

Production of interleukin-12 by monocytes and interferon- γ by natural killer cells in allergic patients during rush immunotherapy

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Background: Allergen specific immunotherapy modifies the immunologic response to allergen exposure; however, the role of cells composing the innate immune system, such as monocytes and natural killer (NK) cells, in this mechanism is still unclear.

Objective: To examine the effect of rush immunotherapy (RIT) on early allergen-induced cytokine production by peripheral blood mononuclear cells from treated cat- and birch-allergic patients.

Methods: Twelve allergic patients received RIT, and another 4 served as controls. Blood samples were taken before the start and after 3 days, 1 week, 3 weeks, and 3 months of RIT. Allergen-induced production of interleukin-12 (IL-12) by monocytes and interferon- γ (IFN- γ) by NK cells was evaluated by means of flow cytometry.

Results: Before the start of RIT, allergic patients had significantly lower numbers of IL-12⁺ monocytes compared with healthy subjects ($P = .01$). The percentage of IL-12⁺ monocytes increased after 3 months of RIT ($P = .003$). In the allergic control group, the proportion of IL-12⁺ monocytes evaluated after 3 months was not different from baseline and was significantly lower compared with that in the RIT group ($P = .005$). Before treatment, the percentage of IFN- γ ⁺ NK cells was lower in allergic patients than in healthy subjects ($P = .04$). The percentage of IFN- γ ⁺ NK cells increased after 3 weeks ($P = .03$) and 3 months ($P = .01$) of RIT.

Conclusions: Restoration of the cytokine imbalance by immunotherapy is not only restricted to the cells of the adaptive immune system but also concerns cells composing the innate immune system.

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INTRODUCTION

The clinical efficacy of allergen specific immunotherapy (SIT) in cat- and birch-allergic rhinoconjunctivitis and asthma has been demonstrated in numerous controlled studies.^{1–3} The mechanism of its action remains unclear. A variety of immunologic changes have been observed in patients undergoing SIT. During immunotherapy, blunting of seasonal increases in serum allergen specific IgE levels has been demonstrated.^{4,5} Also, an increase in IgG levels after SIT seems to be a consistent finding.^{6,7}

Immunotherapy considerably reduced the in vitro expression of interleukin-4 (IL-4) in allergen-stimulated T cells or mixed peripheral blood mononuclear cells (PBMCs) and significantly increased the production of interferon- γ (IFN- γ).^{8–10} Specific immunotherapy also increased the frequency of CD4⁺ IFN- γ ⁺ cells after polyclonal stimulation and raised the levels of IFN- γ in serum.¹¹ Accordingly, a shift in the allergen-specific T-cell population from the IL-4-producing T_H2 type to the IFN- γ -expressing T_H1 type has been pro-

posed. The development of naive T cells into effector cells during antigen presentation is regulated by several factors, including the nature and activation of the antigen-presenting cells together with the local cytokine milieu. It has been suggested that cells of the innate immune system engaged in antigen presentation and rapidly generating key cytokines regulate T-cell responses to the specific antigen.^{12,13}

Monocytes and natural killer (NK) cells generate immunoregulatory mediators that determine the effector phase of the adaptive immune response. The presence of IL-12 diverts activated T cells to develop into T_H1 type, whereas the absence of IL-12 or the presence of IL-4 promotes T_H2 development. Monocyte-derived IL-12 has been implicated in the stimulation of IFN- γ production from T cells.¹⁵

Natural killer cells are also a potent source of IFN- γ , although little is known about the role of NK cells in regulation of the allergic process. The ongoing debate on the role of innate immunity in allergic inflammation and the limited data concerning the modulation of monocyte and NK cell functions by SIT has led us to examine the effect of rush immunotherapy (RIT) on cytokine production in monocytes and NK cells.

METHODS

Patients

Fourteen allergic patients with rhinoconjunctivitis with or without asthma with cat exposure and 2 with a history of

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Table 1. Characteristics of the Study Participants

Characteristics	RIT patients (n = 12)	Allergic controls (n = 4)	Healthy controls (n = 7)
Sex, F/M, No.	3/9	2/2	6/1
Age, mean (range), y	30 (22–37)	36 (29–44)	28 (23–49)
SPT reaction, mean (range), mm	5.7 (4.5–8.5)	7.2 (5–12)	0
Specific IgE, mean (range), kU/L	9.7 (0.6–53)	7.1 (0.6–12.6)	<0.35

Abbreviations: RIT, rush immunotherapy; SPT, skin prick test.

