

Grass pollen immunotherapy decreases the number of mast cells in the skin

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Summary

Background Allergen injection immunotherapy is effective for summer hay fever and reduces cutaneous sensitivity to grass pollen.

Objective We have addressed whether this effect of immunotherapy may be due to a decrease in mast cell numbers in the skin.

Methods Total mast cells and mast cell subtypes in the dermis were measured by dual immunocytochemistry in 40 adult patients who had received either 'active' grass pollen immunotherapy or placebo injections for 9 months in a double-blind clinical trial.

Results Clinical improvement in hay fever was accompanied by a greater than 10-fold reduction in the immediate cutaneous response to grass pollen ($P = 0.0002$) and a sevenfold decrease in mast cell numbers in the skin ($P = 0.0001$). The number of mast cells after immunotherapy correlated with the clinical response in terms of seasonal symptoms ($r = 0.61$, $P = 0.001$) and rescue medication use ($r = 0.75$, $P = 0.0001$). Specific double immunostaining showed that the majority of mast cells (greater than 60%) were tryptase/chymase-positive (MC_{TC}) and the remainder tryptase-only (MC_T) cells. Following immunotherapy both subtypes were equally reduced.

Conclusion One mechanism by which immunotherapy may act is to reduce mast cell numbers with a consequent reduction in immediate allergic sensitivity.

Keywords: immunocytochemistry, immunotherapy, mast cells, skin tests

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Introduction

Mast cells play a central role in allergic disease of the respiratory tract and skin. They can be subdivided on the basis of their granule content of the enzymes tryptase and chymase [1]. In general, mucosal surfaces have a preponderance of tryptase-only containing mast cells (MC_T) believed to be the equivalent of mucosal-type mast cells in rodents, whereas mast cells containing both tryptase and chymase (MC_{TC}) are more associated with connective tissues [2]. Allergen-induced immunoglobulin (Ig) E-dependent release of preformed mediators from mast

cells results in the immediate response, manifest in the skin as a weal and flare at 10–30 min [3]. More recently, pro-inflammatory cytokines including interleukin (IL) -4 and IL-5 have been shown to be present in human mast cells from various tissue sites [4–6] and could initiate the recruitment of cells including eosinophils which characterize the late response after allergen challenge [7]. These observations place mast cells at the centre of inflammatory responses to allergen, and focus attention upon factors which might influence mast cell numbers as well as mast cell sensitivity to allergen and mediator releasability.

Although immunotherapy treatment has been used since 1911, the mechanisms by which it exerts its beneficial effect remain unclear [8]. One way in which immunotherapy might act is by reducing mast cell numbers. A previous

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study found a decrease in metachromatic cells in nasal brushings following successful immunotherapy in mite-sensitive patients [9]. In the present study we have investigated cutaneous mast cell numbers and mast cell subtypes (MC_T and MC_{TC}) before and after grass pollen immunotherapy in patients with severe summer hay fever. Mast cell numbers and the mast cell subtypes (MC_{TC} and MC_T) were related to cutaneous allergen sensitivity and the clinical response to immunotherapy.

Materials and methods

Patients

Forty patients were recruited from the Allergy clinic at the Royal Brompton Hospital London or in response to an advertisement placed in a local newspaper. Their clinical features and response to immunotherapy have been reported previously [10]. Inclusion criteria included: (1) a history of severe summer hay fever (defined by symptoms for more than 10 weeks and poor response to anti-allergic drugs); and (2) a positive skin prick test (>5 mm) to grass pollen (*Phleum pratense*, Soluprick, ALK Abelló, Hørsholm, Denmark). Patients were excluded if they had: (1) a history of chronic perennial asthma; (2) a history of perennial allergic symptoms; (3) a histamine PC₂₀ < 2 mg/mL. Patients with a history of peak seasonal wheezing effectively controlled by occasional inhaled bronchodilators were allowed to participate, provided their baseline PC₂₀ met the above criterion. A further 11 immunotherapy-naïve hay fever patients (eight male, mean age 35 ± 3.2 years) were recruited in order to compare the number of mast cells in the skin of allergic rhinitic patients and normal nonallergic individuals (six male:six female, mean age 35 ± 2.7 years). The inclusion/exclusion criteria for these hay fever patients were identical to those used for the immunotherapy patients.

