

Intralymphatic allergen-specific immunotherapy: An effective and safe alternative treatment route for pollen-induced allergic rhinitis

Terese Hylander, MSc,^a Leith Latif, MD,^b Ulla Petersson-Westin, MD, PhD,^b and Lars Olaf Cardell, MD, PhD^{a,b} *Stockholm and Malmö, Sweden*

Background: Allergen-specific immunotherapy is the only causative treatment of IgE-mediated allergic disorders. The most common administration route is subcutaneous, which may necessitate more than 50 allergen injections during 3 to 5 years. Recent evidence suggests that direct intralymphatic injections could yield faster beneficial results with considerably lower allergen doses and markedly reduced numbers of injections.

Objective: To evaluate the effects of intralymphatic allergen-specific immunotherapy in pollen-allergic patients.

Methods: In an open pilot investigation followed by a double-blind, placebo-controlled study, patients with allergic rhinitis were treated with 3 intralymphatic inguinal injections of ALK Alutard (containing 1000 SQ-U birch pollen or grass pollen) or placebo (ALK diluent). Clinical pre- and posttreatment parameters were assessed, the inflammatory cell content in nasal lavage fluids estimated, and the activation pattern of peripheral T cells described.

Results: All patients tolerated the intralymphatic immunotherapy (ILIT) treatment well, and the injections did not elicit any severe adverse event. Patients receiving active treatment displayed an initial increase in allergen-specific IgE level and peripheral T-cell activation. A clinical improvement in nasal allergic symptoms upon challenge was recorded along with a decreased inflammatory response in the nose. In addition, these patients reported an improvement in their seasonal allergic disease. No such changes were seen in the placebo group.

Conclusions: Although this study is based on a limited number of patients, ILIT with grass-pollen or birch-pollen extracts appears to reduce nasal allergic symptoms without causing any safety problems. Hence, ILIT might constitute a less time-consuming and more cost-effective alternative to conventional subcutaneous allergen-specific immunotherapy. (*J Allergy Clin Immunol* 2013;131:412-20.)

Key words: Allergen-specific immunotherapy, allergic rhinitis, intralymphatic immunotherapy, seasonal allergic rhinitis

Abbreviations used

ILIT: Intralymphatic immunotherapy
MFI: Mean fluorescence intensity
NAL: Nasal lavage
NPT: Nasal provocation test
SCIT: Subcutaneous immunotherapy
SPT: Skin prick test

Allergic rhinitis is a health problem that causes worldwide disability. It affects social life, sleep, school, and work.^{1,2} Allergen-specific immunotherapy is the practice of administering gradually increasing quantities of an allergen extract to an allergic subject to ameliorate symptoms after exposure to the causative allergen. This is the only treatment that diminishes symptoms, improves quality of life, prevents new sensitizations, and reduces the development of asthma in patients suffering from allergic rhinitis.³⁻⁵ The dominating route for the administration of allergen-specific immunotherapy is subcutaneous injections, and although the maintenance dose can be reached through various regimens, the current treatment protocol requires numerous immunotherapeutic injections and may take several years to complete.^{6,7} Despite its proven benefits, only 5% of allergic patients with insufficient symptom control undergo subcutaneous immunotherapy (SCIT)^{8,9} and there is a considerable interest in finding alternative routes to shorten the duration of the treatment and to increase the attractiveness of the therapy.

When antigens are administered subcutaneously, only small fractions of the antigenic epitopes reach the lymph nodes.^{10,11} As immune responses are initiated in secondary lymphoid organs, it seems reasonable that the direct administration of antigens into the highly immunocompetent environment of the lymph nodes induces greater immunogenicity.^{12,13} Animal studies have revealed that vaccines can be given in lower concentrations and with a reduced number of injections when administered directly into the lymph node.¹⁴⁻¹⁶ In mice, intralymphatic immunization has been demonstrated to cause enhanced allergen-specific IgG and T-cell responses when compared with subcutaneous injections. Moreover, only the intralymphatic immunization stimulated the production of the T_H1-dependent subclass IgG2a, which is associated with improved protection against allergen-induced anaphylaxis.¹¹ Three clinical trials have shown a regime of only 3 intralymphatic injections to be effective in induction of tolerance against grass pollen, cat dander extract, and bee venom.¹⁷⁻¹⁹ The present double-blind placebo-controlled study was designed to further assess the effect of intralymphatic immunotherapy (ILIT) on allergen-related symptoms and nasal inflammation in patients with pollen-induced seasonal allergic rhinitis.

From ^athe Department of Clinical Sciences, Intervention and Technology, Division of ENT Diseases, Karolinska Institutet, Stockholm; and ^bthe Department of Otorhinolaryngology Malmö, Skåne University Hospital, Lund University, Malmö.

Disclosure of potential conflict of interest: L. O. Cardell has received grants from the Swedish Medical Research Council. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication April 27, 2012; revised October 23, 2012; accepted for publication October 31, 2012.

Corresponding author: Lars Olaf Cardell, MD, PhD, Department of Clinical Sciences, Intervention and Technology (CLINTEC), Division of ENT Diseases, Karolinska Institutet, SE-141 86 Stockholm, Sweden. E-mail: lars-olaf.cardell@ki.se.

0091-6749/\$36.00

© 2013 American Academy of Allergy, Asthma & Immunology
<http://dx.doi.org/10.1016/j.jaci.2012.10.056>

METHODS

Study population

Study subjects (28 in total) were recruited among patients at the Allergy Department of Skåne University Hospital, Malmö, Sweden. They all had a history of moderate to severe birch-pollen/grass-pollen-induced rhinoconjunctivitis with symptoms including itchy nose and eyes, sneezing, nasal congestion, and secretion. The diagnosis was verified by positive skin prick test (SPT) results, presence of serum-specific IgE antibodies toward birch and/or grass (minimum 0.35 kU/L), and positive nasal provocation test (NPT) results. None of the patients suffered from seasonal asthma, but 7 subjects reported exercise-induced asthma with mild to moderate symptoms.

General contraindications were pregnancy or nursing, wish for pregnancy, autoimmune and collagen disease, cardiovascular disease, current persistent asthma, upper airway disease (nonallergic sinusitis, nasal polyps), chronic obstructive and restrictive lung disease, hepatic and renal disease, cancer, previous immune- or chemotherapy, major metabolic disease, alcohol or drug abuse, mental incapability of coping with the study, or medication with a possible side effect of interfering with the immune response. The local ethics committee approved the study, and all participants, those undergoing ILIT as well as those undergoing SCIT, gave their written informed consent.

Study design

Six patients (3 men and 3 women) and 15 additional patients (10 men and 5 women, Table I) were recruited to participate in an open pilot study and a randomized double-blind, placebo-controlled study of intralymphatic grass-pollen and birch-pollen immunotherapy, respectively (Fig 1).

