

IL-10 production in circulating T cells differs between allergen-induced isolated early and dual asthmatic responders

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Background: IL-10 is an anti-inflammatory cytokine released from various cells, including T cells. The role of IL-10 in asthma pathogenesis remains uncertain. Allergen inhalation by atopic asthmatic subjects results in 2 bronchoconstrictor phenotypes: isolated early response and dual response. Persistence of allergen-induced airway inflammation is a feature of dual responders. **Objectives:** The kinetics of IL-10 production in circulating T cells were investigated to examine a potential role of IL-10 in allergen-induced responses and airway inflammation. **Methods:** Fourteen subjects with mild asthma (7 isolated early and 7 dual responders) were challenged with allergen. PBMCs taken before and 24 hours after allergen challenge were processed for intracellular IL-10 staining with fluorescent-conjugated anti-IL-10 antibody. The frequency of IL-10-producing cells was assessed for CD4⁺ and CD8⁺ T-cell subsets by using flow cytometry. **Results:** Before allergen administration, the frequency of IL-10-producing CD4⁺ cells was significantly higher in dual than in isolated early responders. IL-10-producing CD4⁺ cells significantly increased after allergen in early responders, whereas IL-10-producing CD4⁺ cells significantly decreased in dual responders. Simultaneous assessments of IL-5-producing T cells did not show any differences between each group before or after allergen administration. **Conclusions:** These results suggest that the contrasting profiles of IL-10 production may be associated with the different time course of allergen-induced airway inflammation between allergen-induced early and dual responders. (*J Allergy Clin Immunol* 2002;109:281-6.)

Key words: IL-10, IL-5, allergens, asthma, early responders, dual responders

Abbreviations used

BAL: Bronchoalveolar lavage
GSEM: Geometric SEM
IQR: Interquartile range

Allergen inhalation by atopic asthmatic subjects provokes rapid bronchoconstriction, which appears within 30 minutes and usually ends by 2 hours (early response).¹ The early response can be followed by bronchoconstriction beginning 3 to 7 hours after the exposure (late response) that is associated with increases in eosinophilic airway inflammation^{2,3} and airway hyperresponsiveness,¹⁻³ which can persist for more than a week.⁴ The resolution of allergen-induced responses may be an active process mediated by anti-inflammatory mechanisms, which may differ in dual responders and isolated early responders.

Various types of inflammation are caused by biased interactions of cytokines among T-cell subsets skewed toward either a T_H1 or a T_H2 profile. IL-10 was originally isolated as a cytokine produced from T_H2 cells, which inhibited T_H1 responses.⁵ However, in human subjects not only T_H2 but also T_H1 cells are able to produce IL-10.⁶ IL-10 inhibits the production of proinflammatory cytokines, such as TNF- α , IL-4, and IL-5, from activated T cells^{7,8} from granulocytes and inhibits the survival of eosinophils.^{9,10} Thus the major role of IL-10 in atopic diseases may be anti-inflammatory as a part of the feedback inhibitory mechanism.

IL-10 is released from various types of cells, including antigen-presenting cells, mast cells, eosinophils, B cells, and T cells, in human subjects.¹¹ Allergen-specific T cells play a crucial role in the pathogenesis and regulation of atopic asthma.¹² T cells circulate from the affected organs to the blood stream through the lymphatic system.¹³ This profile enables the detection of T cells, their cytokines, and kinetics by using peripheral blood samples. This study tested the hypothesis that the profiles of IL-10 and IL-5 production by circulating T cells differ between allergen-induced dual responders and isolated early responders, as measured at the single-cell level.¹⁴⁻¹⁶

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TABLE I. Subject characteristics

Subject	Age (y)	Sex	Baseline FEV ₁ (% predicted)	Methacholine PC ₂₀ (mg/mL)	Inhaled allergen	Allergen dilution
IER						
1	22	M	96.6	11	Ragweed	1:2000
2	23	M	87	1.04	HDM	1:64
3	24	F	85.2	21.5	Grass	1:128
4	27	F	88.4	5.75	HDM	1:32
5	22	F	92.4	3.03	HDM	1:512
6	24	F	81.5	11.31	Cat	1:4
7	30	F	72.1	1.17	HDM	1:16
Mean ± SEM	24.6 ± 1.1		86.2 ± 3.0	4.78 (GSEM, 1.56)		
DR						
1	22	F	82.1	12.7	Ragweed	1:64
2	22	F	83.3	6.64	Ragweed	1:128
3	50	M	100.3	1.19	Cat	1:32
4	23	F	91.2	1.87	Ragweed	1:4096
5	23	F	82.3	0.14	Cat	1:512
6	21	F	92	0.71	HDM	1:56
7	24	F	81.5	0.12	HDM	1:32
Mean ± SEM	26.4 ± 3.9		87.5 ± 2.7	1.68 (GSEM, 1.74)		

IER, Isolated early responders; HDM, house dust mite; GSEM, geometric SEM; DR, dual responders.

