

Basophil Interleukin 4 and Interleukin 13 Production Is Suppressed during the Early Phase of Rush Immunotherapy

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Key Words

Rush immunotherapy · Basophil activation · CD203c expression · Histamine release

Abstract

Background: Studies using rush immunotherapy (RIT) have shown that rapid protection can be achieved using protocols allowing a fast increment of allergen dose. We examined the early effects of RIT on basophil numbers and expression of CD203c, production of interleukin (IL)-4 and IL-13 and histamine release by basophils in the peripheral blood of patients treated with immunotherapy and controls. **Methods:** Twelve patients treated with RIT and 4 untreated controls were included in the study. Any adverse events were evaluated during the incremental phase of RIT. Mononuclear cells were isolated before the start of RIT and 3 days, 1 week, 4 weeks and 3 months after the beginning of the treatment. Histamine release upon allergen stimulation, expression of CD203c and allergen-induced production of IL-4 and IL-13 by basophils were examined. **Results:** Significant decreases in blood basophil count ($p = 0.02$) were observed early in the treatment, returning to baseline values 1 week after the start of RIT. Similarly, histamine release decreased at day 3 ($p = 0.02$), but returned to pretreatment levels after 1 week. Also, the percentage of IL-4+ and IL-13+ basophils and levels of

CD203c expression were markedly reduced early in the treatment. IL-4 and IL-13 production correlated with histamine release and CD203c expression. Histamine release and production of IL-4 and IL-13 by basophils before the treatment correlated with the severity of adverse events during the incremental phase of RIT. **Conclusion:** We report the decrease in blood basophil numbers, their lower activation status and the reduced production of IL-4 and IL-13 early in the course of RIT. This early suppression of basophil activation could be one mechanism behind the protective effect of RIT.

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Introduction

Several placebo-controlled studies have found significant effects of specific immunotherapy on symptoms, use of medication and allergen-specific and nonspecific hyperresponsiveness in cat- and birch-allergic rhinoconjunctivitis and asthma [1–3].

Studies using ultra-rush and rush immunotherapy (RIT) schedules have shown that rapid protection may be achieved using protocols allowing a fast increment of allergen dose. However, the mechanisms underlying the early effects of RIT, occurring in the course of hours or days, are still poorly understood. Clearly, this short time

frame is insufficient to induce alterations in T-cell populations or changes related to T-cell cytokine profiles. Therefore, other cells such as mast cells and basophils, expressing high-affinity immunoglobulin E (IgE) receptors (FcεRI), may be the first line of response to the injected allergen. The participation of mucosal mast cells is difficult to investigate, but a study of RIT to bee venom indicated that the treatment does not induce rapid mediator depletion or surface receptor blockade in skin mast cells [4]. Rather, a significant reduction in basophil histamine and sulfidoleukotriene release was observed following ultra-rush immunotherapy [5].

Upon allergen stimulation, basophils secrete immunoreactive cytokines interleukin (IL)-4 and IL-13 promoting the differentiation of CD4⁺ cells towards the Th2 phenotype and enhancing eosinophil recruitment to the bronchial mucosa via upregulation of adhesion receptors on endothelial cells [6]. In addition, a previous study found that cultured basophils but not mast cells were able to induce IgE synthesis from B cells after antigenic stimulation [7]. The present study examined the early effects of ultra-rush immunotherapy on the number of basophils, expression of the activation marker CD203c on their surface, as well as the kinetics of IL-4 and IL-13 production and histamine release from basophils of immunotherapy-treated cat- and birch-allergic patients and untreated controls.

Methods

Patients

Fourteen allergic subjects with rhinoconjunctivitis, with or without asthma triggered upon cat allergen exposure, and 2 subjects with a history of rhinoconjunctivitis during the birch pollen season participated in the study. Ten cat-allergic subjects received RIT with cat extract and the 2 birch-allergic subjects received RIT with birch extract (Alutardd ALK, Hørsholm, Denmark); subjects included 3 females and 9 males, with a mean age of 30 years (range 22–37). Those treated with immunotherapy (RIT group) had a positive skin prick test (SPT) for the relevant allergen (median 5.7 mm, range 4.5–8.5) and elevated plasma levels of specific IgE (median 9.7 kU/l, range 0.6–53).

Four cat-allergic subjects served as a control group, including 2 females and 2 males, with a mean age of 36 years (range 29–44), with positive SPT for cat allergen (median 7.2 mm, range 5–12) and elevated specific IgE (median 7.1 kU/l, range 0.6–12.6).