At the first visit, patient eligibility was determined, SPTs and NPTs were performed, blood was sampled, and nasal lavage (NAL) fluids were collected. After approximately 1 week, patients in the double-blind study were randomly allocated (1:1, based on recruitment order) to receive either placebo (n = 8) or active (n = 7) intralymphatic treatment. At visits 2 to 4, the study subjects received three 0.1-mL injections with either placebo (allergen diluents without aluminum hydroxide; ALK-Abelló, Hørsholm, Denmark) or 1000 SQ-U of a standardized, aluminum hydroxide-adsorbed, depot birch-pollen or grass-pollen vaccine²⁰ (Alutard; ALK-Abelló) with approximately 4-week intervals. On the basis of the outcome of the allergy tests, patients were challenged and vaccinated with either birch or grass. The vaccines used were packed and blinded by staff with no connection to the study, and thus both patients and physicians were blind. At visit 5 (~4 weeks after the last injection) and at visit 6 (after the next consecutive pollen season), patients were evaluated by means of SPTs and NPTs, NAL fluids, and immunological parameters and the subjects were asked to answer a questionnaire regarding their seasonal allergic symptoms as compared with the previous pollen season. All patients completed the treatment, the emergency envelopes were unbroken, and the study remained double-blind.

Immunization protocol for SCIT patients

As open controls, 7 patients were subcutaneously injected with incremental doses of birch-pollen vaccine (Alutard; ALK-Abelló) over 14 weeks (week 1, 50 SQ-U; week 2, 100 SQ-U; week 3, 200 SQ-U; week 4, 400 SQ-U; week 5, 800 SQ-U; week 6, 1,500 SQ-U; week 7, 3,000 SQ-U; week 8, 6,000 SQ-U; week 9, 10,000 SQ-U; week 10, 20,000 SQ-U; week 11, 40,000 SQ-U; week 12, 60,000 SQ-U; week 13, 80,000 SQ-U; and week 14, 100,000 SQ-U). Thereafter, maintenance injections (100,000 SQ-U) were given every 6 to 8 weeks over 3 years. These patients finished treatment in 2010 and were not part of the initial study. However, they were evaluated by the same parameters as for the patients receiving intralymphatic injections but for the NPTs. In addition, another 10 patients receiving subcutaneous injections were asked to score the discomfort associated with the injection by using an arbitrary scale ranging from 0 to 10 (0 = completely painless and 10 = worst pain ever).

Intralymphatic injections

Using ultrasound guidance and a 25-gauge needle, a superficial inguinal lymph node in either the left or the right groin was aseptically injected for 30

TABLE I. Baseline characteristics of patients included in the placebo study

	Active ILIT	Placebo ILIT
No. of participants	7	8
Age (y), median (range)	34 (22-51)	29 (19-53)
Gender (male vs female)	4:3	6:2
Allergen-specific IgE (kU/L)	3.5-50	0.35-50
Birch vs grass vaccination	2:5	3:5
No. of monosensitized subjects	2	3
No. of patients with exercise-induced asthma	2	3

seconds. The same side was targeted during all 3 injections. Aspirations were made before the injections to avoid inadvertent intravascular administration. The peak expiratory flow was measured before and after each injection, and all patients were monitored at the ward for no less than 60 minutes after each injection. All signs of local and/or systemic reactions in conjunction to the injections were recorded by the trial staff, and patients were then asked to record and report all indications of late reactions during the following 24 hours. In addition, patients were requested to estimate the pain associated with the injection by using an arbitrary scale ranging from 0 to 10 (0 = completely painless and 10 = worst pain ever) and relate it to the discomfort associated with a venous puncture (more painful, same pain, less painful, no pain at all).

Skin prick tests

SPTs were performed with a standard panel of 11 common airborne allergens (ALK-Abelló) including pollen (birch, timothy, mugwort, and ragweed), house-dust mite (*Dermatophagoides pteronyssinus* and *D farinae*), molds (*Cladosporium* and *Alternaria*), and animal allergens (cat, dog, and horse). SPTs were administered on the volar side on forearms, with saline buffer as negative control and histamine chloride (10 mg/mL) as positive control. All patients presented a wheal reaction diameter of more than 3 mm toward birch and/or grass.

Nasal provocations and symptom scores

Before and after the ILIT treatment, all patients were challenged with 10,000 SQ-U birch-pollen or grass-pollen extract per nostril.^{21,22} The occurrence and severity of nasal allergic symptoms (itching, secretion, and congestion) were self-recorded during the first 30 minutes after each challenge by using a scale ranging from 0 to 3 (0 = no; 1 = mild; 2 = moderate; 3 = severe symptoms).²³ The combination of these scores is referred to as the total symptom score, which could maximally reach 9 points.

Blood sampling

Venous blood was obtained from participants at baseline, after completion of the treatment, and at the end of the upcoming pollen season. Blood collected in tubes containing EDTA (Vacuette 454209) was used for total and differential leukocyte counts in a Coulter LH750/GenS cell counter (Beckman Coulter, Marseille, France), and allergen-specific IgE and IgG₄ levels were determined by using the Phadia CAP system (Uppsala, Sweden). Blood collected in tubes containing buffered trisodium citrate solution (BD Vacutainer 367704) was used for flow cytometry analyses and *in vitro* experiments.

NAL fluids

Before NPTs were performed, NAL fluids were collected as previously described.^{21,22,24} Briefly, after clearing excess mucus by exsufflation, a sterile saline solution was aerosolized into the nostrils and nasal fluids were passively collected in a test tube until 7 mL was recovered. The number of living cells (leukocytes and epithelial cells) was determined in a Burkner chamber by using

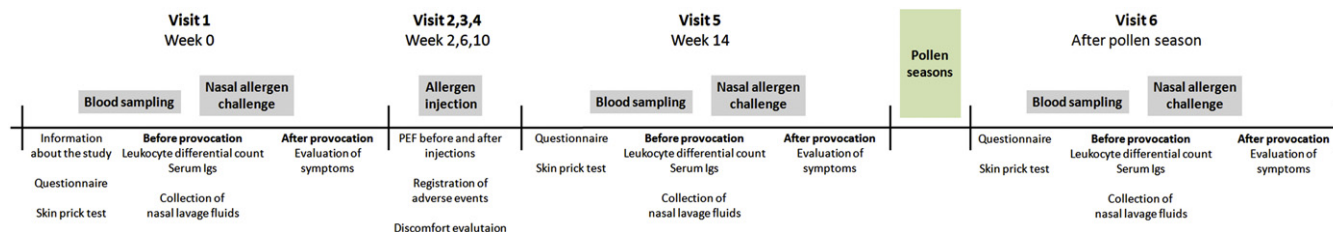


FIG 1. Study outline of the placebo-controlled study. At visit 1, patients were screened and enrolled to the study; at visits 2 to 4, the study subjects received intralymphatic immunotherapeutic allergen injections; and at visit 5 (after treatment) and visit 6 (after pollen season), patients were evaluated.

tryptan staining. IL-8 level in NAL was assessed by using ELISA (R&D Systems, Minneapolis, Minn).