Study design

Patients were stratified into two groups according to disease severity (as judged by reported symptom levels) and randomized to receive twice-weekly injections with Alutard SQ (*Phleum pratense*, an aluminium adsorbed depot grass pollen extract) or matched placebo injections (allergen diluent containing histamine 0.01 mg/mL). Clinical improvement was documented [10] by diary cards recording symptoms and medication requirements for 11 weeks during the peak pollen season. Symptoms (nose, eye, throat and chest) were recorded daily on a 0–3 severity scale (0 = no symptoms, 1–3 = mild to severe symptoms) and rescue medication as one acrivastine 8 mg capsule (= 2), sodium cromoglycate eye drops or nasal spray (= 1) and one prednisolone 5 mg tablet (= 2). Prednisolone was prescribed

as a 7-day course, once daily in the morning as 30 mg, 30 mg, 25 mg, 20 mg, 15 mg, 10 mg, 5 mg, only if patients symptoms failed to respond to the above measures. A visual analogue score (scale 0–10) was completed every 2 weeks during the grass pollen season, in response to the question 'How has your hay fever been in the previous week?'. Patients received up-dosing treatment twice weekly over 6–8 weeks to a maintenance dose Alutard 100 000 SQ units, followed by monthly injections of maintenance 100 000 SQ units for a period of 9 months. Skin prick tests with *Phleum pratense* (Soluprick, ALK Abelló) were performed on the volar aspect of the forearm in duplicate from 100 to 100 000 BU/mL. Intradermal allergen challenge was performed on the extensor surface of the forearm using *Phleum pratense* 30 BU/mL in 0.02 mL allergen diluent and allergen diluent (0.02 mL) in the opposite arm. The immediate response was recorded at 15 min and the late response at 24 h (the time of biopsy). Skin tests were performed before and 9 months after starting treatment. A permanent record was obtained by transferring a ball point pen trace of the weal size by means of adhesive transparent tape to a permanent record sheet and measured as the mean diameter of the response measured at right angles (the mean of duplicate skin tests). The mean diameter of the early response (15 min) was plotted against log concentration *Phleum pratense*. Immediate allergen sensitivity was expressed as the provocation concentration of *Phleum pratense* (PC₆) which caused a 6-mm skin weal and was obtained by linear interpolation of the log dose response curve. Skin tests were performed and measured by the same individual before and after immunotherapy independent and blind to the treatment given to the patients.

Skin biopsies

Skin biopsies were obtained from control sites and at 24 h from the centre of the late-phase response after allergen for each patient using a 4-mm disposable punch. Local anaesthesia was obtained using 1% lignocaine and the wound was closed with a single stitch. Biopsies for immunohistology were immediately placed in embedding medium (O.C.T compound BDH Merck, Dagenham, UK) on a small piece of card and snap-frozen in isopentane precooled in liquid nitrogen and stored at –80 °C.

Immunohistology

Six micron sections were cut on a cryostat (Bright Instruments, Huntingdon, UK) and consecutive sections placed on microscope slides. The slides were air dried for 1 h, fixed in 50/50 acetone/methanol for 5 min and air dried for a further 1 h. The slides were wrapped in aluminium foil and stored at –20 °C. Immunostaining was performed as described [11]

and was briefly as follows: mast cells were identified with an alkaline phosphatase-conjugated murine antitryptase mAb (G3) and a biotinylated murine antichymase mAb (B7). Slides were defrosted, fixed in Carnoy's fluid (ethanol, 60 mL; chloroform, 30 mL; and glacial acetic acid, 10 mL) for 30 min, and washed in hydrogen peroxide/methanol (2 mL hydrogen peroxide 30% in 98 mL of methanol) for 30 min to block endogenous peroxidase. After three washes in 0.05% Tween/TBS, slides were incubated overnight at +4 °C with B7 (antichymase) Ab diluted in 20% NHS/TBS and used at 1:1000 dilution. After an additional three washes with Tween/TBS, the slides were incubated for 1 h with peroxidase-conjugated streptavidin (1:50 dilution in 20% NHS/TBS) and washed (three times) in Tween/TBS. Freshly made diaminobenzidine tetrahydrochloride (6 mg in 10 mL of TBS plus 5 µL of 30% hydrogen peroxide) was filtered onto the sections and incubated for 8 min, followed by two washes in Tween/TBS. For the detection of tryptase-positive cells, the sections were then incubated with alkaline phosphatase-conjugated murine antitryptase mAb overnight at +4 °C (1:1000 dilution in 20% NHS/TBS). After two additional washes, the standard alkaline phosphatase substrate with fast red was filtered directly onto the sections and incubated for 20 min. After an additional wash in buffer, followed by a wash in tap water, the slides were counter-stained with Gill's haematoxylin for 10 s. Slides were then mounted in Kaiser's medium after a final wash in water. Tonsillar tissue was used as a positive control for these Abs. Slides were coded and counted using an eye piece graticule using a BH-2 microscope (Olympus Corp, Lake Success, NY, USA) at ×100 magnification. Counts of positively stained cells were made in the dermis immediately beneath the dermo-epidermal junction, to a depth of 1.0 mm. The full length of the epidermis was assessed but areas containing sebaceous glands, hair shafts, etc. were disregarded. At least two sections from each biopsy were double stained. Results were expressed as cells/power field.