None of the subjects had received specific immunotherapy within the last 15 years. Cat-allergic patients refrained from contacts with cats.

The study was performed outside the birch pollen season. Depending on the severity of the side effects during RIT, the following medication was given: antihistamine (acrivastin capsule, Semprex®), short acting β₂-agonists (salbutamol, Ventoline Dis-

kus®), steroids (betametasolone, Betapred™) and adrenaline. If oral steroids had to be used, blood sampling was omitted.

Study Design

The following treatment regime was applied in the RIT group: day 1 – 10, 100, 1,000 and 5,000 standardized quality units (SQ); day 2 – 10,000 and 20,000 SQ; day 3 – 40,000 and 65,000 SQ; day 5 – 100,000 SQ. The maintenance dose (100,000 SQ) was repeated on days 9, 14 and 23 and 4 weeks from the start of treatment. Thereafter, injections of 100,000 SQ were given every 6 weeks. Blood sampling was performed before RIT, as well as 3 days, 1 week, 4 weeks and 3 months after the start of treatment. Samples were always taken before the injection of allergen. Subjects included in the control group did not receive any treatment. Blood samples were obtained at the same intervals as for the RIT group.

Side Effect Score

The side effects of immunotherapy were evaluated during the incremental phase of RIT. Adverse events were classified as local, generalized or systemic according to the following scale: 0 – no symptoms; 1 – mild, transient symptoms from eyes and nose, local skin reaction <10 cm, no use of medication required; 2 – marked symptoms from eyes, nose and/or lower airways, local skin reaction >10 cm and/or local urticaria, medications include antihistamines and short-acting β₂-agonists; 3 – marked symptoms from eyes, nose and/or lower airways, general skin urticaria and/or angioedema, medications include adrenaline and oral steroids; 4 – severe reaction, anaphylactic reaction, adrenaline administered.

Skin Prick Test

SPT was performed on the volar side of the forearms before the start of RIT and during consecutive visits, before the injection of allergen. The cat or birch extract, histamine solution (10 mg/ml) as a positive control and diluent were applied in duplicate. The wheal size was measured 15 min after allergen challenge and expressed as the average of the two largest diameters, according to the formula: (d1 + d2)/2. A wheal reaction of ≥5 mm in diameter was considered positive.

Differential Blood Cell Count

Differential blood cell counts were performed using a Cell-Dyn 4000 analyzer (Abbot Diagnostics, St. Clara, Calif., USA).

Blood Sampling and Isolation of Mononuclear Cell Fraction

Heparinized blood diluted 1:1 with phosphate-buffered saline (PBS) was applied to isotonic Percoll (density 1,084 g/ml; Amersham Pharmacia Biotech AB, Uppsala, Sweden). After centrifugation for 30 min at 1,000 g, the cell fraction containing mononuclear leukocytes and basophils was carefully removed from the interface for surface staining and cell culture.

Histamine Release

Isolated mononuclear cells (MNC) were resuspended in histamine release buffer, i.e. PBS buffer with 1 mM CaCl₂, 1 mM MgCl₂, 0.1% glucose and 0.1% (w/v) BSA (Sigma-Aldrich Sweden AB, Stockholm, Sweden), at a concentration of 1×10^6 cells/ml. The cells were held for 15 min at 37°C before the addition of allergen at a concentration of 200 ng/ml or an equal volume of buffer. Stimulation was performed using cat or birch allergen (2 µg/ml

Fel d 1 or Bet v 1, respectively; Alk-Abelló, Hørsholm, Denmark). After 60 min at 37°C, cell suspensions were centrifuged for 7 min at 600 g (4°C) and the supernatant frozen at -20°C. The amount of histamine in samples was determined using an HR-Test Kit (Reflab ApS, Copenhagen, Denmark).

Staining of Surface Markers

Aliquots of MNC (10^5 cells/100 µl) were treated with antibody against IgE (Dako A/S, Glostrup, Denmark) followed by secondary peridinin chlorophyll protein-conjugated anti-mouse IgG1 antibody (BD Pharmingen Int., San Diego, Calif., USA) and CD203c-phycoerythrin (Immunotech Coulter, Marseille, France). After staining, cells were resuspended in 1% (v/v) paraformaldehyde in PBS and held at 4°C before analysis by flow cytometry.