Treatment outcome

At the end of the first allergy season after the given treatment, patients were asked to compare their allergic symptoms during the recent pollen season with the symptoms they experienced during the season just before the cure was started. To this end, a visual analog scale ranging from 0 (unchanged symptoms, no improvement) to 10 (total symptom relief, complete recovery) was used.

Immunocytochemistry

Flow cytometry analyses were performed on a Coulter Epics XL flow cytometer (Beckman Coulter). A total of 150,000 events were collected, and lymphocytes were gated on the basis of forward and side scatter properties. Data were analyzed by using Expo32 Analysis software (Beckman Coulter). To ensure flow cytometric standardization, the voltage settings were updated daily by using FlowSet calibration beads (Beckman Coulter). All antibodies were titrated before use, and staining intensity data were controlled on a weekly basis.

For the immunocytochemical analyses, 50 μ L of whole blood was incubated for 20 minutes at room temperature with an antibody cocktail containing monoclonal CD98-FITC (clone 44D7) from Serotec (Oxford, United Kingdom) and CD69-ECD (clone TP1.55.3) and CD4-PCy5 (clone 13B8.2) from Beckman Coulter. Erythrocytes were lysed by mixing with 600 μ L of 0.1 (v/v) formic acid for 3 to 4 seconds, and the ionic strength was rendered isotonic by the addition of 280 μ L of 51 mM Na_2CO_3 , 0.20 mol/L Na_2SO_4 , and 0.22 mol/L NaCl. Cells were thereafter washed in isoflow (Beckman Coulter) and resuspended in 1% formaldehyde (in isoflow) prior to analysis.

Cell isolation and cell culturing

Freshly drawn peripheral blood was diluted 1:1 in PBS, and PBMCs were isolated by density-gradient centrifugation by using Ficoll-Paque (Amersham Bioscience, Uppsala, Sweden). The lymphocyte-enriched interphase fraction was collected and centrifuged, and the pellet was resuspended in complete medium consisting of RPMI 1640 (PAA, Pasching, Austria) supplemented with 0.3 g/L of L-glutamine, 10% FBS (PAN, Aidenbach, Germany), and 100 U/mL of penicillin and 100 μ g/mL of streptomycin (Invitrogen, Carlsbad, Calif). Freshly isolated cells were thereafter cultured (1×10^6 cells/mL) at 37°C in 5% CO_2 in complete medium with different concentrations of Aquagen (0, 10, 100, 1,000, or 10,000 SQ-U; ALK-Abelló). In all experiments, phorbol 12-myristate 13-acetate (0.1 ng/mL) and ionomycin (0.5 μ g/mL) were used as positive controls.

Proliferation and cytokine measurements

Allergen-specific proliferation of PBMCs cultured in triplicates with various concentrations of allergen was quantified after 144 hours by using an 18-hour pulse period. Incorporated radioactivity was determined in a Wallac 1450 MicroBeta Liquid Scintillator Counter (Perkin Elmer, Waltham, Mass).

Supernatants of allergen-stimulated PBMCs were assessed for IL-10 secretion (detection limit 3.9 pg/mL) by using ELISA plates from R&D Systems.

Statistical analyses

Statistical analysis was performed by using Graphpad Prism 5.01 (San Diego, Calif). Distribution of data was assessed by using D'Agostino and Pearson omnibus normality test. Parametric paired data were analyzed with paired *t* test, whereas nonparametric data were analyzed with Wilcoxon matched pairs signed 2-sided rank test. For unpaired observations, unpaired *t* test or Mann-Whitney test was used and Fisher exact test was used for evaluation of the number of patients with improved symptoms upon allergen challenge after the end of treatment. Data are presented as individual values, where mean is represented by a horizontal line or as mean $\pm$ SEM. *n* equals the number of independent donors. A *P* value of .05 or less was considered statistically significant.

RESULTS

The pilot study

As a first evaluation of the safety and technical aspects of ultrasound-guided intralymphatic injections, 6 patients were recruited to receive active treatment in an open pilot study. These patients were evaluated before treatment (autumn 2009), 4 weeks after treatment (before the start of the pollen season, spring 2010), and after the upcoming pollen season (autumn 2010). Seven patients who had received conventional SCIT for 3 years were used for open comparison. The superficial inguinal lymph nodes were relatively easy to locate with the help of ultrasound guidance. All patients tolerated the ILIT treatment well, and no acute or late reactions were observed. Among the patients receiving conventional SCIT, 5 of 7 exhibited mild local reactions (redness/itch/edema) in proximity to the administration site. Some of them also experienced tiredness the evening after the injection. This was not reported after ILIT.

Both groups reported that the injections (intralymphatic or subcutaneous) were virtually painless, ranked as 0.65 ± 0.4 for ILIT injections and 1.15 ± 0.5 for SCIT injections on a scale where 0 represented no pain and 10 the worst experienced pain. The allergen-specific IgE levels in sera showed a tendency toward reduction 3 months after the completion of the treatment (before 23.86 ± 8.50 kU/mL vs after 11.03 ± 8.86 kU/mL; *P* = .06). This was similar to what was seen for the SCIT patients after 3 years of treatment (43.26 ± 12.42 kU/mL vs 25.76 ± 9.59 kU/mL; *P* = .03). Both groups reported improvement in symptoms during the first season after the end of the treatment. The ILIT group estimated its improvement to 5.5 ± 1.40 (*P* = .05 over baseline) on a scale ranging from 0 to 10, where 0 stands for "no improvement" and 10 stands for "complete recovery of allergic symptoms." The SCIT patients estimated their improvement to 6.1 ± 0.30 (*P* = .03). In addition, half of

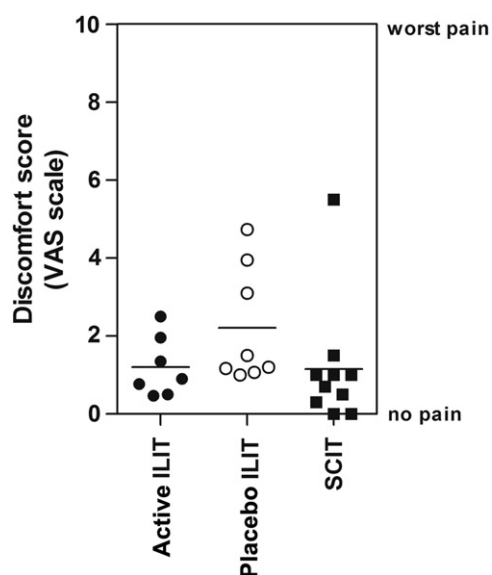


FIG 2. Patient-recorded discomfort associated with intralymphatic and subcutaneous allergen administration. After the injections, patients were asked to estimate the discomfort by using a visual analog scale ranging from 0 (no pain) to 10 (worst experienced pains). VAS, Visual analog scale.

the ILIT patients reported that they used less rescue medication as a result of the treatment. Patients given ILIT were also evaluated by nasal allergen provocation. After treatment, the allergic symptoms following the challenge were reduced by 23% after 5 minutes, 47% after 15 minutes, and 59% after 30 minutes.

