de novo- 3000.

392

393In ILIT after SCIT- 10 000, one placebo patient completed the questionnaire only 42% of the
394days at the baseline season, due to an unforeseen travel for 3 weeks that included the peak
395pollen season. This patient was excluded from the before-after analyses of the symptoms
396and medication scores (SMS). One patient replied > 50% of the questionnaires and the
397remaining replied >70% of the days at the registration periods. At the pollen season after
398treatment one active and one placebo patient answered >50% of the days, and the
399remaining completed >70% of the days of registration.

400

401At the peak pollen season before treatment, all patients replied $\geq 50\%$ of the days. During the
402peak season after treatment, one placebo patient completed only 43% of the days due to
403poor adherence and was excluded from the peak pollen analysis. All other patients replied
404>70% of the peak pollen days.

405

406In ILIT de novo- 3000, all patients completed the questionnaires >70% of the days during the
407baseline season. The year after treatment, one placebo patient completed only 48% of the
408days, due to poor adherence, and was excluded from the analysis of the symptoms and
409medication scores. Two active patients completed >50% of the days and all remaining
410patients completed >70% of the questionnaires.

411

412At the peak pollen period before treatment, all patients replied >70% of the days. During the
413peak pollen season after treatment, one active patient was away for travel during the entire
41414 days peak period and was excluded from the peak pollen period analyses. That patient
415had 55% completed days during the rest of the study period and remained in the analyses of
416the entire pollen season. Two active patients replied 50% of the 14 peak pollen days and one
417placebo patient replied 57% of the days. All the remaining patients responded >70% of the
418peak period.

419

420

421Primary outcome measure

422

423In ILIT after SCIT- 10 000, when we compared the average value of CSMS for each patient
424over the season, instead of AUC, the results also showed an improvement in the active group
425with reduced CSMS by 22% from mean 1.59 (SD 0.79 [95% CI 1.12-2.07]) to mean 1.24 (SD
4260.76 [95% CI 0.78-1.70]), $p=0.03$. The placebo group did not improve, with mean CSMS 1.03
427before treatment (SD 0.64[95%CI 0.59-1.46]) and mean CSMS after treatment 0.91 (SD 0.72
428[95% CI 0.43-1.39]), $p=0.17$.

429

430Comparing the mean value of CSMS for each patient over the season showed similar results
431with reduced CSMS by 22% in the active group but no improvement in the placebo group.

432

433

434Secondary outcome measures

435

436SPT

437In ILIT after SCIT- 10 000, the SPT measured increased size in the placebo group at the follow
438up 4 weeks after treatment, from median 5.8 mm (IQR 5.0-6.9) to median 7.0 (IQR 5.1-8.8),
439 $p=0.04$, $n=12$. At the follow up 8 months after treatment the SPT reactivity was the same as
440at baseline; median wheal size 6.0 (IQR 4.6-8.0), $p>0.99$. The active group did not change;
441the measured median size was, at the different time points, 6.5-6.5-5.5 (IQR [5.0-9.0]- [5.5-
4428.0]- [5.0-6.5]), $p=0.7$, $n=13$.

443

444The SPT did not change in any of the groups in ILIT de novo- 3000. Before treatment the
445active group had median wheal size 9.5 (IQR 5.4-11.9), 4 weeks after treatment 8.5 (IQR 6.8-
4469.5) and 8 months after treatment 8.8 (IQR 8.4-10.5), $p=0.8$, $n=16$. Before treatment, the
447placebo group had median wheal size 8.5 (IQR 6.0-9.0), 4 weeks after treatment 7.0 (IQR 5.0-
4489.0) and 8 months after treatment 8.0 (IQR 6.0-11.5), $p=0.2$, $n=19$.

449

450Global estimation of improvement on VAS

451In ILIT after SCIT- 10 000, the active patients scored median improvement 1.9 (IQR 0.8-4.0),
452 $n=13$, compared to the placebo patients that scored median improvement 2.0 (IQR 0.2-6.8),
453 $n=11$, $p=0.85$. In ILIT de novo- 3000, the median improvement in the active group was 0.7
454(IQR 0.1-6.3), $n=16$, compared to the placebo group that scored median improvement 3.1
455(IQR 0.5-6.5), $n=19$, $p=0.27$. (See Figure E3.)