Cell Culture Conditions and Stimulation

Isolated MNC were resuspended at a concentration of 2×10^6 cells/ml in RPMI, supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine and 50 µg/ml gentamicin (complete medium; Invitrogen Life Technologies Ltd., Paisley, UK).

Stimulation was performed by addition of cat or birch allergen (2 µg/ml Fel d 1 or Bet v 1, respectively; Alk-Abelló) or an equivalent volume of PBS in the negative control, followed by incubation for 5 h at 37°C in the presence of the protein-release inhibitor brefeldin A (GolgiPlug, BD Pharmingen Int.).

Intracellular Staining of Cytokines and Flow Cytometry Analysis

Following culture, cells were surface stained by treatment with monoclonal antibody anti-IgE (Dako A/S) followed by anti-mouse IgG1 peridinin chlorophyll protein. Subsequently, cells were fixed in phosphate-buffered 2% paraformaldehyde (v/v) and permeabilized with saponin (Perm-Wash solution, BD Pharmingen Int.). Intracellular staining was performed with mouse and rat monoclonal phycoerythrin-conjugated anti-IL-4 and IL-13 antibodies (0.12 µg/ 10^6 cells; BD Pharmingen Int.) and samples were subjected to flow cytometry analysis.

Flow Cytometry Analysis

All samples were analyzed on a Becton Dickinson FACS Calibur (Becton Dickinson, San Jose, Calif., USA). Fifty to eighty thousand events were counted in the flow cytometer so that a minimum of 500 basophils could be analyzed in each sample. Basophils were identified by their characteristic forward scatter pattern, between the lymphocyte and monocyte gates, and bright staining with the anti-IgE antibody (fig. 1). The results are presented as median fluorescence intensity (surface marker expression) or relative number (%) of positive basophils (cytokine production). The IL-4 and IL-13 production upon allergen stimulation was dose-dependent and no increase in the number of cytokine-positive basophils was detected when the cells were cultured in the absence of protein release inhibitor.

Statistical Analysis

The Wilcoxon signed rank test for paired data and the Mann-Whitney U test for nonpaired data were applied. Correlations were calculated using the Spearman rank order correlation test. A p value < 0.05 was considered statistically significant. All statistical tests were performed on a personal computer using the Statistica® software package (StatSoft Inc., Tulsa, Okla., USA).

Results

Patients and Clinical Data

Of the 14 patients who were included in the study and gave a blood sample before the start of treatment, 6 had a side effect score of 3, i.e. after reaching the full allergen dose (1 ml of 100,000 SQ/ml), when assessed at V1. Five patients received oral steroid and were therefore omitted at V1, and 2 of these patients did not wish to continue their participation in the study. None of the patients experienced any anaphylactic reaction during the course of RIT treatment.

Skin Prick Test

The median SPT wheal size measurements are presented in table 1. A significant decrease in SPT wheal size was observed in RIT-treated subjects 4 weeks and 3 months after the start of treatment ($p = 0.02$ and $p = 0.003$, respectively). SPT wheal size was significantly smaller in the RIT group compared with the controls at 4 weeks and 3 months ($p < 0.01$).

Blood Basophil Count

In the immunotherapy-treated subjects, blood basophil counts decreased significantly ($p = 0.02$) on the third day after the start of treatment. After 1 week of RIT, basophil numbers increased again, returning to the baseline level (table 1). An unaltered blood basophil count throughout the study was found in the control group.

Histamine Release

In the RIT group, the in vitro allergen-stimulated histamine release in mixed MNC decreased significantly at day 3 compared with the baseline values ($p = 0.02$); but after 1 week, it returned to the pretreatment level. No changes in HR were observed in the control group. Histamine release did not correlate with the blood basophil count and the proportion of basophils in MNC.