The placebo study

As a second step, 15 new patients were recruited (during winter 2010-2011) and randomized to receive either placebo (8 patients) or active (7 patients) ILIT (Table I). Both ILIT and SCIT injections were found to be associated with mild to moderate discomfort (active ILIT, $P = .01$ over baseline; placebo, $P = .007$ over baseline; and SCIT, $P = .01$ over baseline) (Fig 2). The inguinal allergen administration was generally reported to be less painful than a venous puncture.

Safety assessment of intralymphatic allergen administration

To ensure early detection of respiratory adverse reactions, peak expiratory flow was measured before and after each injection. No evidence of treatment-associated asthma was observed (data not shown). One of the disadvantages of conventional SCIT is the risk of adverse allergic reactions,^{6,7} and therefore all treatment-induced side effects were recorded throughout the study. Two patients receiving active ILIT experienced itch during the first hour in proximity to the administrations site (after 1 of the 3 injections given). In the placebo group, 1 patient reported itch at the injection site along with mild nasal symptoms. Another patient in the placebo group sneezed, and a third exhibited local lymph node swelling. Over the next 24 hours, late therapy-induced reactions were almost exclusively reported by the study subjects receiving active treatment (5 patients receiving active treatment and 1 patient obtaining placebo treatment) (Table II). None of the late symptoms required treatment, and all patients were able to complete the entire immunization protocol as originally planned.

TABLE II. Adverse events associated with the intralymphatic injections

	Active ILIT (21 injections)		Placebo ILIT (24 injections)	
	Early reactions	Late reactions	Early reactions	Late reactions
Local reactions				
Lymph node swelling	Mild: 0 Moderate: 0 Severe: 0	Mild: 3 Moderate: 0 Severe: 0	Mild: 1 Moderate: 0 Severe: 0	Mild: 1 Moderate: 0 Severe: 0
Itch	Mild: 2 Moderate: 0 Severe: 0	Mild: 5 Moderate: 0 Severe: 0	Mild: 2 Moderate: 0 Severe: 0	Mild: 1 Moderate: 0 Severe: 0
Redness	Mild: 0 Moderate: 0 Severe: 0	Mild: 5 Moderate: 0 Severe: 0	Mild: 0 Moderate: 0 Severe: 0	Mild: 0 Moderate: 0 Severe: 0
Systemic reactions				
Nasal symptoms	Mild: 0 Moderate: 0 Severe: 0	Mild: 1 Moderate: 0 Severe: 0	Mild: 4 Moderate: 0 Severe: 0	Mild: 2 Moderate: 0 Severe: 0
Pulmonary symptoms	Mild: 0 Moderate: 0 Severe: 0	Mild: 0 Moderate: 0 Severe: 0	Mild: 0 Moderate: 0 Severe: 0	Mild: 0 Moderate: 0 Severe: 0
Urticaria and angioedema	Mild: 0 Moderate: 0 Severe: 0	Mild: 0 Moderate: 0 Severe: 0	Mild: 0 Moderate: 0 Severe: 0	Mild: 0 Moderate: 0 Severe: 0
Abdominal symptoms	Mild: 0 Moderate: 0 Severe: 0	Mild: 0 Moderate: 0 Severe: 0	Mild: 0 Moderate: 0 Severe: 0	Mild: 0 Moderate: 0 Severe: 0

Patient-recorded treatment outcome

After the pollen season of 2011, study subjects were asked to compare their seasonal allergic symptoms with the symptoms they experienced during the pollen season 2010 (before the start of treatment). A clear improvement over baseline was seen in the active ($P = .03$) but not in the placebo group ($P = .125$), and when the 2 groups were compared with each other, the outcome in the active group was markedly better than that in the placebo group ($P = .05$) (Fig 3).

Total leukocyte count, cell distribution, and serum Ig levels

To detect any leukocytic changes induced by the therapy, the total and differential leukocyte counts were determined for each patient pre- and posttreatment. The number of leukocytes, eosinophils, neutrophils, basophils, monocytes, and lymphocytes did not change during the course of the treatment (data not shown). The levels of allergen-specific IgE and IgG₄ were also assessed as these parameters have been suggested to be hallmarks of SCIT. After the end of treatment, a rise in allergen-specific serum IgE levels was observed in the active group ($P = .03$) (Fig 4) whereas the levels of allergen-specific IgG₄ remained unchanged (before active treatment, 0.38 ± 0.55 mg/L vs after active treatment, 0.41 ± 0.68 mg/L; $P = .81$). Analysis of the changes (post-pre) in IgE levels revealed no significant difference between the 2 groups ($P = .46$).

Nasal reactivity upon allergen challenge

One of the key issues of this study was to evaluate the effects of ILIT on allergen tolerance after intranasal provocation.

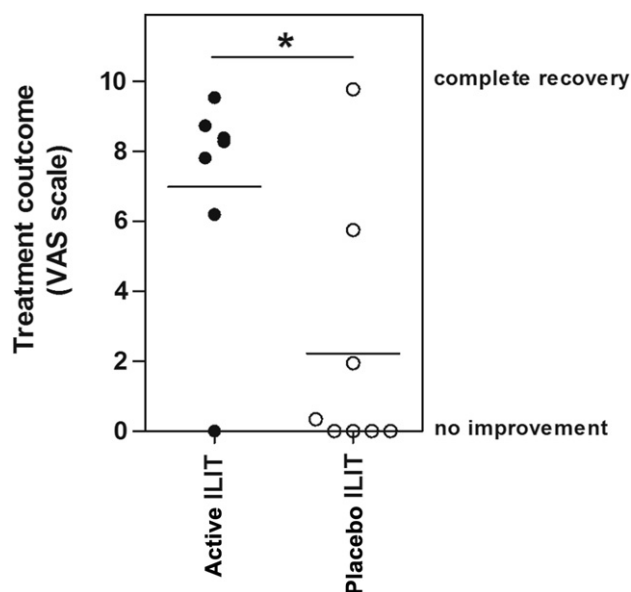


FIG 3. Patient-reported treatment outcome. As compared with the pollen season preceding intralymphatic treatment, the patients were asked to rate the change in their allergic symptoms on a scale ranging from 0 (no improvement) to 10 (complete recovery). * $P \geq .05$. VAS, Visual analog scale.