Mast cells numbers in the skin of untreated hay fever patients were compared with nonatopic controls using an antitryptase antibody, AA1 (Dakopatts, High Wycombe, UK) following the standard APAAP procedure [12].

Analysis

All counts were performed blind, independent of the clinical protocol. Within group comparisons (before vs after immunotherapy) were made using the Wilcoxon matched pairs signed-ranks test. Between group comparisons (immunotherapy vs placebo-treated patients) were made using the Mann-Whitney *U*-test. Correlation coefficients were obtained using Spearman's rank method. *P*-values <0.05 were considered significant. Immediate (15 min) skin

tests were analysed using Minitab software (Minitab Inc., University Park, PA, USA).

Results

Clinical effects of immunotherapy

Immunotherapy- and placebo-treated groups were well-matched for age: median (range) for active group 34 (19–51) years vs 37 (23–52) years for placebo group. Groups were also well matched for disease severity and duration. There was a small excess of males within the immunotherapy treated group (14/21 vs 8/19; *P* = NS). Clinical results have been reported previously [10]. Thirty-seven of the 40 patients (93%) completed the study. The three dropouts were all in the placebo-treated group. The actively treated group showed significant decreases in seasonal symptoms (125.5 vs 291.5; *P* < 0.001) rescue medication (128 vs 607; *P* < 0.002) and visual analogue scores (2.5 vs 5.5; *P* < 0.02) [10].

Immediate cutaneous allergen sensitivity

Before treatment patients were well matched for immediate (15 min) cutaneous sensitivity to grass pollen. Median values (± 95% confidence intervals [CIs]) for grass pollen PC₆ were 500 (CIs: 200, 950) and 550 (CIs: 180, 1240) BU/mL for immunotherapy and placebo-treated patients, respectively (*P* = not significant). Following 9 months' treatment the immunotherapy-treated group demonstrated a greater than 10-fold increase in allergen PC₆ (10 000 (confidence intervals 4,700, 55 300) BU/mL) (*P* = 0.0002). In contrast, for the placebo-treated group the allergen PC₆ (1000 [380, 1630] BU/mL) did not significantly change. The increase in allergen PC₆ (decrease in immediate allergen sensitivity) was highly significant for the immunotherapy-treated group when both groups were compared (*P* = 0.0002) (Fig. 1).

Skin mast cells and mast cell subtypes

The total number of mast cells and mast cell subtypes before treatment were virtually identical for both groups (Fig. 2). Following 9 months' immunotherapy a highly significant fivefold reduction in total mast cell numbers was observed only in the immunotherapy-treated group (*P* = 0.01). In contrast, total mast cell numbers for the placebo-treated group demonstrated no significant change. Before treatment the principle mast cell subtype in both groups was the MC_{TC} subtype (immunotherapy group MC_T:MC_{TC} 32:68 and placebo treated group MC_T:MC_{TC} 40:62). Following immunotherapy comparable reductions in both MC_{TC} and MC_T cells were observed (Fig. 2) leaving the ratio of