CD203c Expression on Basophils

In the RIT group, the median fluorescence intensity of CD203c staining on basophils decreased significantly from 136 (range 67–282) before treatment to 105 (range 77–189) on the third day of treatment ($p = 0.04$). The CD203c level 1 week, 3 weeks and 3 months after the start of immunotherapy was 110 (range 79–223), 106 (range 85–194) and 112 (range 46–232), respectively. Median fluorescence intensity of CD203c on basophils correlated with histamine release ($r = 0.5$, $p < 0.001$). The CD203c staining on basophils of the control group did not show

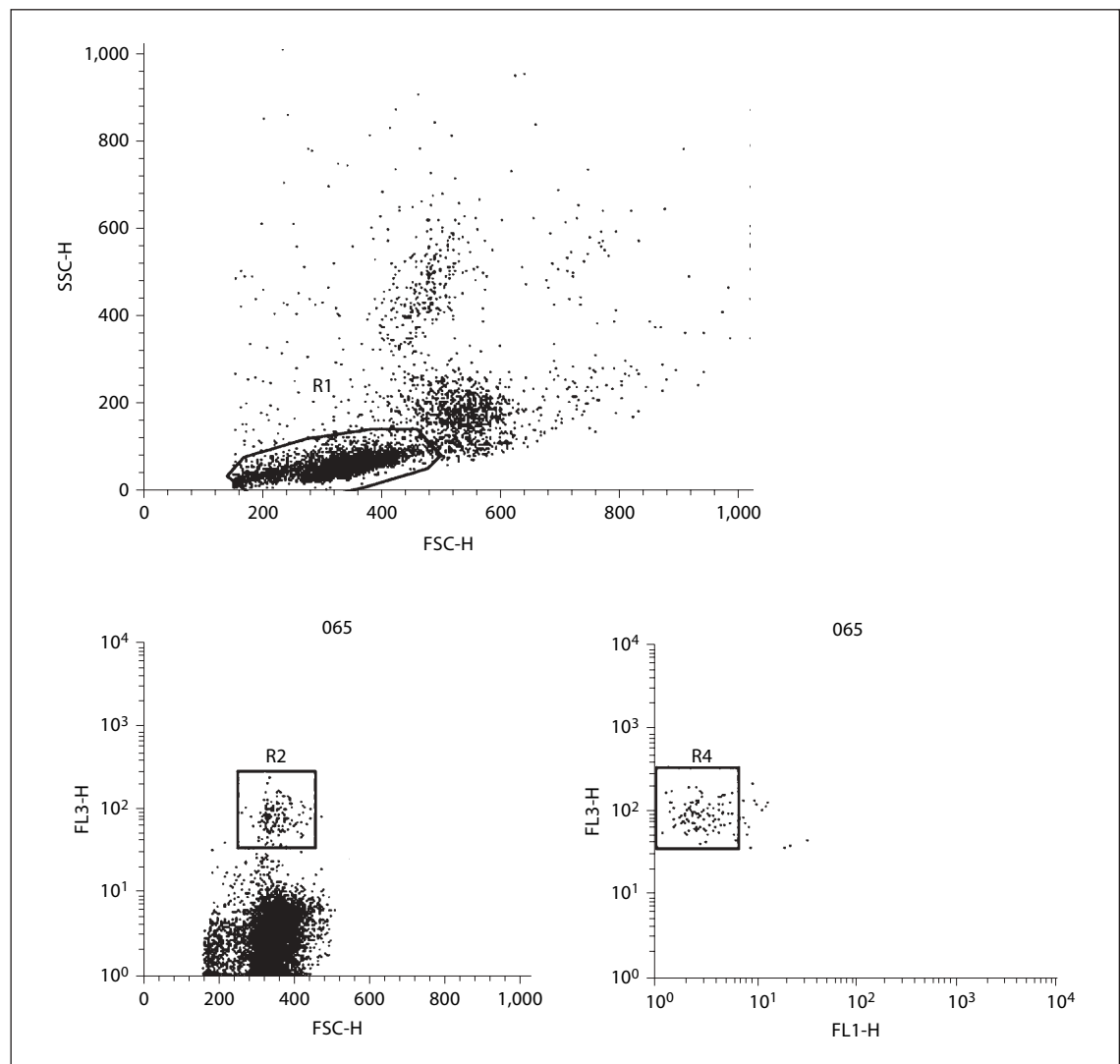


Fig. 1. Flow cytometric analysis of MNC and differentiation of inflammatory cell populations. Original gating for basophils. Basophils were identified by a characteristic forward scatter pattern, positioned between the lymphocyte and monocyte gates and bright staining with anti-IgE antibody. Basophils are enclosed within a box or encircled.