Therefore, nasal symptoms were assessed after allergen challenge. In patients who had received active injections, the allergic symptoms declined by 12% after 5 minutes, 23% after 15 minutes, and 44% after 30 minutes whereas the corresponding changes in the placebo group were 0.5%, 15%, and 0%, respectively (Fig 5). Five of 7 actively treated patients showed reduced allergic symptoms upon challenge after finishing treatment, whereas this number was 2 of 8 in the placebo group ($P = .13$). When the changes (post-pre) between the 2 groups were compared with each other, a trend toward a difference was observed 30 minutes after allergen challenge ($P = .12$, data not shown).

Nasal inflammatory response

To further explore the effects of ILIT on the inflammatory response of the nose, the total number of live leukocytes and epithelial cells along with levels of IL-8 in NAL fluids were assessed. After the end of treatment, a clear reduction in the number of living leukocytes and epithelial cells in NAL fluids was seen in the active group ($P = .01$) (Fig 6, A). A tendency toward a reduction in the IL-8 level was also observed ($P = .07$) (Fig 6, B). No such changes were seen in the placebo group ($P = .87$ and $P = .41$, respectively). Comparison of treatment effect over placebo (post-pre) showed a significantly lower number of live cells in NAL fluids in actively treated patients over placebo ($P = .01$, data not shown) along with a trend toward downregulated levels of IL-8 ($P = .18$, data not shown).

Activation of CD4⁺ T lymphocytes

Last, the effect of ILIT on peripheral T-cell activation was investigated by means of flow cytometry. Four weeks after completion, actively treated patients showed upregulated expressions of CD69 (mean fluorescence intensity [MFI], 39.73 ± 1.98 vs 43.77 ± 0.88 ; $P = .02$) and CD98 (MFI, 33.73 ± 2.83 vs

40.83 ± 0.98 ; $P = .04$) on CD4⁺ lymphocytes. This increase was not seen in patients on placebo treatment (MFI, 42.99 ± 1.03 vs 36.06 ± 1.81 , $P = .0078$, for CD69 and MFI, 36.5 ± 1.59 vs 30.19 ± 1.93 , $P = .07$ for CD98). The difference was even more emphasized when the outcomes (post-pre) of the 2 groups were compared ($P = .001$ for CD69 and $P = .009$ for CD98) (Fig 7). No effects were found on the percentage of CD4⁺ cells and neither PBMC proliferation nor IL-10 secretion was significantly affected by the treatment (data not shown).

DISCUSSION

Allergic rhinitis is a highly prevalent disease in industrialized countries and causes a considerable economic burden.^{25,26} The efficacy of allergen-specific immunotherapy for the treatment of respiratory allergies has been confirmed in numerous clinical studies in which extracts for a wide array of allergens including birch, grass, and ragweed pollen have been subcutaneously administered.²⁷ Important disadvantages of SCIT include long-term commitment of the patients and the risk of systemic adverse reactions in conjunction with allergen administration. Although it is preferable over symptomatic treatment, only a small percentage of allergic patients undergo SCIT.²⁸ In this study, we have evaluated the effects of intralymphatic allergen administration and presented results suggest ILIT to be a safe and effective strategy to treat patients with pollen-induced allergic rhinitis. Data show that the intralymphatic injections were not associated with any major discomfort, and only minor local reactions were observed after allergen administration. Actively treated patients displayed a rise in allergen-specific IgE level, an improvement in nasal allergic symptoms upon challenge, and lowered levels of inflammatory cells and IL-8 in the nose. In addition, a self-recorded treatment outcome showed an improvement in seasonal allergic symptoms after treatment.

In the inguinal regions, lymph nodes are readily detected because of their superficial localization and intralymphatic injections are easily given under ultrasound guidance. It has been noted that the sensory innervations of the lymph nodes are sparse²⁹ and in line with this, the study subjects reported that the injections were not associated with any major discomfort. The estimated pain of inguinal injections was similar to what was seen for subcutaneous injections. All patients tolerated the treatment well, and none suffered from any severe or systemic adverse events. For conventional SCIT using aeroallergens, it has been reported that up to 30% of the patients suffer from systemic reactions after allergen administration.³⁰ Since only 3 injections and a 100-fold lower accumulated allergen dose were given intralymphatically in this study, this seems to be a likely explanation why the subjects experienced only mild local reactions. However, since alum-adsorbed vaccines can be retained locally at the injection site, long-term evaluation is needed to rule out the possibility of side effects due to local chronic inflammation.

Allergic rhinitis is known to markedly affect the quality of life,^{2,25} and therefore the patient-experienced treatment outcome was studied. Six of the 7 patients given active intralymphatic injections reported a marked improvement in their allergic disease during the pollen season after as compared with before treatment, which was significantly different from the patients receiving placebo. It is important to notice that this is based on the patient's recall of the pollen season before the start of treatment. Seasonal fluctuations and recall bias must therefore be taken

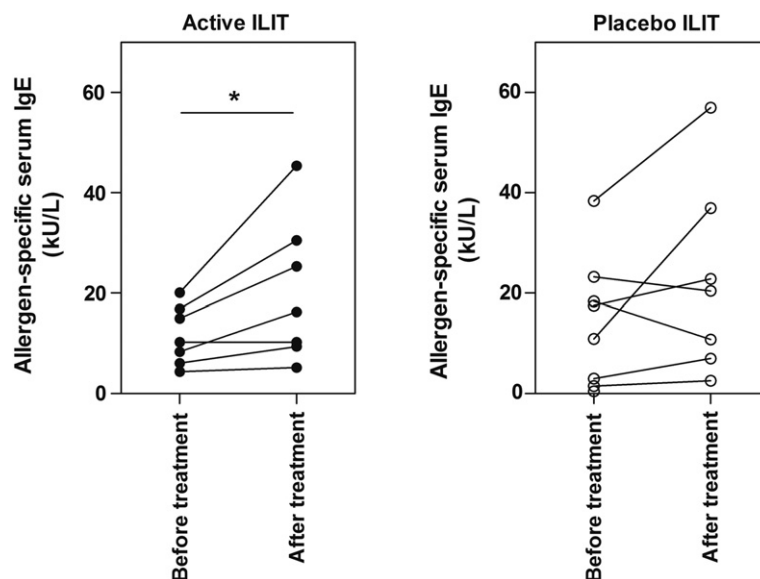


FIG 4. Intralymphatic allergen-specific immunotherapy promotes a rise in allergen-specific serum IgE levels. Before and after treatment, serum levels of birch or timothy grass-specific IgE (kU/L) were analyzed. * $P \geq .05$.