METHODS

Subjects

Fourteen subjects with atopic asthma (7 dual responders and 7 isolated early responders) were studied (Table I). The study was approved by the Ethics Committee of the McMaster University Health Sciences Center, and all subjects gave signed consent. The subjects had mild, stable atopic asthma,¹⁷ requiring only intermittent use of an inhaled β_2 agonist, with baseline FEV₁ values of greater than 70% of predicted value. All were nonsmokers, and none had experienced a respiratory infection for at least 4 weeks. Medications were withheld for at least 8 hours before the investigation. Atopic status was defined by means of positive skin prick test responses (a wheal 3 mm greater than that caused by the physiologic saline control) to allergens. Subjects were classified as dual responders if they developed both early and late asthmatic responses, as defined by a greater than 15% drop in FEV₁ from baseline, or as isolated early responders if they developed only an early fall in FEV₁ of greater than 15% from baseline values.

Study design

Subjects attended the laboratory on 3 occasions. At the initial visit, a medical history was taken, and skin prick tests, spirometry, methacholine inhalation challenges, and induced sputum differential counts were performed. On the next day, subjects underwent an allergen challenge before which a blood sample was taken for baseline measurements. Spirometry was measured until 7 hours after allergen inhalation, at which time sputum samples were obtained. The third visit to the laboratory occurred 24 hours after allergen inhalation, when blood samples were obtained and followed with spirometry, methacholine inhalation challenge, and sputum sampling.

Methacholine inhalation challenge

Methacholine inhalation challenge was performed, as described previously by Cockcroft.¹⁸ Briefly, subjects inhaled normal saline and then doubling concentrations of methacholine phosphate from a Wright nebulizer for 2 minutes. Increasing concentrations of methacholine were administered until the FEV₁ fell by greater than 20% of the baseline value. Results were expressed as PC₂₀.

Allergen inhalation challenge

Allergen challenge was performed, as described by O'Byrne et al.¹ The allergen extract was selected by using the results from the skin test, and dilutions for inhalation were determined from a formula described by Cockcroft et al.¹⁹ The early bronchoconstrictor response was the largest fall in FEV₁ within 2 hours after allergen inhalation, and the late response was the largest fall in FEV₁ between 3 and 7 hours after allergen inhalation.

Sputum analysis

Sputum was induced and processed, as described previously.³ Subjects inhaled 3%, 4%, and 5% saline solution for 7 minutes each. The induction was stopped when an adequate sample was obtained or if the FEV₁ dropped 20% from baseline. Differential cell counts were obtained from the mean of 2 slides, with 400 cells counted per slide stained with Diff-Quik (American Scientific Products, McGaw Park, Ill). The percentage of eosinophils was used as an index of eosinophilic airway inflammation.

Monoclonal antibodies

All mAbs were purchased from PharMingen (San Diego, Calif). Phycoerythrin-conjugated anti-human IL-10 mAb, JES3-19F1 (rat IgG_{2a}) anti-human IL-5 mAb, JES1-39D10 (rat IgG_{2a}), and isotype control rat IgG_{2a} were used for the study. Positive staining was detected by means of flow cytometry, which could be inhibited when the mAb was preincubated with 25 μ g/mL recombinant human IL-10 and IL-5 (PharMingen) or with an excessive dose (50 μ g/mL) of fluorescent-unlabeled anti-human IL-10 mAb and anti-human IL-5 mAb. As a positive control for IL-10, a commercially available cell sample, HiCK-2 (PharMingen), was used. In nonpermeabilized cells positive staining for IL-10 decreased by 30% of the paired sample with permeabilization, showing that 70% of positive signal is of intracellular origin. FITC-conjugated anti-CD4 mAb (RPA-T4; mouse IgG₁, κ), anti-CD8 mAb (RPA-T8; mouse IgG₁, κ), and CyChrome-conjugated anti-CD3 mAb (HIT3a; mouse IgG_{2a}, κ) were used for identifying T-cell subsets.