Table 1. RIT, control group and number of subjects at each test point

	Before RIT		3 days		7 days		1 month		3 months	
	RIT (n = 14)	control (n = 4)	RIT (n = 9)	control (n = 4)	RIT (n = 11)	control (n = 4)	RIT (n = 11)	control (n = 4)	RIT (n = 11)	control (n = 4)
SPT, mm	5.7 (5–9)	7.2 (5–12)	6.5 (3–8)	7.2 (5–10)	5.5 (4–14)	11 (5–13)	4.2 ^{a, c} (0–10)	10 (8–12)	3 ^{b, c} (0–5)	11.5 (9–14)
Basophils, 10 ⁶ /l	31.3 (16–62)	23.4 (7–36)	12.8 ^a (0–40)	14.2 (0–25)	27.6 (6–72)	28 (21–62)	29 (0–99)	42 (0–115)	37.1 (9–61)	22.1 (5–52)

Values are presented as medians and ranges in parentheses.

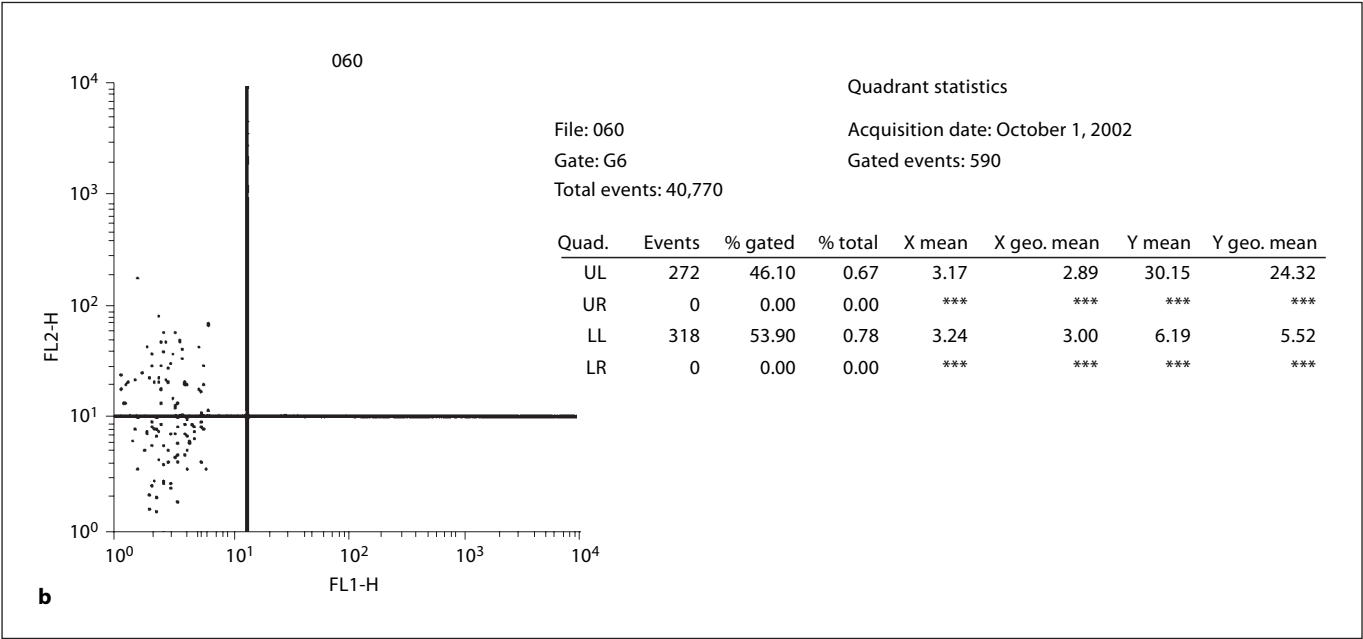
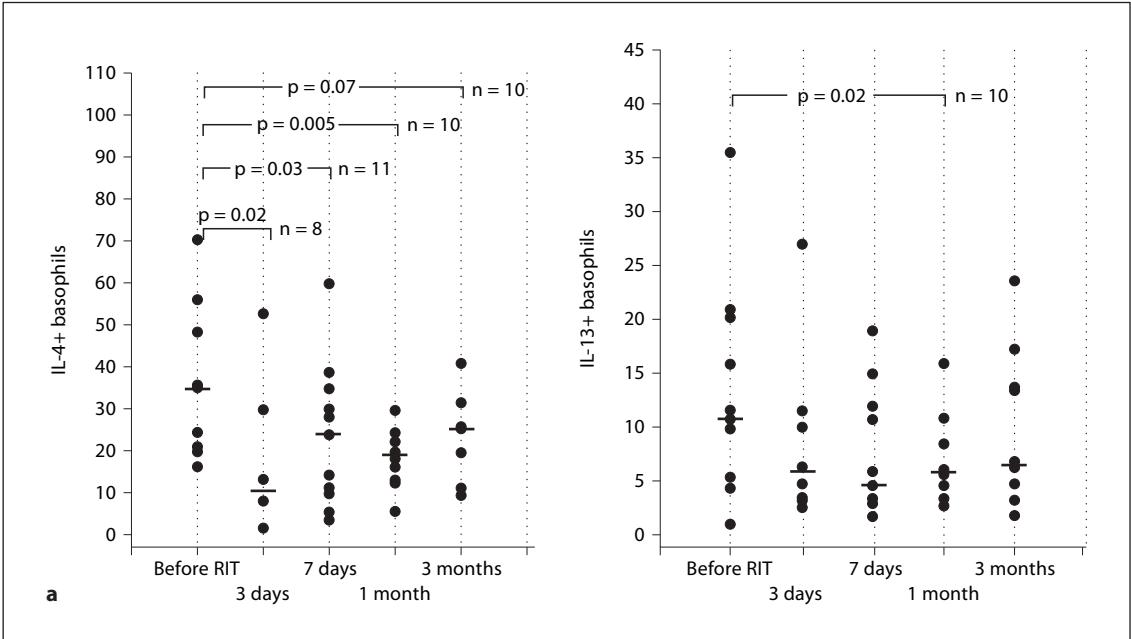
^a $p < 0.05$, ^b $p < 0.01$, significant differences in relation to the start of RIT.