rhinoconjunctivitis during the birch pollen season participated in the study (Table 1). Ten of the cat-allergic patients received RIT with cat extract (Alutard; ALK-Abelló, Hørsholm, Denmark), and the 2 birch-allergic patients received RIT with birch extract (Alutard). Depending on the severity of the adverse effects during RIT, the following medication was given: antihistamine (acrivastine capsules [Semprex; Glaxo Wellcome Operations, Greenford, England]), short-acting β_2 -agonist (salbutamol [Ventolin Diskus; GlaxoSmith-Kline, Philadelphia, PA]), or corticosteroid (betamethasone [Betapred; Swedish Orphan International, Stockholm, Sweden]). If oral corticosteroid had to be used, blood sampling was omitted. The allergic control group consisted of the 4 remaining cat-allergic patients. None of the patients had received SIT in the past 15 years. They were not allowed to have daily contact with cats. The study was performed outside the birch pollen season. Seven healthy, nonatopic individuals with negative skin prick test (SPT) results to allergens, including cat and birch, served as a reference group. All the patients provided written informed consent. The ethics committee of Sahlgrenska University Hospital approved the study.

Study Design

The following treatment regimen was applied in the RIT group: day 1 to 10 SQ-U/mL, 100 SQ-U/mL, 1,000 SQ-U/mL, and 5,000 SQ-U/mL; day 2 to 10,000 SQ-U/mL and 20,000 SQ-U/mL; day 3 to 40,000 SQ-U/mL and 65,000 SQ-U/mL; and day 5 to 100,000 SQ-U/mL; the maintenance dose (100,000 SQ-U/mL) was then repeated on days 9, 14, and 23 and 4 weeks after the start of treatment. Thereafter, injections of 100,000 SQ-U/mL were given every 6 weeks. Blood sampling was performed before the start of RIT and 3 days, 1 week, 3 weeks, and 3 months after the start of treatment. Samples were always collected before the injection of allergen. Patients included in the control group did not receive any treatment, and blood samples were obtained at the same intervals as in the RIT group. Blood samples from the healthy subjects were obtained once and served as a reference.

SPT, Blood Sampling, and Isolation of Mononuclear Cell Fraction

The SPT was performed before the start of RIT and during all consecutive visits, always before the injection of allergen. The cat or birch extract, 10 mg/mL of histamine solution (positive control), and diluent (negative control) were applied

to the volar side of the forearm, and the reactions were read after 15 minutes (Soluprick SQ; ALK-Abelló). The 2 largest diameters (d) were used to estimate the wheal size according to the following formula: $(d_1 + d_2)/2$. A wheal diameter greater than 5 mm was considered a positive SPT result.

Heparinized blood diluted 1:1 in phosphate-buffered saline was applied to isotonic Percoll (density, 1.084 g/mL) (Amersham Pharmacia Biotech AB, Uppsala, Sweden). After centrifugation for 30 minutes at 1,000g, the mononuclear cell fraction was carefully removed from the interface for surface staining and cell culture.

PBMC Culture Conditions and Stimulation

Isolated PBMCs were resuspended in RPMI, supplemented with 10% heat-inactivated fetal calf serum, 2-mmol/L L-glutamine, and 50 μ g/mL of gentamicin (complete medium) (Invitrogen Life Technologies Ltd, Paisley, England) at a concentration of 2×10^6 cells/mL. The PBMC stimulation was performed by the addition of cat or birch allergen (2 μ g/mL; Fel d 1 or Bet v 1, respectively) (ALK-Abelló) or an equivalent volume of phosphate-buffered saline in the negative control. After 15 minutes at 37°C, protein-release inhibitor–brefeldin A (GolgiPlug; BD PharMingen International, San Diego, CA) was added to the PBMC suspension, and incubation was continued for 5 hours. To determine the kinetics of cytokine release within the first 24 hours of allergen stimulation, an additional experiment was performed. Briefly, PBMCs were stimulated with allergen for various periods followed by 14 hours of culture in the presence of brefeldin A. After 5 hours of allergen stimulation, the population of cells producing IFN- γ was identified by means of immunostaining and flow cytometry as CD2⁺/CD56⁺/CD3[−] NK cells. When PBMCs were stimulated with allergen for 7 hours followed by 14 hours of culture with brefeldin A, only a few NK cells were positive for IFN- γ .

Staining of Surface Markers

After culture, PBMCs (10^5 cells/100 μ L) were stained for surface markers. Monocytes were stained with anti-CD14–fluorescein isothiocyanate–conjugated antibody. Natural killer cells were identified as CD3[−] cells (with anti-CD3–fluorescein isothiocyanate–conjugated) in the CD2⁺ population (with anti-CD2–peridin-chlorophyll protein) and further by staining with anti-CD56–phycoerythrin, the selected population of NK cells was thus CD2⁺/CD56⁺/CD3[−]. Monoclonal, anti-human antibodies were all purchased from (BD PharMingen International).