Cell cultures

PBMCs were isolated from 20 mL of heparinized peripheral blood by means of Lymphoprep (Nycomed, Oslo, Norway) density gradient separation and washed twice with RPMI-1640 (Gibco BRL, Gaithersburg, Md). Cells were suspended in RPMI-1640 with 10% FCS and 25 mmol/L HEPES at a density of 2×10^6 cells/mL. Cells were then cultured in 6-well culture plates in a 5-mL volume without any stimulation for 4 hours at 37°C in 5% CO₂ in an incubator with 85% humidity. Breferrdin A (Sigma) at 10 µg/mL was added over the incubation time to inhibit cytokine secretion. At harvest, cells were gently removed from the plate with a cell scraper. The contents of each well were placed in a 15-mL V-bottom polypropylene tube and incubated for 5 minutes to allow disaggregation of cells. The cell viability was assessed by means of the trypan blue dye method. The cells were then centrifuged at 500g for 10 minutes and washed once in ice-cold PBS with 0.2% FCS and 0.1% sodium azide (PBS⁺; BDH Inc, Toronto, Canada). The cells were transferred to 5-mL polystyrene tubes at 1×10^6 cells/tube in PBS⁺ and then processed for staining.

Staining

Cells were washed in PBS⁺ and resuspended at a density of 1×10^6 cells in 100 µL of PBS with 0.1% sodium azide and 5% normal mouse serum incubated for 10 minutes on ice and followed by surface staining with anti-CD3 mAb and anti-CD4 mAb/anti-CD8 mAb for 20 minutes on ice in the dark. For intracellular cytokine staining, the cells were washed once in simple PBS and then fixed in 100 µL of ice-cold fixation buffer containing 4% paraformaldehyde for 20 minutes on ice. After an additional wash with simple PBS, cells were resuspended in 100 µL of permeabilization buffer containing 0.1% saponin with 5% normal rat serum for 10 minutes at room temperature and then added with anti-IL-10 mAb, anti-IL-5 mAb, or isotype control IgG_{2a} for 30 minutes at room temperature in the dark. After a final wash in simple PBS, cells were resuspended in PBS with 1% paraformaldehyde and kept in the dark at 4°C until evaluated.

Flow cytometric analysis

A FACScan flow cytometer (Becton Dickinson, San Jose, Calif) equipped with a 15-mA argon ion laser and filter settings for FITC (530 nm, FL-1), phycoerythrin (585 nm, FL-2), and CyChrome (650 nm, FL-3) was used. Ten thousand events were acquired in list mode, with debris excluded by the forward scatter threshold. The data were analyzed with CELLQuest software. An analysis gate was set on the lymphocyte population based on the forward and side scatter plot. First, CD3⁺-stained cells falling within the gated area were identified by means of quadrant analysis with FL-3 and the side scatter on the dot plots. The gated population was further analyzed for the identification of CD3⁺CD4⁺ (CD4⁺) or CD3⁺CD8⁺ (CD8⁺)-stained cells by FL-1 and the side scatter. CD3⁺ cells only brightly positive for CD8 were counted as a CD8⁺ T-cell subset. Monocytes were excluded by lack of CD3 expression. This gating strategy enabled us to count approximately 5000 ± 500 CD3⁺ cells, 3000 ± 500 CD4⁺ cells, and 1500 ± 500 CD8⁺ cells from each sample. The IL-10-producing or IL-5-producing cells were analyzed by means of detection of FL-2 on FL-1, FL-3-positive lymphocytes. The cut-off level for definition of positive cells was set so that less than 2% of isotype antibody-stained cells were positive. The frequency of true-positive cells was obtained by subtracting the value of isotype control from that stained with anti-IL-10 or anti-IL-5 mAb.

Statistical analysis

Statistical analysis was done with standard statistical software (Stat View J4.02; Abacus Concepts Inc, Berkeley, Calif). Data with normal distribution were expressed as means ± SEM, and nonpara-

metric data were expressed as the medians and interquartile ranges (IQRs). Methacholine PC₂₀ values were log₁₀ transformed before analysis, and the results were expressed as geometric means and geometric SEMs (GSEM). Preallergen versus postallergen comparisons were performed with Student paired *t* tests. Comparisons of sputum cells and flow cytometry between groups used the Mann-Whitney *U* test, whereas preallergen versus postallergen comparisons used the Wilcoxon signed-rank test. The Spearman rank correlation coefficient was used to examine relationships among FEV₁, methacholine PC₂₀, sputum cells, and flow cytometric variables. *P* values of less than .05 were accepted as being statistically significant.