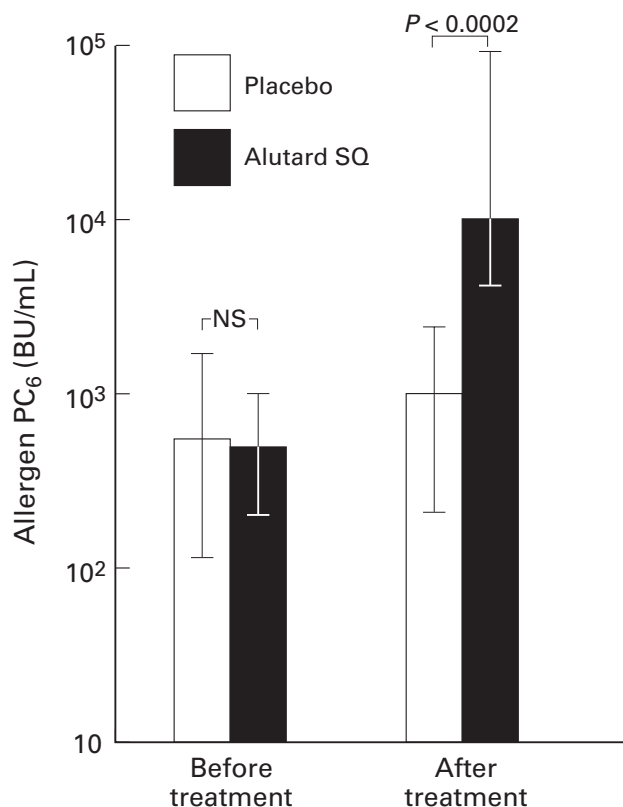


Fig. 1. Cutaneous grass pollen sensitivity, expressed as PC₆ (provocation concentration of allergen used for skin prick testing that caused a 6-mm skin weal at 15 min). Results for immunotherapy (Alutard SQ; $n = 20$) and placebo-treated ($n = 16$) groups are shown as median $\pm$ interquartile range

subtypes unchanged (immunotherapy group MC_T:MC_{TC} 33:67 and placebo-treated group MC_T:MC_{TC} 38:62).

In the biopsies obtained after intradermal allergen provocation, no significant changes from diluent biopsies were observed in either total mast cells or mast cell subtypes both within treatment groups or between treatment groups (data not shown).

Mast cell numbers in immunotherapy-naïve hay fever patients compared with normal skin of nonatopic individuals

The number of total mast cells/high power field present in the dermis was identical in both groups. Median (interquartile range [IQR]) atopic group: 11.2 (8.8, 14.9) and normal group: 11.4 (9.1, 14.6); $P = 1.0$.

Relationship to clinical response

Associations were sought between mast cell numbers and the clinical response to immunotherapy. No significant

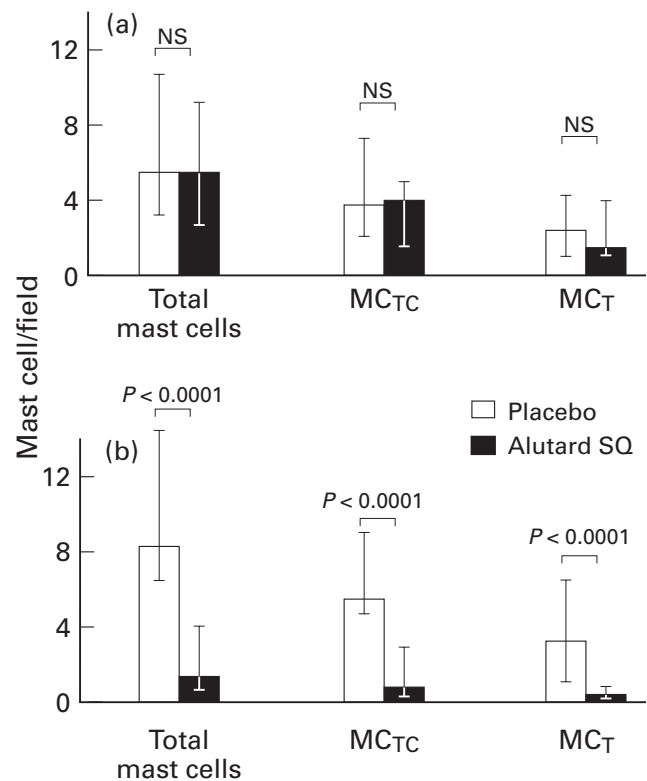


Fig. 2. Cutaneous mast cell numbers (a) before and (b) after 9 months' subcutaneous immunotherapy (Alutard SQ; $n = 20$) or placebo (histamine; $n = 16$) treatment. Total mast cells MC_{TC} (tryptase- plus chymase-containing, connective tissue-type mast cells) and MC_T (tryptase only-containing mast cells, mucosal-type) are expressed as median values $\pm$ interquartile ranges. Levels of statistical significance (P -values) refer to between group comparisons