456

457Quality of Life

458In ILIT after SCIT- 10 000, the total RQLQ scores did not change in the placebo or in the active
459group. The median total score in the active group at the season before treatment was 2.4
460(IQR 1.2-3.0) and at the season after treatment 2.1 (IQR 1.1-2.7) $n=12$, $p=0.22$. The median
461total RQLQ scores in the placebo group before treatment was 1.8 (IQR 0.8-2.1) and after
462treatment 1.3 (IQR 0.3-1.9), $n=9$, $p=0.57$. At the pollen season after treatment there was no
463difference between the active and the placebo group, the active group had median score 2.0
464(IQR 1.0-2.7), $n=13$, and the placebo group had median score 1.3 (IQR 0.4-1.9) $n=11$, $p=0.15$.

465In ILIT de novo- 3000, the active group had a trend for a small improvement that did not
466reach statistical significance; the median total score before treatment was 2.2 (IQR 1.6-2.6)
467and after treatment 1.5 (IQR 0.7-2.3), $p=0.09$, $n=15$. Instead, the RQLQ scores in the slightly
468larger placebo group showed a moderate but highly statistically significant improvement at
469the year after treatment compared to the season before treatment; at the baseline season
470the placebo group had median total score 2.8 (IQR 1.7-3.3) and after treatment median
471score 1.5 (IQR 0.9-2.6), $p<0.0001$, $n=19$. All the patients that were included in the before-
472after analysis within each group in ILIT de novo-3000, were also part of the analysis
473between the active and placebo group after treatment; there was no difference in total
474RQLQ scores between the groups, $p=0.65$.

475

476*Nasal provocation test*

477The reactivity at NPT did not change after active or placebo treatment, in any of the studies.
478

479In ILIT after SCIT- 10 000, the median total AUC score before treatment in the active group
480was 118 (IQR 66-160), 4 weeks after treatment 80 (IQR 44-136) ($p=0.16$) and 8 months after
481treatment 108 (IQR 66-159) ($p>0.99$), $n=13$. The corresponding results for the placebo group
482was median total AUC score before treatment 104 (IQR 44-131), 4 weeks after treatment 55
483(IQR 38-90) ($p=0.06$) and 8 months after treatment 89 (IQR 65-128) ($p>0.99$), $n=12$ (See
484Figure E3).

485

486In ILIT de novo- 3000, before treatment the active group had mean total AUC score 171 (SD
48761 [95% CI 139-204]), 1 month after treatment 158 (SD 60 [95% CI 126-189]) ($p=0.52$) and 8
488months after treatment 151 (SD 60 [95% CI 121-181]) ($p=0.41$), $n=16$. The placebo group
489had before treatment mean score 180 (SD 51 [95% CI 155-204]), 1 month after treatment
490154 (SD 70 [95% CI 122-186]) ($p=0.10$) and 8 months after treatment 180 (SD 56 [95% CI 153-
491207]) ($p>0.99$) $n=19$. (See Figure E3.)

492

493*DC activation in lymph node and blood*

494In ILIT de novo- 3000, the presence of CD80, CD86 and CD141 expression in dendritic cells
495was determined with flow cytometry. The detection of dendritic cells was based on the
496expression of CD45+, SSC++ and CD11C+. In analysis of blood we also used CD14 to exclude
497monocytes. (See Figure E5). Due to low yield of DCs in fine needle aspiration before
498treatment, values from both placebo and active ILIT were pooled and compared with values
499after treatment. There were no significant differences in the expression levels between
500placebo and active ILIT before treatment.

501

502In patients treated with active ILIT, DCs in the lymph node showed increased activation after
503treatment, with upregulated expression of T-cell co-stimulatory molecules CD80 and CD86,
504compared to samples from non-treated patients. Mean MFI for CD80 increased from 555.5
505(SD 691.0 [95% CI 91.32-1020]) to 1050 (SD 652.2 [95% CI 548.4-1515]), $p=0.02$, Kruskal-
506Wallis test with Dunns correction. Mean MFI for CD86 increased from 4158 (SD 3931 [95% CI
5071517-6799]) to 6710 (SD 1806 [95% CI 5322-8098]), $p=0.02$, Kruskal-Wallis test with Dunns
508correction. DCs increased the expression of CD141, mean MFI increased from 4048 (SD 1496
509[95% CI 3043-5053]) to 6257 (SD 1990 [95% CI 4727-7786]), $p=0.007$. Flow cytometry
510histogram for a representative staining is presented in Figure E5. No changes in DCs from
511lymph node samples were detected in the placebo treated patients. DCs in PBL did not

change in any of the groups, 2-way ANOVA with Sidak's multiple comparisons test (See Fig. 5 in the main text.)