^c $p < 0.01$, significant differences between RIT-treated patients and control subjects.

any marked changes throughout the study (results not shown).

IL-4 and IL-13 Production by Basophils
The in vitro allergen-stimulated production of IL-4 and IL-13 by basophils was expressed as the percentage of cytokine-positive cells (fig. 2a–c). In the RIT-treated group, a significant decrease in the proportion of IL-4+

blood basophils was observed on the third day of RIT ($p = 0.02$), as well as after 1 week ($p = 0.03$) and 3 weeks ($p = 0.005$) of treatment (fig. 2a). The tendency towards a lower number of IL-4+ basophils was still observed 3 months after the start of RIT ($p = 0.07$).
The proportion of IL-13+ basophils showed a similar pattern to IL-4+ cells and was significantly reduced 3 weeks after the start of RIT ($p = 0.02$; fig. 2a).



The relative numbers of IL-4+ and IL-13+ basophils after in vitro allergen stimulation correlated with each other when counts from samples collected at all visits were considered ($r = 0.69$, $p < 0.0001$). The numbers of IL-4+ and IL-13+ basophils also correlated with CD203c expression ($r = 0.41$, $p < 0.006$ and $r = 0.53$, $p < 0.001$, respectively) and histamine release ($r = 0.38$, $p < 0.01$ and $r = 0.31$, $p < 0.03$, respectively).

Correlation between Side Effect Score and Activation of Basophils

The adverse reactions to RIT during the incremental phase, expressed as the side effect score, correlated with the percentage of allergen-stimulated IL-4+ and IL-13+ basophils before the start of treatment (fig. 3; table 2). The side effect score also correlated with histamine release from allergen-stimulated mixed MNC, assessed before RIT ($r = 0.64$, $p = 0.03$) (table 2).