correlation was observed between either total mast cells or mast cell subtypes and the decrease in immediate allergen sensitivity (allergen PC₆) for either group. However, the number of mast cells in the skin following immunotherapy correlated with the patients' clinical response during the pollen season expressed as either total diary symptom scores ($r = 0.61$, $P = 0.001$) or as rescue medication use ($r = 0.75$, $P = 0.0001$) (Fig. 3).

Discussion

We have shown that grass pollen immunotherapy which resulted in a reduction in seasonal symptoms and medication requirements was accompanied by a decrease in cutaneous immediate allergen sensitivity and a reduction in total mast cells and mast cell subtypes in the skin. Immunotherapy may act, at least in part, by reducing mast cell numbers with a consequent decrease in the immediate response to allergen exposure.

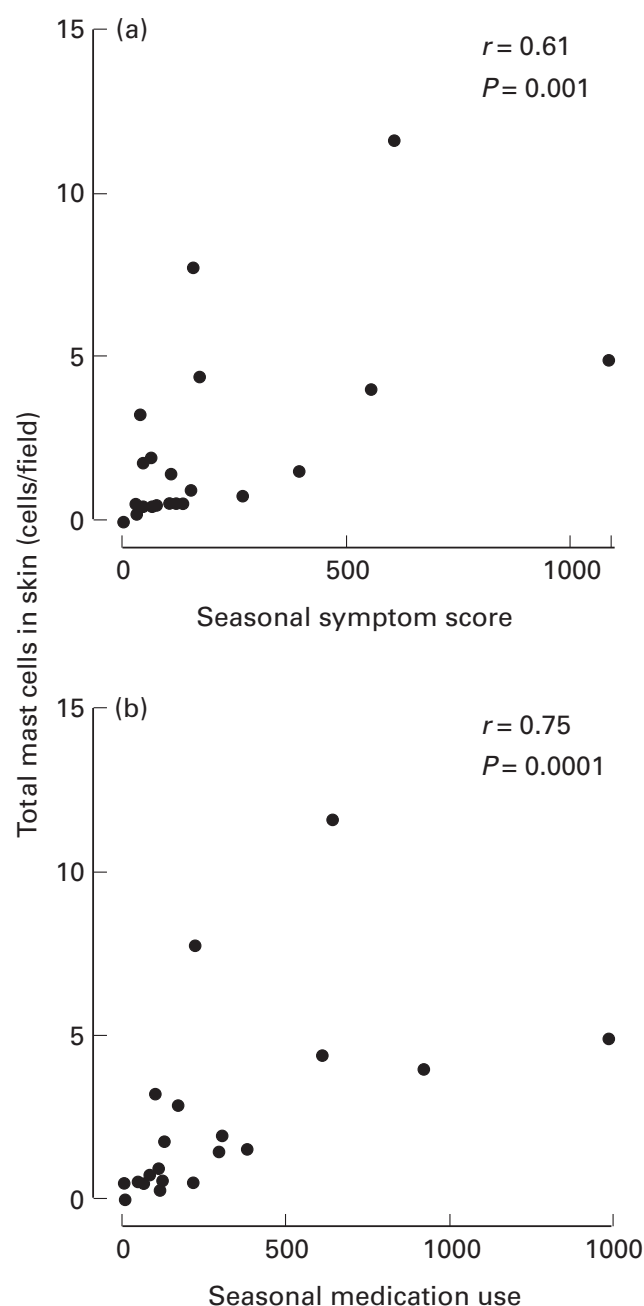


Fig. 3. Total mast cell numbers (cells/field) in the skin of patients who received immunotherapy (Alutard SQ) are compared with (a) seasonal hay fever symptoms (area under curve analysis of total diary symptom scores during the peak 11 week pollen season) and (b) use of rescue medication (antihistamine [acrivastine 8 mg] and topical sodium cromoglycate eye drops). Correlation coefficients obtained using Spearman's rank method