514

515 *CD4 T-cells*

As a continuation of our previous ILIT studies (4), in ILIT de novo-3000, we analyzed changes in central memory (CM) CCR7-CD45RA+CD4+CCR5+ T-cells and the amount of effector memory (EM) CD4 T-cells with high expression of CD25⁺⁺. No significant changes in placebo or active ILIT could be detected. In the active group the mean amount of CM T-cells before treatment was 1,31% (SD 0.73% [95% CI 0.75%-1.87%]), and after treatment 2,50% (SD 1,60% [95% CI -1.02%-6.04%]), p=0.41. The amount of EM CD25⁺⁺ T-cells before treatment was 72.51% (SD 11.61% [95% CI 63.93%-81.09%]) and after treatment 70.41% (SD 11.32% [95% CI 61.71%-79.11%]), p=0.82. See Figure E6 for detailed flow cytometry gating procedure.

525

At the follow up 4 weeks after treatment analysis of allergen activated CD4 T-cells was also included. The detection of activated CD4+CD69+CD154+ cells were determined by flow cytometry. See Figure E7 for detailed flow cytometry gating procedure. In the placebo group 11 of 11 patients showed increased activation in allergen stimulated samples compared to control samples. The mean activation in the control samples was 0.030% (SD 0.026% [95% CI 0.012%-0.047%]), the mean activation for stimulated samples was 0.168% (SD 0.225% [95% CI 0.017%-0.319%]), p=0.001. 9 of 10 samples from active ILIT patients showed increased activation compared to control. The mean activation for control samples was 0.023% (SD 0.024% [95% CI 0.006%-0.041%]), the mean activation for stimulated samples was 0.104% (SD 0.077% [95% CI 0.050%-0.160%]), p=0.004. No significant difference in the amount of activation (allergen stimulation– negative control) could be detected between placebo 0.139% (SD 0.205% [95% CI 0.001%-0.276%]) and active ILIT 0,081% (SD 0.083% [95% CI 0.021%-0.141%]), p= 0.86. (See Figure E2.)

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541 *References*

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Table E1. The duration, course and intensity of the pollen seasons and study periods.

	Duration of pollen season (peak date)	Study period	Maximum concentration	Seasonal pollen index†	Study period pollen index§	Peak pollen period	Peak season index§§
ILIT after SCIT- 10 000							
Stockholm							
2015	May 24 – Sept 14 (July 3)	May 26 - July 28	143	1512	1399	June 24- July 7	864
2016	May 20 – Sept 15 (June 22)	June 3 - August 5	62	1302	1104	June 19- July 2	458
Gothenburg							
2015	May 18- Sept 10 (July 11)	May 26 - July 28	130	2195	1815	June 23- July 6	883
2016	May 12 – Oct 4 (June 26)	June 3 – August 5	105	1854	1505	June 3- June 16	713
Malmö							
2015	May 6 – Sept 7 (June 29)	May 26– July 28	160	2443	2176	June 20- July 3	1084
2016	May 10 – Aug 26 (June 5)	June 3 - August 5	226	2189	1810†	June 4- June 17	972
ILIT de novo- 3000							
Stockholm							
2016	May 20 - Sept 15 (June 22)	June 4- July 29	62	1302	1043	June 19- July 2	458
2017	June 4 – Sept 25 (June 18)	June 12- Aug 6	71	933	781	June 17- June 30	352
Malmö							
2016	May 10 - Aug 26 (June 5)	June 4- July 29	226	2189	1730†	June 4- June 17	972
2017	May 17 - Sept 4 (June 11)	June 12- Aug 6	106	1438	940	June 12- June 25	563

†In 2016, there was a gap in the data series from Malmö during 19-21 July 2016, due to a technical error. ‡The sum of the measured daily pollen counts during the year. §The sum of the measured daily pollen counts during the study period. §§The sum of the measured daily pollen counts during the 14 peak pollen days of the study period.