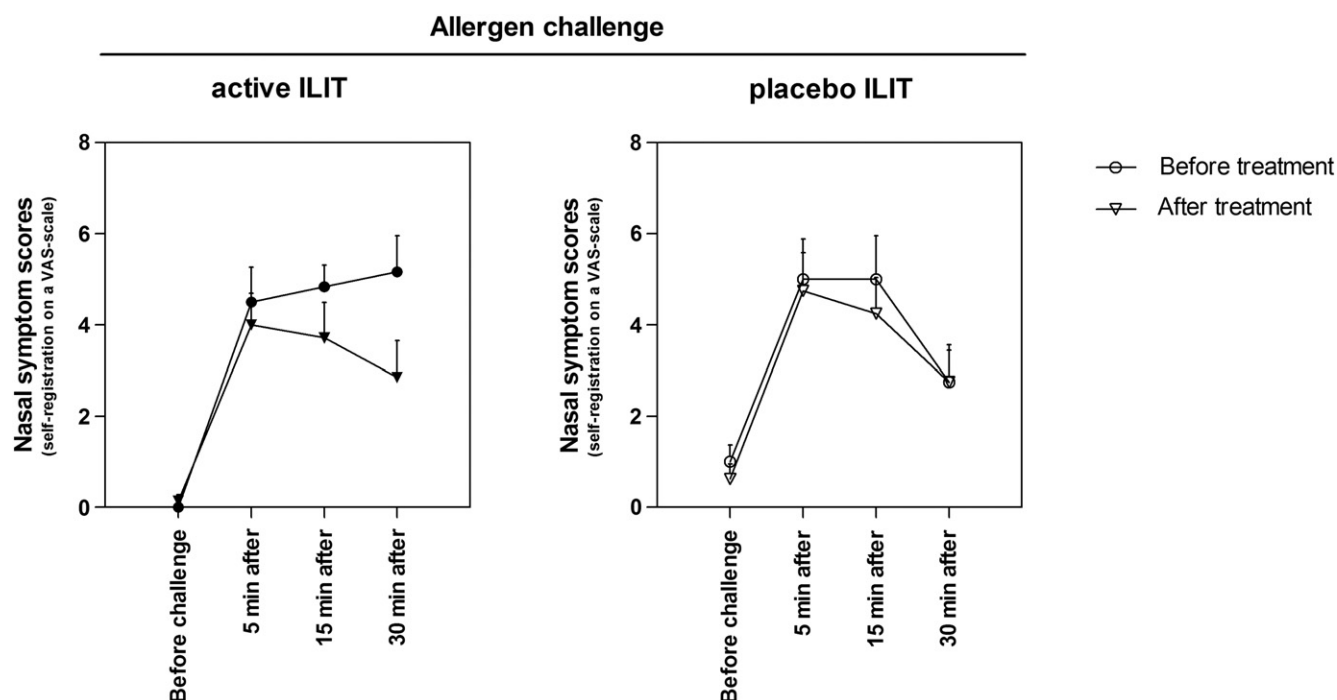


FIG 5. Intralymphatic allergen-specific immunotherapy induces tolerance against the administered allergen. Before and after treatment, patients were intranasally challenged with allergen. During the next consecutive 30 minutes, the patients were asked to score their nasal symptoms by using a 4-step scale ranging from 0 to 3 (0 = no; 1 = mild; 2 = moderate; 3 = severe symptoms). VAS, Visual analog scale.

into account. Nevertheless, the success rate of pollen ILIT appears to be comparable to that of SCIT, which has been reported to be 70% to 80%.¹⁹

A hallmark of successful SCIT is a long-term reduction in allergen-specific IgE level in favor of IgG (especially IgG₄).³¹⁻³³

We observed an induction of allergen-specific serum IgE and no effects on IgG₄ production as the result of treatment. This finding is consistent with the first report on intralymphatic immunization of grass-pollen allergic patients, which showed decreased IgE levels at the 3-year follow-up.¹⁸ Several studies have during the

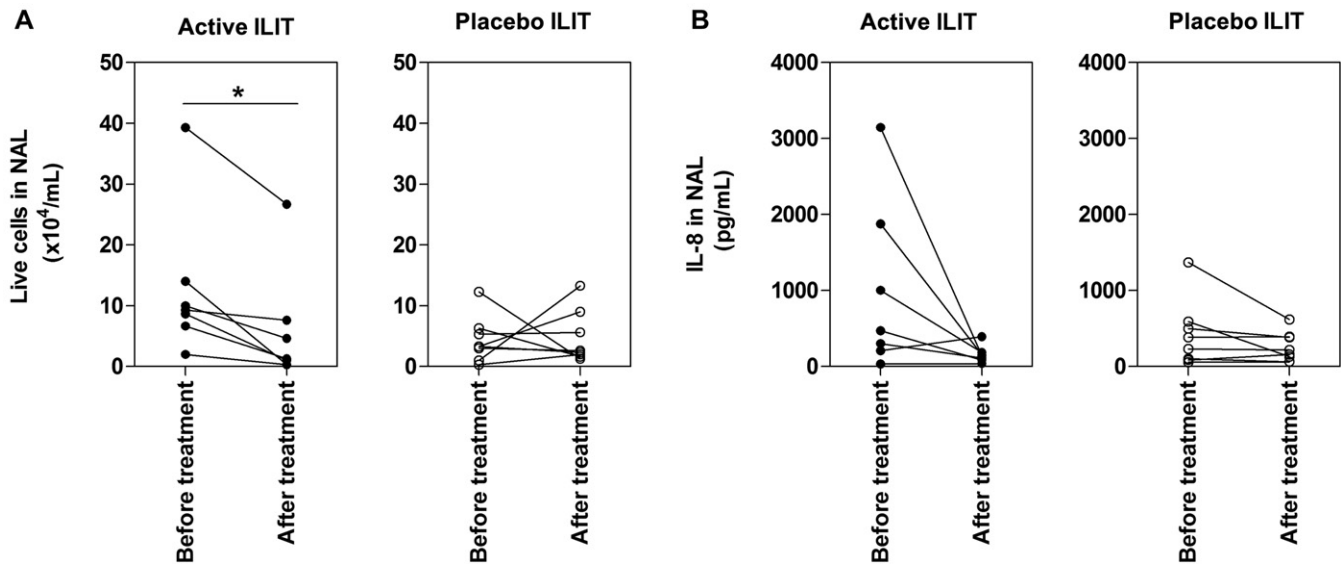


FIG 6. Intralymphatic allergen-specific immunotherapy reduces the inflammatory response of the nose. Before and after treatment, the number of live leukocytes and epithelial cells (**A**) along with levels of IL-8 in NAL fluids (**B**) were assessed by using ELISA. * $P \geq .05$.