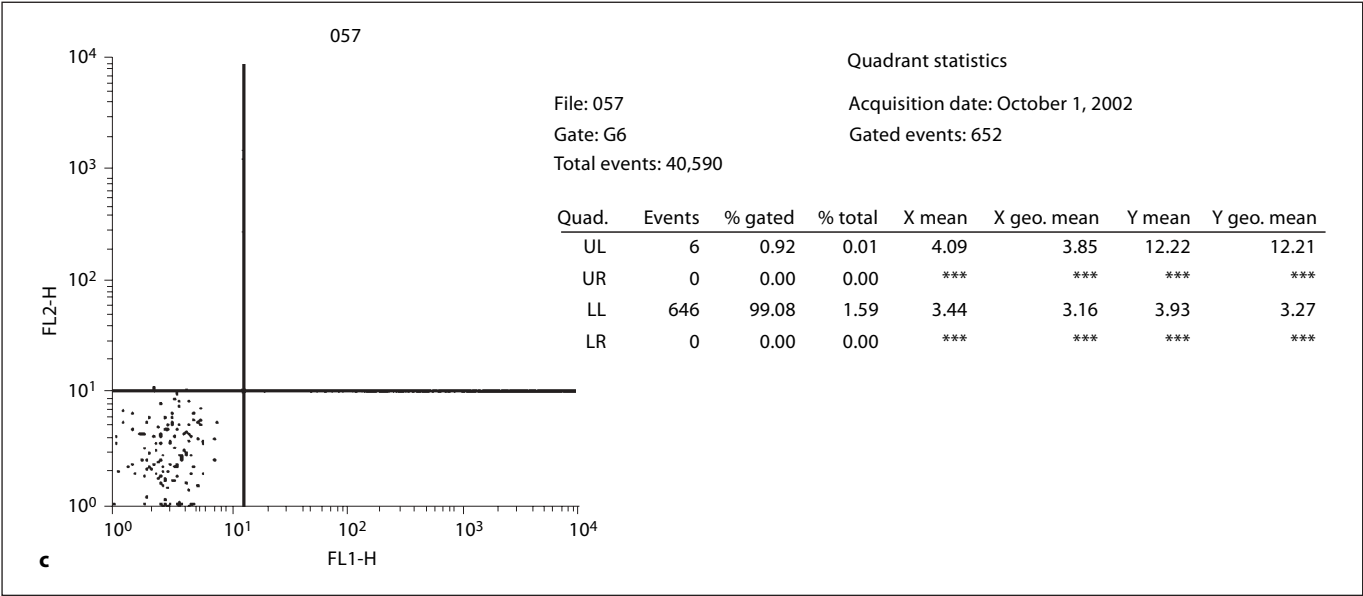


Fig. 2. a Allergen-induced production of IL-4 and IL-13 expressed as the percentage of basophils positive for these cytokines. Results obtained from subjects treated with RIT. Median values and significant differences between visits are indicated. **b, c** Flow cytometry scans showing in vitro allergen-stimulated basophils producing IL-4 (**b**) and IL-13 (**c**). Quad. = Quadrants; geo. = geometric.

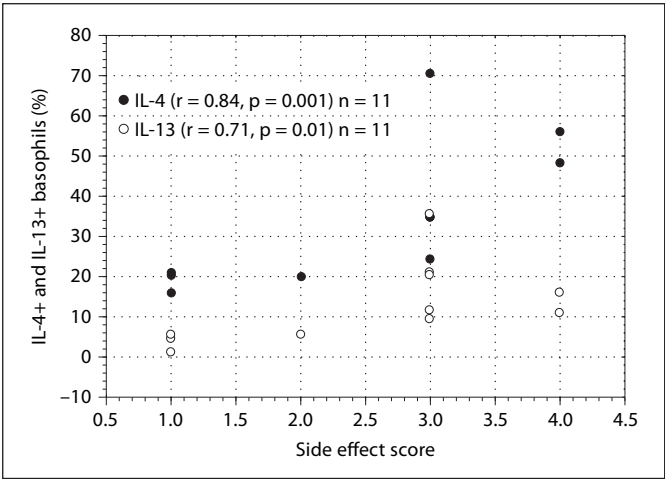


Table 2. Correlations between inflammatory mediators and side effect score

	IL-4	IL-13	Histamine release
Side effects	$p = 0.001$ $r = 0.84$	$p = 0.01$ $r = 0.71$	$p = 0.03$ $r = 0.64$

Fig. 3. Relationship between the percentage of IL-4- and IL-13-producing basophils (before the start of treatment) and the side effect score during RIT.

Discussion

The present study demonstrates the marked reduction in blood basophil number, histamine release, expression of CD203c, and proportion of IL-4+ and IL-13+ basophils during the initial phase of RIT. On continuation of RIT, basophil numbers and histamine release returned to pretreatment levels, while the expression of CD203c and the proportion of IL-4+ and IL-13 cells remained reduced compared with baseline values. Moreover, the baseline levels of histamine release and the relative numbers of IL-4+ and IL-13+ basophils upon allergen stimulation correlated significantly with the frequency of adverse events during the incremental phase of RIT. This study also confirmed that IT treatment leads to decreased skin mast cell reactivity within the first month.