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Table E2. CSMS, SS and MS at the peak pollen seasons

ILIT after SCIT- 10 000 peak pollen season		CSMS peak season mean (SD [95%CI]), median (IQR)	SS peak season mean (SD [95%CI]), median (IQR)	MS peak season mean (SD [95%CI]), median (IQR)
Active N= 13	Before treatment	26.0 (13.4 [18.0-34.1]), 29.1 (15.4-34.6)	12.0 (6.1 [8.3-15.7]), 14.1 (6.9-15.3)	14.0 (8.7 [8.8-19.3]), 13.5 (7.5-21.0)
	After treatment	22.2 (13.5 [14.0-30.3]), 23.9 (7.4-34.2)	12.3 (7.3 [7.9-16.7]), 12.3 (4.4-18.9)	9.9 (8.3 [4.9-15.0]), 10.0 (0.75-16.5)
	P-value before vs. after treatment †	0.19	0.45	0.02
Placebo N=11	Before treatment, mean (SD [95%CI])	17.9 (9.9 [11.3-24.6]), 21.4 (9.1-24.5)	8.2 (3.6 [5.8-10.6]), 7.1 (4.7-11.5)	9.7 (7.7 [4.6-14.9]), 10.5 (2.0-14.0)
	After treatment, mean (SD [95%CI])	18.3 (11.8 [10.4-26.2]), 19.8 (9.6-21.7)	8.4 (5.0 [5.1-11.8]), 8.5 (4.4-12.3)	9.9 (8.0 [4.5-15.2]), 9.5 (1.5-13.0)
	P-value before vs. after treatment †	0.76	0.76	>0.99

ILIT de novo- 3000 peak pollen season		CSMS peak season mean (SD [95%CI]), median (IQR)	SS peak season mean (SD [95%CI]), median (IQR)	MS peak season mean (SD [95%CI]), median (IQR)
Active N=15	Before treatment	27.2 (11.7 [21.1-34.2]), 24.7 (20.0-39.5)	15.7 (5.9 [12.4-19.9]), 15.8 (9.3-19.5)	12.0 (8.8 [7.1-16.9]), 11.3 (5.6-19.8)
	After treatment,	24.3 (11.8 [17.6-30.6]), 25.4 (14.1-35.0)	13.4 (6.9 [9.6-17.3]), 12.8 (7.7-18.5)	10.7 (7.3 [6.7-14.8]), 12.0 (2.0-14.5)
	P-value before vs. after treatment †	0.31	0.13	0.40
Placebo N=18	Before treatment	29.7 (10.3 [24.6-34.8]), 30.0 (21.8-37.7)	14.9 (5.3 [12.3-17.5]), 15.3 (9.9-19.4)	14.8 (6.9 [11.4-18.21]), 13.5 (9.1-21.8)
	After treatment	23.1 (11.0 [17.7-28.6]), 22.0 (14.5-33.5)	12.5 (4.9 [10.1-14.9]), 11.9 (9.2-16.5)	10.6 (7.8 [6.7-14.5]), 7.5 (4.4-17.1)
	P-value before vs. after treatment†	0.02	0.07	0.03

† Wilcoxon sign rank test.

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Table E3. Grass specific IgE and IgG4.

ILIT after SCIT- 10 000

		Timothy specific IgG4 mg/L median (IQR), n	Timothy specific IgE kU/L median (IQR), n
Active	Before treatment	3.4 (2.7-6.9), n=13	11.9 (5.0-24.0), n=12
	4 weeks after treatment	8.3 (5.2-10.8), n=13	36.5(14.5-64.8), n=12
	8 months after treatment	3.3 (1.9-5.9), n=13	15.8 (6.8-43.3), n=12
	P-value before vs. 4 weeks after treatment †	0.007	0.002
	P-value before vs. 8 months after treatment †	0.48	0.61
Placebo	Before treatment	3.9 (3.0-8.4), n=11	13.9 (5.2-36.7), n=11
	4 weeks after treatment	2.9 (2.4-3.7), n=11	9.3 (3.5-24.0), n=11
	8 months after treatment	2.3 (1.7-3.5), n=11	17.9 (7.1-42.0), n=11
	P-value before vs. 4 weeks after treatment †	0.02	0.18
	P-value before vs. 8 months after treatment†	<0.0001	0.57

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ILIT de novo- 3000

		Timothy specific IgG4 mg/L median (IQR), n	Timothy specific IgE kU/L median (IQR), n
Active	Before treatment	0.24 (0.11-0.33), n=16	5.2 (2.7-12.4), n=16
	4 weeks after treatment	0.34 (0.19-0.61), n=16	16.1 (9.5-31.3), n=16
	8 months after treatment	0.25 (0.14-0.48), n=16	10.7 (6.0-24.9), n=16
	P-value before vs. 4 weeks after treatment†	0.0001	<0.0001
	P-value before vs. 8 months after treatment†	0.03	0.009
Placebo	Before treatment	0.18 (0.09-0.26), n=19	11.0 (4.4-24.6), n=19
	4 weeks after treatment	0.16 (0.05-0.27), n=19	9.1 (4.3-17.0), n=19
	8 months after treatment	0.15 (0.08-0.33), n=19	12.4 (4.4-24.1), n=19
	P-value before vs. 4 weeks after treatment †	0.21	0.83
	P-value before vs. 8 months after treatment†	>0.99	0.15

IQR= interquartile range, † Friedman test.