Intracellular Staining of Cytokines and Flow Cytometry Analysis

After cell culture and surface staining, cells were fixed in phosphate-buffered 2% (vol/vol) paraformaldehyde and permeabilized with saponin (Perm-Wash solution; BD Pharmingen International). Intracellular staining was performed with mouse phycoerythrin-conjugated monoclonal antibody anti-IL-12 and IFN- γ antibodies ($0.12 \mu\text{g}/10^6$ cells) (BD Pharmingen International). All the samples were analyzed using a Becton Dickinson FACS Calibur (Becton Dickinson, San Jose) recognizing 3 colors. Cell gates were established on the basis of characteristic side vs forward scatter patterns.

Statistical Analysis

The Wilcoxon signed rank test for paired data, the Mann-Whitney test for nonpaired data, and Friedman 2-way analysis of variance (ANOVA) were applied. $P < .05$ was considered statistically significant. All statistical tests were performed on a personal computer using a software package (Statistica; StatSoft Inc, Tulsa, OK).

RESULTS

Skin Prick Testing

A significant decrease in SPT wheal size (Friedman ANOVA, $P < .004$) from 5.7 mm (range 4.5–8.5 mm) at baseline to 4.2 mm (range, 0–10 mm) was observed 3 weeks after the start of RIT ($P = .02$).¹⁶ further decrease was noted after 3 months of RIT (3 mm; range, 0–5 mm) ($P = .003$). The median SPT wheal size was significantly smaller in the RIT group compared with controls at the 3-week and 3-month visits ($P < .01$).

IL-12 Production by Monocytes

The production of IL-12 by allergen-stimulated monocytes was determined (Fig 1). Before the start of RIT, allergic patients had significantly lower numbers of IL-12⁺ monocytes compared with healthy subjects ($P = .01$). The percentage of IL-12⁺ monocytes increased after 3 months of RIT ($P = .003$) to a level similar to that of healthy subjects (Friedman ANOVA, $P < .02$). In the allergic control group, the proportion of IL-12⁺ monocytes evaluated at the 3-month visit was not different from that at baseline and was significantly lower compared with that of the RIT group ($P = .005$).

IFN- γ Production by NK Cells

Before the start of immunotherapy, the relative number of IFN- γ ⁺ NK cells after in vitro allergen stimulation was lower in allergic patients compared with healthy subjects ($P = .04$). The percentage of IFN- γ ⁺ NK cells was significantly ($P = .03$) increased after 3 weeks and 3 months of RIT (Friedman ANOVA, $P < .02$) (Fig 2). In the control group, the percentage of IFN- γ ⁺ NK cells did not show any marked changes throughout the study.

DISCUSSION

We showed an increase in the proportion of IL-12⁺ monocytes and IFN- γ ⁺ NK cells in the peripheral blood of immu-

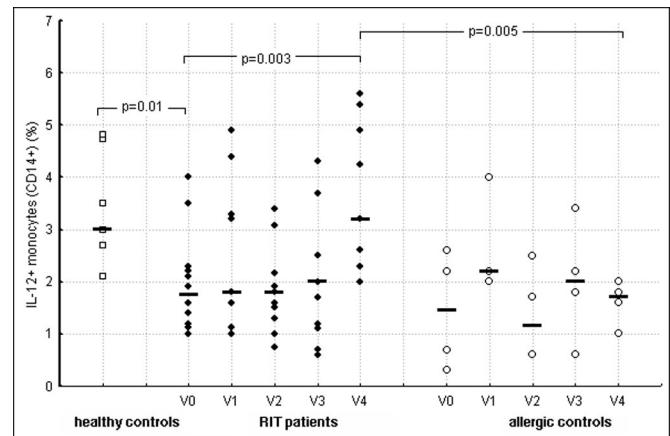


Figure 1. The percentage of interleukin-12 (IL-12)-producing monocytes in peripheral blood of rush immunotherapy (RIT) patients, allergic controls, and healthy subjects. Median values (horizontal bars) and significant differences between the visits are indicated. Significant differences between healthy subjects and allergic patients and between the RIT group and the allergic control group are also shown. V0 indicates baseline; V1, after 3 days of RIT; V2, after 1 week of RIT; V3, after 3 weeks of RIT; and V4, after 3 months of RIT.

notherapy-treated patients. The number of IL-12⁺ monocytes increased significantly after 3 months of treatment, whereas the earliest effect of RIT on the percentage of IFN- γ ⁺ NK cells was already observed after 3 weeks.