Immunotherapy protocols using a fast increase in allergen dose have been clinically proven to be effective in treating venom hypersensitivity and perennial allergic rhinitis, but the mechanisms behind the fast protection achieved with RIT and ultra-rush immunotherapy are largely unknown. The conversion from a Th2-type to a Th0/Th1-type response has been proposed based on data from allergen-specific T-cell clones and cultured MNC from patients treated with RIT, where decreased IL-4 production and induction of interferon- γ has been demonstrated [8–10]. In cat-allergic patients, decreased levels of IL-4 in MNC and T-cell line supernatants as well as lower numbers of IL-4+ lymphocytes were observed in studies using conventional immunotherapy protocols [11–13]. It is now well established that basophils rapidly secrete IL-4 and IL-13 following IgE-dependent activation preceding cytokine release from MNC [14, 15]. Moreover, the concentration of allergen required for optimal cytokine production is much lower in the case of basophils than for MNC [16]. Thus, the early release of IL-4 and IL-13 from allergen-stimulated basophils may be crucial in promoting IgE synthesis and enhancing the Th2 response.

We found a suppression of allergen-induced IL-4 and IL-13 production in basophils by the third day of RIT. At the same time, we observed a decrease in basophil activation as evaluated by CD203c expression. CD203c is a basophil-specific ectonucleotide pyrophosphatase/phosphodiesterase 3 that is upregulated in sensitized subjects after appropriate allergen challenge [17]. The expression of CD203c has been associated with activation of basophils and correlated with histamine release; thus, it has been proposed as a suitable marker to assess the progress of immunotherapy [18]. To our knowledge, no previous studies have investigated the early kinetics of IL-4 and IL-

13 production in basophils at a single cell level during RIT. In one study, a significant reduction in the IL-4 level during the first 24 h of venom RIT was shown, but this was thought to depend on the downregulation of T-cell responsiveness to allergen rather than changes in the release of IL-4 by basophils [19]. A decrease in total histamine in mixed peripheral blood leukocytes and a reduction in histamine release from basophils were previously observed 1 day and 1 week after the initiation of venom RIT [5]. The immediate effect on total histamine content was attributed to the decreased number of basophils in peripheral blood. Accordingly, in our study, the reduced histamine release observed on the third day of RIT paralleled the decrease in basophil count. One reason for the depletion of circulating basophils could be the upregulation of adhesion molecules and migration of cells to the sites of subsequent reaction in the skin [20]. Thus, reduced histamine release could be explained by the disappearance of sensitized basophils from the bloodstream, leaving less responsive or immature basophils in the circulation. Another explanation for the decreased HR could be the induction of IL-10 and interferon- γ from lymphocytes, which has been previously reported in the course of RIT [21].

Interestingly, the production of IL-4 and IL-13 by basophils examined before the start of RIT correlated strongly with the severity of adverse events observed during the rapid allergen dose increase. IL-4 and IL-13 promote inflammatory cell recruitment to the tissues by upregulation of adhesion molecules (VCAM-1) on vascular endothelial cells [22] and the induction of eotaxin release [23].

IL-4 also upregulates the high-affinity IgE receptor (Fc ϵ RI) on cultured human mast cells, potentially leading to increased IgE-dependent mediator release and local allergic inflammation [24]. Moreover, a study in mice showed that endogenously produced IL-4 and IL-13 enhance the severity of anaphylaxis by increasing the effects of vasoactive mediators such as histamine, platelet-activating factor and leukotriene C₄. This effect was largely attributed to the exacerbation of vascular leakage [25]. IL-4 not only contributes to the development of late-phase reaction to allergen but also promotes the differentiation of antigen-specific lymphocytes into the Th2 type; thus, reduced levels of this cytokine in the milieu could favor a switch towards Th1-type cells. While earlier studies found that IL-4 production in long-term MNC cultures [26] and IL-4 mRNA expression in bronchial biopsies [27] were not related to symptom score in atopic asthma, a correlation between IL-4 and IL-13 production from blood basophils and the radioallergosorbent test score was reported [16].

Inhibition of basophil activation, IL-4 and IL-13 production and histamine release, could be the explanation for decreased antigen sensitivity in the early course of RIT. Furthermore, it is possible that IL-4 derived from basophils could influence the severity of the side effects of immunotherapy treatment.

In conclusion, we have identified an early suppression of basophil numbers, their activation status and production of IL-4 and IL-13 during the early phase of RIT. Interestingly, we also found that the ability of basophils to

produce IL-4 and IL-13 was related to the individual's reaction to the immunotherapy regime.

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