Table E4. The relationship between pollen levels and allergic symptoms in ILIT after SCIT- 10 000.

	Placebo		Active	
	Estimate +/- SE	p-value	Estimate +/- SE	p-value
2015 nasal CSMS	0.0027±0.0014	n.s.	0.0016±0.0007	0.025
2016 nasal CSMS	-0.009856±0.0012	n.s.	0.0006±0.0009	n.s.
2015 ocular CSMS	0.0030±0.0014	0.028	0.0018±0.0007	0.029
2016 ocular CSMS	0.0004±0.0015	n.s.	0.0002±0.0011	n.s.
2015 nasal + ocular CSMS	0.0030±0.0014	0.036	0 . 0 0 1 7 ± 0 . 0 0 0 0 7	0.029
2016 nasal + ocular CSMS	-0.000659±0.0014	n.s.	0.0005±0.0001	n.s.

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581**Figure E1.** The flow of patients in ILIT after SCIT- 10 000 and ILIT de novo- 3000.

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583**Figure E2. ILIT induced no detectable changes in CD4 T-cells in flow cytometry analyses.**

584**Fig. A-B.** The percent of positive cells before and after treatment. **Fig. C-D.** The percent of
585CD69⁺CD154⁺ T-cells in negative control and after allergen (timothy) stimulation. **P<0.01,
586***P<0.001, ns=not significant.

587

588**Figure E3. A.** There were no differences in the global estimation of improvement on VAS
589between the active and placebo group in any of the studies. **B.** The QoL was unchanged in
590ILIT after SCIT- 10 000 in both groups. In ILIT de novo- 3000 the placebo, but not the active
591group, showed improved scores the year after treatment. **C.** The reactivity at nasal
592provocation test did not change in any of the groups in the two studies. n.s.= not significant,
593****p<0.0001, AUC=area under the curve, horizontal lines at the median values and
594interquartile ranges.

595

596**Figure E4. The relationship between pollen levels and allergic symptoms in ILIT after SCIT-**

597**10 000.** A time-series analysis including all patients from the Malmö-Lund area, using a
598generalized additive model (GAM). Both the active and the placebo group showed increased
599relative risk (RR) of symptoms measured as CSMS (y-axis) at increased pollen levels (x-axis)
600before treatment. The year after treatment, no significant relationship between pollen levels
601and symptoms could be verified, in any of the groups. The dotted lines show the 95% CI.

602

603**Figure E5. Gating strategy for DCs. A.** The analyses for DC in fine needle aspirates.

604Leukocytes were detected by expression of CD45, lymphocytes were excluded based SSC and
605FCS. For detection of DC:s we used high expression of CD11C. **B.** Analyses for dendritic cells
606in blood. Leukocytes were detected by expression of CD45, lymphocytes and neutrophils
607were excluded based SSC and FCS. For detection of dendritic cells we used high expression of
608CD11C and also negative for expression of CD14. **C.** The staining for CD80, CD86 and CD141.
609Dotted line represents fluorescence minus one control and red histograms illustrates
610positive staining.

611

612**Figure E6. Gating strategy for CD4+ cells.** CD4 and CD3 were used to identify CD4 T-cells.

613FSC-H and FSC-A were used to exclude aggregates from the analysis. CD45RA and CCR7 were
614used to divide central memory (CM) T-cells CCR7⁺CD45RA⁻, naïve T-cells CCR7⁺CD45RA⁺ and
615effector memory (EM) T-cells CCR7⁻. On CM T-cells we analyzed the expression of CCR5. For
616enrichment of regulatory cells, we used CD4⁺ and CD25⁺⁺. T-regulatory cells were further
617divided into CM, naïve and EM.

618

619**Figure E7. Gating strategy for allergen activated T-cells.** Top row represents staining of
620control samples. Bottom row represents staining of allergen stimulated samples. SSC and CD4
621were used for detection of CD4 T-cells. FSC-H and FSC-A were used to exclude aggregates
622from the analysis. Double positivity for CD69 and CD154 were used for detection of activated
623cells.

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625