Immunotherapy modifies T-cell response and decreases allergen-induced T-cell proliferation.¹⁷ The previously proposed switch from a T_H2-type response to a T_H1-type response has been confirmed in cultured blood mononuclear cells obtained from patients treated with RIT.^{8–10} Establish-

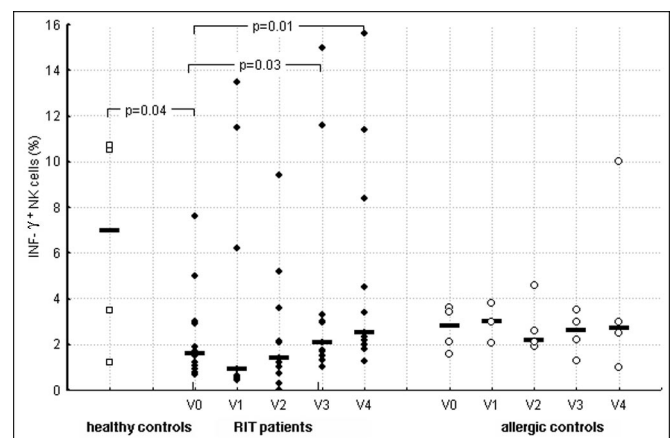


Figure 2. The percentage of interferon- γ (IFN- γ)-producing natural killer (NK) cells in peripheral blood of rush immunotherapy (RIT) patients, allergic controls, and healthy subjects. Median values (horizontal bars) and significant differences between visits are indicated. Significant differences between healthy subjects and allergic patients are also shown. V0 indicates baseline; V1, after 3 days of RIT; V2, after 1 week of RIT; V3, after 3 weeks of RIT; and V4, after 3 months of RIT.

ment of the T_H1/T_H2 balance is determined by many factors, including the local cytokine milieu during antigen presentation and T-cell response.¹⁴ Cytokines produced by the cells of the innate immune system, including monocytes and NK cells, can have a potent regulatory effect on adaptive immunity, affecting the course of immunologic response and the balance between T_H1 - and T_H2 -type T cells. The presence of IL-12 in the milieu during antigen presentation suppresses transcription of IL-4 and promotes the differentiation of T_H1 -type cells, whereas the absence of IL-12 favors T_H2 development.¹⁸ Interleukin 12 stimulates IFN- γ release, and, in turn, IFN- γ can augment IL-12 production and enhance T-cell responsiveness to IL-12 by increasing expression of the high-affinity IL-12 receptor (IL-12R β 2).¹⁹ Thus, a reduced capacity of antigen-presenting cells to produce IL-12 may favor the T_H2 -type response. We confirmed in the present study the previous finding that monocytes from allergic individuals produce less IL-12 on allergen exposure than those from healthy subjects.²⁰ In line with these results, the decreased production of IL-12 by monocytes, after lipopolysaccharide stimulation, was shown in patients with severe asthma.²¹

An important finding of the present study is the restoration of IL-12 production by monocytes to a level comparable with that of healthy controls after 3 months of RIT. In agreement with the present results, Magnan et al²² reported the induction of spontaneous IL-12 release from monocytes by day 15 of venom immunotherapy.

The ability of IL-12 to promote the in vitro development of T_H1 -type cells is at least partly due to its stimulatory effect on IFN- γ production.¹⁸ In the present study, IFN- γ^+ cells were found after 5 hours of in vitro allergen stimulation and were identified as NK cells. The onset of IFN- γ production in NK cells was rapid and lasted up to 12 hours of allergen stimulation (data not shown). Previous studies reporting the effect of immunotherapy on blood mononuclear cell cytokine production examined IFN- γ release in the supernatant 24 hours after allergen stimulation at the earliest.^{23,24} In the study by Meissner et al,²³ the allergen-induced IFN- γ production in the nonatopic controls was significantly higher compared with the allergic group, but no changes in the level of IFN- γ after immunotherapy were observed. We found a higher percentage of IFN- γ^+ NK cells in the blood of healthy subjects compared with allergic patients and increased numbers of IFN- γ^+ NK cells 3 weeks after the start of RIT. The present study suggests that investigations of immunotherapy-induced changes in IFN- γ production should consider NK cells as a possible source of this cytokine. The capability of NK cells to migrate rapidly in response to cytokines and chemokines supports the notion that they can infiltrate tissues before T cells and thus participate in directing T-cell responses.²⁵ A study²⁶ in mice showed that IFN- γ^+ NK cells are among the first cells detected at the site of immunization, some time before T cells appear. In conclusion, we demonstrated an increase in the frequency of IL-12⁺ monocytes and IFN- γ^+ NK cells after in vitro allergen stimulation in patients under-

going RIT. These results support the idea of a regulatory role for innate immunity in allergen-specific T-cell responses. Furthermore, the present data indicate that the restoration of cytokine imbalance by immunotherapy treatment involves not only the cells of the adaptive immune system but also cells participating in innate immunity.

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