RESULTS

Bronchoconstrictor responses

The maximal percentage fall in FEV₁ during the early response was 26.2% ± 3.1% in early responders and 33.8% ± 4.2% in dual responders (*P* = .06). The maximal percentage fall in FEV₁ during the late response was 2.8% ± 1.5% in early responders and 20.0% ± 3.0% in dual responders (*P* = .002).

Airway hyperresponsiveness

Methacholine airway hyperresponsiveness developed in the dual responders, but not in the early responders, 24 hours after allergen challenge. The methacholine PC₂₀ values in the dual responders fell from 1.67 mg/mL (GSEM, 1.74) before to 0.41 mg/mL (GSEM, 1.87) after allergen challenge (*P* = .01), whereas in the early responders the values were 4.78 mg/mL (GSEM, 1.56) before and 3.66 mg/mL (GSEM, 1.80) after allergen challenge (*P* = .30). The difference between the 2 groups in the allergen-induced change in PC₂₀ values was significant (*P* = .04).

Changes in sputum inflammatory cells by allergen challenge

Inhaled allergen significantly increased the percentage of sputum eosinophils at 7 and 24 hours after allergen challenge in both early and dual responders (Table II). There was no significant difference in the percentage of eosinophils between the groups at baseline or 7 or 24 hours after allergen challenge (*P* = .78, *P* = .57, and *P* = .09, respectively). No significant changes were observed for the other types of inflammatory cells (Table II).

Changes in frequency of CD4⁺ and CD8⁺ cells

Cell viability at the beginning of incubation varied from 97% to 100%, and no significant difference was observed between groups. There was no significant difference in the proportions of CD4⁺ and CD8⁺ cells between groups either before or after allergen challenge (Table II).

Frequency of IL-10/IL-5-producing cells at baseline

The frequency of IL-10-producing CD4⁺ cells, but not CD8⁺ cells, was significantly higher in the dual responders when compared with that in the early responders (2.00% [IQR, 1.42%] vs 0.25% [IQR, 0.61%], *P* = .03, Fig 1). The frequencies of neither IL-5-producing CD4⁺ cells nor CD8⁺ cells differed between the groups.

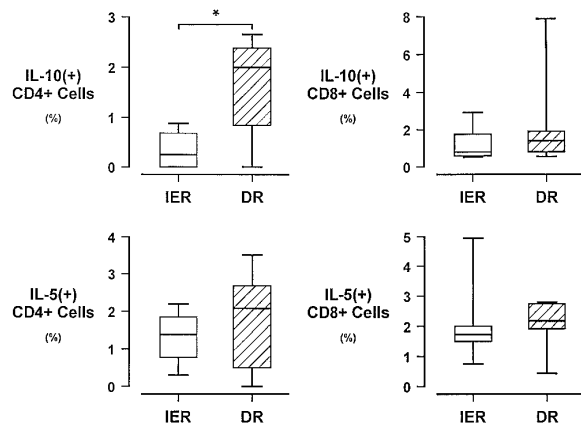


FIG 1. Frequency of IL-10- or IL-5-producing T cells at baseline (before allergen challenge). Data are presented as box plots. The boxes span the 25th and 75th percentiles, with medians noted by inside bars. Length of whiskers is based on maximal and minimal data. The frequency of IL-10-producing CD4⁺ cells in the dual responders (DR) was significantly higher than that in the isolated early responders (IER). * $P < .05$.

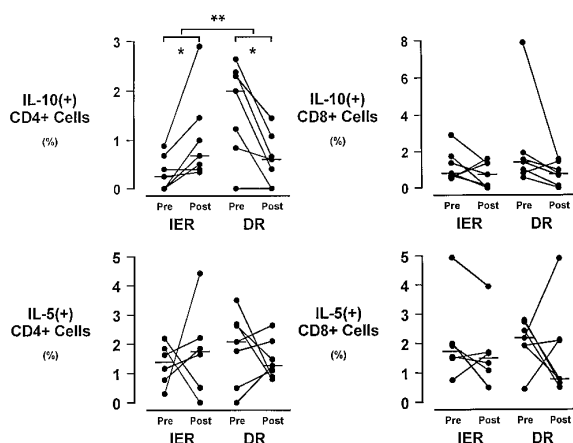


FIG 2. Changes in the frequency of IL-10- or IL-5-producing T cells before and 24 hours after allergen challenge. Horizontal lines indicate median values. In the isolated early responders (IER) the frequency of IL-10-producing CD4⁺ cells significantly increased after allergen challenge. In the dual responders (DR) the frequency of IL-10-producing CD4⁺ cells significantly decreased after allergen challenge. There was a significant difference between groups in the allergen-induced shift in the frequencies of IL-10-producing CD4⁺ cells. * $P < .05$; ** $P < .01$.