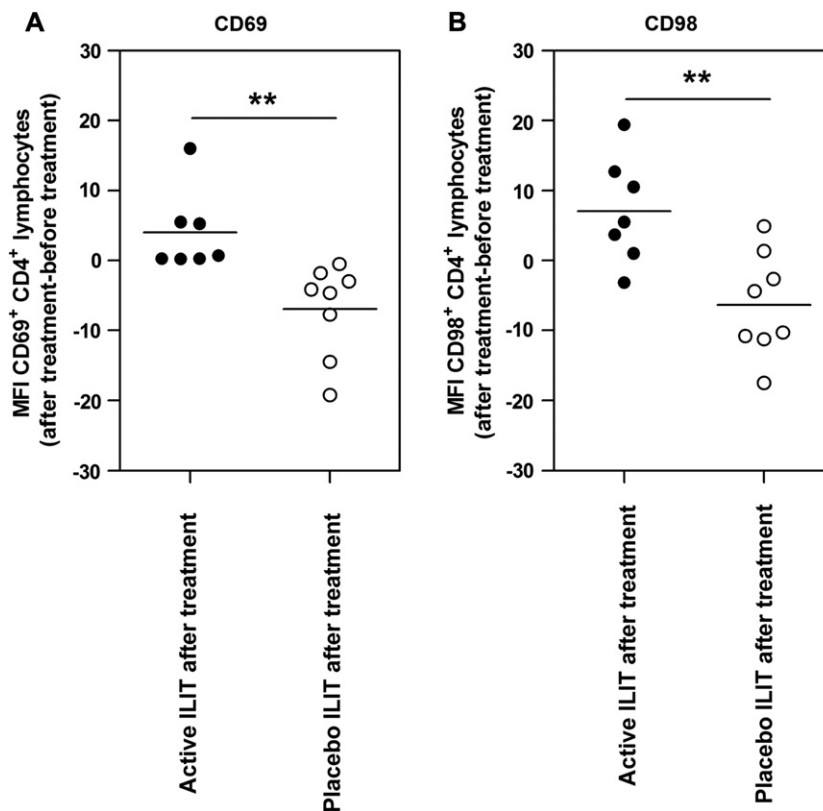


FIG 7. Increased activation of circulating CD4⁺ lymphocytes. Before and 4 weeks after the end of treatment, peripheral blood was collected and analyzed for the expression of CD69 (**A**) and CD98 (**B**) on CD4⁺ T cells by using flow cytometry. Results are presented as post-pre values of MFI of positive cells after versus before treatment. ** $P \geq .01$.

course of SCIT demonstrated an initial rise in the IgE level followed by a slow decline.^{34,35} In line with a previous SCIT study from our laboratory,³⁶ no changes in the total number of blood leukocytes or in leukocyte subset compositions were detected.

Even though we, 4 weeks after the end of treatment, did not observe an induction of serum IgG₄, we found that the actively treated group, over a 30-minute period after an allergen challenge, exhibited a 44% reduction in symptoms reported compared with

the challenge outcome before treatment. This reduction did not reach statistical significance, but it is important to keep in mind that improvements exceeding 30% have been suggested to be clinically relevant for allergy vaccination during real-life conditions, even though statistical significance cannot be reached.^{23,37} The limited numbers of patients included in the study along with interpersonal variations of treatment outcome likely contribute to the lack of statistical significance. According to a power-calculation of these data, approximately 40 individuals need to be included in each group to detect statistical differences between active and placebo treatment upon allergen challenge. However, we find these results encouraging since the majority of actively treated patients showed reduced allergic symptoms upon challenge after the end of treatment.

Upon allergen exposure, neutrophils are recruited to the nasal cavity where they constitute important players in maintaining the so-called minimal persistent inflammation seen among allergic patients.³⁸ We have previously demonstrated that patients with allergic rhinitis display higher NAL fluid cytokine levels and leukocyte numbers than do healthy controls.²⁴ To strengthen the finding of reduced nasal inflammation as the result of ILIT, the number of living cells along with IL-8, the most abundant inflammatory cytokine in the nose, was determined in NAL fluids. In accordance, reduced levels of living cells and IL-8 were observed in the active group after treatment, perhaps reflecting a decline in local nasal inflammation and possibly a skewing toward a more nonallergic, noninflammatory profile.

Although the beneficial effects of SCIT are well documented, it is still a matter of controversy which of the underlying immunological mechanisms are crucial for symptom amelioration.³⁹ We have previously demonstrated that SCIT induces several phenotypic changes in leukocytes including an increased activation of circulating T cells.³⁶ In line with these findings, we now show that active ILIT induces peripheral CD4⁺ T cells' expression of CD69 and CD98, which have been demonstrated to be markers of cellular activation and proliferation.⁴⁰⁻⁴² It has been suggested that an induction of IL-10 secretion by regulatory T cells and a reduced proliferation upon allergen stimulation are necessary for successful allergen-specific immunotherapy. However, other studies have failed in their attempts to reproduce this.²⁷ In the present study, we were not able to detect ILIT effects on proliferation or on IL-10 secretion. This stands in contrast to Senti et al¹⁷ and Martinez-Gomez et al¹¹ who have demonstrated IL-10 induction after the end of ILIT with recombinant cat dander allergen and bee venom allergen. One explanation for these discrepancies might include differences in the type of allergen extracts used for immunization.²⁷

The present study confirms that a series of 3 intralymphatic injections, with 4 weeks in between, might be given to patients with moderate to severe pollen-induced allergic rhinitis. The treatment reduces allergic symptoms upon challenge and during the pollen season, decreases nasal inflammatory responses, and enhances the activation of peripheral T lymphocytes. Together, this supports the idea of using the intralymphatic route to further develop and improve allergen-specific immunotherapy.

In conclusion, ILIT might constitute a new paradigm in allergen-specific immunotherapy from an economical, societal, and health economic perspective. As compared with SCIT, ILIT requires fewer treatment visits, fewer injections, and considerably lower allergen doses. On the basis of current prices in Sweden, we estimate that the 4 treatment visits required for ILIT along with allergen expenses cost approximately 800 euros per patient and

allergen whereas a complete SCIT treatment with more than 50 visits over a 4-year period is estimated to cost approximately 9000 euros. Although more complex factors such as loss of income due to recurrent absence from work are not included in the estimations, the possible socioeconomic benefits of ILIT over SCIT stand clear.

We thank Ann Reutherborg, Josefine P. Riikonen, Anna Karin Bastos, and Eva Thylander for logistic support.

Clinical implications: In this study, intralymphatic allergen-specific immunotherapy is further highlighted as a safe and effective administration route for the treatment of patients with pollen-induced allergic rhinitis.