Measurement of skin prick tests and immunohistological studies on skin biopsies were performed blind and independent of the clinical protocol. Serum total IgE and specific IgE were unchanged before compared with after treatment although small transient increases for both groups were observed during the pollen season (data not shown). No significant correlation was observed between the decrease in mast cell numbers and skin sensitivity to allergen. However, the decrease in skin sensitivity may depend on other factors such as decreased mast cell sensitivity or releasability, as well as a decrease in mast cell numbers. Mast cell numbers were not affected by allergen challenge for either group of subjects. This is in contrast with our previous findings of allergen-induced increases in cutaneous CD4⁺ T cells and eosinophils in placebo-treated patients which were inhibited following immunotherapy [12]. Although MC_{TC} mast cells predominated (greater than 60% total mast cells) their proportion was not as high as that previously reported (88–99% in normal skin [1,13]). This difference may be explained either by the larger number of patients studied here, or more likely, the fact that we studied only atopic patients. The latter is supported by the observation that higher percentages of MC_T mast cells were found by the same investigators in skin biopsies from patients with atopic eczema [14]. The significant relation between mast cell numbers after immunotherapy and reduction in seasonal symptoms and medication requirements provides evidence that the decrease in mast cells may be 'protective' rather than occurring as a 'bystander' event.

Alternative mechanisms of immunotherapy have been proposed. For example, studies have identified changes in antibody levels with either a decrease in IgE or an increase in so-called 'blocking' IgG antibodies [15,16]. However, changes in serum antibodies have not in general correlated with the clinical response to immunotherapy. Other putative mechanisms include decreases in mediator release [17], and a reduction in tissue eosinophils [18] and eosinophil activation [19]. T lymphocyte subsets in peripheral blood have been shown to be altered, with an increase in CD8⁺ cells [20]. A characteristic feature of subcutaneous injection immunotherapy is its ability to inhibit late as well as early cutaneous responses after allergen provocation [10,21]. Our previous *in situ* hybridization studies of late cutaneous biopsies taken 24 h after allergen suggest that immunotherapy may induce a 'TH1 response' [12] with allergen-induced local increases in cells cytokine messenger RNA-positive for interferon gamma. A 'switch' from TH2 to TH1-T lymphocyte responses is also supported by measurements of cytokine concentrations in supernatants from cultures of peripheral blood T lymphocytes obtained from allergic patients before and after immunotherapy. These studies have revealed both a decrease in IL-4 [22] and an increase in interferon gamma [23] production after

immunotherapy. Rather than inducing TH1-type responses, it has been suggested that immunotherapy may act by inducing T-cell unresponsiveness, so-called 'T-cell anergy' [24,25].

The mechanism by which immunotherapy reduced cutaneous mast cell numbers in the present study is unknown. Mast cell growth and development is dependent on a number of cytokines [26]. Human stem cell factor (also called Steel factor, mast cell growth factor, or kit ligand) is a major growth factor in the differentiation of MC_T and, to a lesser extent, MC_{TC} human mast cells [27–29]. Other cofactors, including IL-3, IL-4 and IL-10 [30] and IL-13 [31], also appear to be required. In murine systems IL-9 and nerve growth factor induce and promote mast cell growth and differentiation [32,33]. There is some controversy regarding the role of IL-3 and IL-4 in the human system since one group has reported inhibition of stem cell factor-dependent mast cell formation by these cytokines [34]. Whatever the precise mechanism involved in the skin of atopic subjects the observed reduction in mast cell numbers following immunotherapy may be explained on the basis of downregulation of critical cytokine growth factors.

Mast cells produce a range of inflammatory mediators including histamine, tryptase and leukotrienes [1,35]. More recently both *in vitro* [36,37] and *in vivo* [4–6] mast cells have been identified as an alternative source of cytokines including IL-4 and IL-5. These cytokines have been localized to the intracytoplasmic granules. It is possible that immediate release of preformed cytokines from mast cells may augment the allergic response by stimulating local IgE synthesis (IL-4), recruiting and activating eosinophils (IL-5) and stimulating differentiation of TH2-type T lymphocytes (IL-4) for subsequent release of newly formed cytokines. It follows that reduction in mast cells following immunotherapy, in addition to inhibiting early responses, may remove an essential 'priming' mechanism for the development of late responses after allergen exposure. Further studies are required to determine the factors leading to decreased mast cell numbers after immunotherapy.

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