Change in IL-10/IL-5 production after allergen challenge

In the early responders the frequency of IL-10-producing CD4⁺ cells, but not CD8⁺ cells, significantly increased after allergen challenge from 0.25% (IQR, 0.61%) to 0.68% (IQR, 0.92%; $P = .03$, Fig 2). By contrast, the frequencies of IL-10-producing CD4⁺ cells, but not CD8⁺ cells, significantly decreased after allergen challenge in the dual responders from 2.00% (IQR, 1.42%) to 0.60% (IQR, 0.88%; $P = .03$). For the CD4⁺ subset, the allergen-induced changes were significantly different between the groups ($P = .003$). Allergen-induced changes in the fre-

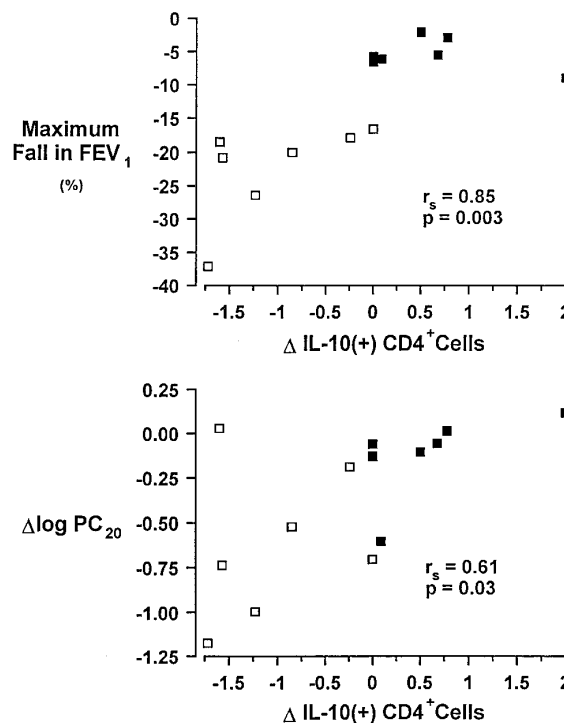


FIG 3. Correlations between allergen-induced changes in the frequency of IL-10-producing CD4⁺ cells and the maximal fall in FEV₁ during late response and allergen-induced change in log PC₂₀: filled squares, isolated early responders; open squares, dual responders.

quency of IL-10-producing CD4⁺ cells was significantly related to the maximal falls in FEV₁ during late response ($r_s = 0.85$, $P = .003$) and to the changes in methacholine PC₂₀ ($r_s = 0.61$, $P = .03$; Fig 3). However, no significant correlation was observed between the change in IL-10-producing CD4⁺ cells and the change in eosinophils ($r_s = -0.25$, $P = .38$). The frequencies of IL-5-producing CD4⁺ and CD8⁺ cells were not changed either in early responders or in dual responders.

DISCUSSION

This study has demonstrated that numbers of IL-10-producing CD4⁺ cells in the peripheral blood of dual responders are significantly higher than those in early responders before allergen challenge. Inhaled allergen caused a significant increase in IL-10-producing CD4⁺ cells in isolated early responders but a significant decrease in dual responders. By contrast, IL-5-producing T cells were not different between the groups before or after allergen challenge.

A previous study has reported an increased number of IL-10 mRNA-positive T cells in bronchoalveolar lavage (BAL) cells of atopic asthmatic subjects compared with that in healthy subjects.²⁰ We have recently shown an increase in IL-10-producing T cells in the peripheral blood of atopic asthmatic subjects compared with that in healthy subjects.²¹ In the present study the frequency of