REFERENCES

- Kay AB. Allergy and allergic diseases: first of two parts. *N Engl J Med* 2001;344:30-7.
- Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). *Allergy* 2008;63:8-160.
- Frew AJ. 25. Immunotherapy of allergic disease. *J Allergy Clin Immunol* 2003;111:S712-9.
- Till SJ, Francis JN, Nouri-Aria K, Durham SR. Mechanisms of immunotherapy. *J Allergy Clin Immunol* 2004;113:1025-34; quiz 35.
- Larche M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. *Nat Rev Immunol* 2006;6:761-71.
- Bousquet J, Lockett R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *J Allergy Clin Immunol* 1998;102:558-62.
- Cox L, Nelson H, Lockett R, Calabria C, Chacko T, Finegold I, et al. Allergen immunotherapy: a practice parameter third update. *J Allergy Clin Immunol* 2011;127:S1-S55.
- Frew AJ. Allergen immunotherapy. *J Allergy Clin Immunol* 2010;125:S306-13.
- Holgate ST, Polosa R. Treatment strategies for allergy and asthma. *Nat Rev Immunol* 2008;8:218-30.
- Senti G, Johansen P, Kundig TM. Intralymphatic immunotherapy. *Curr Opin Allergy Clin Immunol* 2009;9:537-43.
- Martinez-Gomez JM, Johansen P, Erdmann I, Senti G, Cramer R, Kundig TM. Intralymphatic injections as a new administration route for allergen-specific immunotherapy. *Int Arch Allergy Immunol* 2009;150:59-65.
- Zinkernagel RM, Ehl S, Aichele P, Oehen S, Kundig T, Hengartner H. Antigen localisation regulates immune responses in a dose- and time-dependent fashion: a geographical view of immune reactivity. *Immunol Rev* 1997;156:199-209.
- Zinkernagel RM. Localization dose and time of antigens determine immune reactivity. *Semin Immunol* 2000;12:163-71; discussion 257-344.
- von Beust BR, Johansen P, Smith KA, Bot A, Stormi T, Kundig TM. Improving the therapeutic index of CpG oligodeoxynucleotides by intralymphatic administration. *Eur J Immunol* 2005;35:1869-76.
- Maloy KJ, Erdmann I, Basch V, Sierro S, Kramps TA, Zinkernagel RM, et al. Intralymphatic immunization enhances DNA vaccination. *Proc Natl Acad Sci U S A* 2001;98:3299-303.
- Johansen P, Haffner AC, Koch F, Zepter K, Erdmann I, Maloy K, et al. Direct intralymphatic injection of peptide vaccines enhances immunogenicity. *Eur J Immunol* 2005;35:568-74.
- Senti G, Cramer R, Kuster D, Johansen P, Martinez-Gomez JM, Graf N, et al. Intralymphatic immunotherapy for cat allergy induces tolerance after only 3 injections. *J Allergy Clin Immunol* 2012;129:1290-6.
- Senti G, Prinz Vavricka BM, Erdmann I, Diaz MI, Markus R, McCormack SJ, et al. Intralymphatic allergen administration renders specific immunotherapy faster and safer: a randomized controlled trial. *Proc Natl Acad Sci U S A* 2008;105:17908-12.
- Kundig TM. Immunotherapy concepts under investigation. *Allergy* 2011;66:60-2.
- Larenas-Linnemann D, Cox LS. European allergen extract units and potency: review of available information. *Ann Allergy Asthma Immunol* 2008;100:137-45.
- Ekman AK, Virtala R, Fransson M, Adner M, Benson M, Jansson L, et al. Systemic up-regulation of TLR4 causes lipopolysaccharide-induced augmentation of nasal cytokine release in allergic rhinitis. *Int Arch Allergy Immunol* 2012;159:6-14.

22. Fransson M, Benson M, Wennergren G, Cardell LO. A role for neutrophils in intermittent allergic rhinitis. *Acta Otolaryngol* 2004;124:616-20.
23. Pfaar O, Kleine-Tebbe J, Hormann K, Klimek L. Allergen-specific immunotherapy: which outcome measures are useful in monitoring clinical trials? *Immunol Allergy Clin North Am* 2011;31:289-309.x.
24. Mansson A, Bachar O, Adner M, Cardell LO. Nasal CpG oligodeoxynucleotide administration induces a local inflammatory response in nonallergic individuals. *Allergy* 2009;64:1292-300.
25. Hellgren J, Cervin A, Nordling S, Bergman A, Cardell LO. Allergic rhinitis and the common cold—high cost to society. *Allergy* 2010;65:776-83.
26. Casale TB. Status of immunotherapy: current and future. *J Allergy Clin Immunol* 2004;113:1036-9.
27. James LK, Durham SR. Update on mechanisms of allergen injection immunotherapy. *Clin Exp Allergy* 2008;38:1074-88.
28. Senna G, Ridolo E, Calderon M, Lombardi C, Canonica GW, Passalacqua G. Evidence of adherence to allergen-specific immunotherapy. *Curr Opin Allergy Clin Immunol* 2009;9:544-8.
29. Popper P, Mantyh CR, Vigna SR, Maggio JE, Mantyh PW. The localization of sensory nerve fibers and receptor binding sites for sensory neuropeptides in canine mesenteric lymph nodes. *Peptides* 1988;9:257-67.
30. Ragusa VF, Massolo A. Non-fatal systemic reactions to subcutaneous immunotherapy: a 20-year experience comparison of two 10-year periods. *Eur Ann Allergy Clin Immunol* 2004;36:52-5.
31. Kay AB. Allergy and allergic diseases: second of two parts. *N Engl J Med* 2001;344:109-13.
32. Shamji MH, Durham SR. Mechanisms of immunotherapy to aeroallergens. *Clin Exp Allergy* 2011;41:1235-46.
33. Eifan AO, Shamji MH, Durham SR. Long-term clinical and immunological effects of allergen immunotherapy. *Curr Opin Allergy Clin Immunol* 2011;11:586-93.
34. Lichtenstein LM, Ishizaka K, Norman PS, Sobotka AK, Hill BM. IgE antibody measurements in ragweed hay fever: relationship to clinical severity and the results of immunotherapy. *J Clin Invest* 1973;52:472-82.
35. Gleich GJ, Zimmermann EM, Henderson LL, Yunginger JW. Effect of immunotherapy on immunoglobulin E and immunoglobulin G antibodies to ragweed antigens: a six-year prospective study. *J Allergy Clin Immunol* 1982;70:261-71.
36. Mansson A, Bachar O, Adner M, Bjornsson S, Cardell LO. Leukocyte phenotype changes induced by specific immunotherapy in patients with birch allergy. *J Investig Allergol Clin Immunol* 2010;20:476-83.
37. Malling HJ. Immunotherapy as an effective tool in allergy treatment. *Allergy* 1998;53:461-72.
38. Storms WW. Minimal persistent inflammation, an emerging concept in the nature and treatment of allergic rhinitis: the possible role of leukotrienes. *Ann Allergy Asthma Immunol* 2003;91:131-40.
39. Cevit O, Kendirli SG, Yilmaz M, Altintas DU, Karakoc GB. Specific allergen immunotherapy: effect on immunologic markers and clinical parameters in asthmatic children. *J Investig Allergol Clin Immunol* 2007;17:286-91.
40. Dorfman DM, Shahsafaei A. CD69 expression correlates with expression of other markers of Th1 T cell differentiation in peripheral T cell lymphomas. *Hum Pathol* 2002;33:330-4.
41. Zola H. Markers of cell lineage, differentiation and activation. *J Biol Regul Homeost Agents* 2000;14:218-9.
42. Ziegler SF, Ramsdell F, Alderson MR. The activation antigen CD69. *Stem Cells* 1994;12:456-65.