TABLE II. Allergen-induced changes in sputum inflammatory cells

		Baseline	7 h after allergen challenge	24 h after allergen challenge
TCC (10 ⁶ /mL)	IER	2.4 (2.1)	3.1 (3.8)	2.3 (5.8)
	DR	3.0 (2.8)	9.4 (14.3)	6.1 (4.6)
	IER vs DR	<i>P</i> = .52	<i>P</i> = .08	<i>P</i> = .15
Eosinophils (%)	IER	2.5 (2.2)	14.8 (7.0)*	10.9 (4.8)*
	DR	2.0 (4.6)	19.8 (35.5)*	13.6 (23.6)*
	IER vs DR	<i>P</i> = .78	<i>P</i> = .57	<i>P</i> = .09
Macrophages (%)	IER	69.4 (36.6)	41.7 (7.0)	55.8 (18.5)
	DR	59.2 (59.1)	32.3 (43.2)	30.1 (29.4)
	IER vs DR	<i>P</i> = .63	<i>P</i> = .63	<i>P</i> = .42
Neutrophils (%)	IER	25.5 (33.4)	35.3 (23.4)	31.3 (29.3)
	DR	20.3 (53.8)	50.8 (78.7)	34.8 (24.6)
	IER vs DR	<i>P</i> = .87	<i>P</i> = .87	<i>P</i> = .63
Lymphocytes (%)	IER	1.4 (0.8)	1.3 (1.0)	1.8 (2.4)
	DR	1.3 (2.1)	1.4 (2.1)	2.2 (0.3)
	IER vs DR	<i>P</i> = .87	<i>P</i> = .93	<i>P</i> = .81
T-cell subset CD4 ⁺ cells (%)	IER	40.5 (14.3)	ND	43.3 (15.2)
	DR	44.0 (15.7)	ND	44.9 (6.9)
	IER vs DR	<i>P</i> = .09	ND	<i>P</i> = .13
T-cell subset CD8 ⁺ cells (%)	IER	20.4 (6.5)	ND	25.6 (8.6)
	DR	21.4 (10.8)	ND	17.8 (12.7)
	IER vs DR	<i>P</i> = .40	ND	<i>P</i> = .24

Values are presented as medians, with IQRs shown in parentheses.

TCC, Total sputum cell count; IER, isolated early responders; DR, dual responders.

**P* < .05, allergen-induced change from baseline.

IL-10–producing CD4⁺ cells was significantly higher in the dual responders than in the isolated early responders. This high frequency suggests an increased number of IL-10–producing T cells in the airways because T cells circulate between the affected organs and systemic vessels.¹³

The increases in sputum eosinophils in isolated early responders is consistent with previous observations from our laboratory²²; however, in previous studies the magnitude of the eosinophilic response and its persistence was greater in dual responders.⁴ It is known that late responses and the associated worsening of eosinophil inflammation are associated with T_H2-skewed activation of T cells.²³ Recently, Haselden et al²⁴ reported that intradermal administration of short peptides derived from the cat allergen Fel d 1, which did not cross-link IgE, elicited an isolated late response without an early response. This study suggests that late responses depend on allergen-specific T-cell responses but not necessarily on an IgE/mast cell-mediated early responses. Accordingly, the higher frequency of IL-10–producing CD4⁺ cells may reflect the more activated status of the T cell-dependent mechanism in the dual responders than in the isolated early responders. This is supported by a previous study showing that, at baseline, dual responders have a higher expression of intercellular adhesion molecule 1, a marker of T-cell activation, on blood CD4⁺ cells when compared with that seen in isolated early responders or healthy subjects.²⁵

The most intriguing result of this study is that allergen inhalation increased IL-10–producing CD4⁺ cells in early responders but decreased IL-10–producing CD4⁺ cells in dual responders. The number of IL-10 mRNA-positive T cells in BAL fluid is increased 24 hours after allergen challenge in atopic asthmatic subjects.²⁰ Also,

allergen inhalation increased IL-10 transcripts in BAL cells obtained during late responses.²⁶ Thus allergen inhalation increases IL-10 production in asthmatic airways. Two patterns of recruitment of IL-10–producing T cells may occur after allergen. One is trafficking of blood T cells into the airways. The other is the recirculation of airway T cells, which reflects active redistribution into other immune organs. Although these recruitments are potentially concurrent after allergen challenge, the overall change in IL-10–producing T cells appears to differ among phenotypes. The decrease of IL-10–producing T cells in the blood of the dual responders suggests a dominant traffic of circulating T cells into the airways. The profile of early responders may correspond with dominant traffic of airway T cells into the circulation. In addition, allergen-induced change in IL-10–producing CD4⁺ cells correlated with the fall in FEV₁ during late response and with the change in PC₂₀ but not with allergen-induced changes in sputum eosinophils. We have recently shown that there is an inverse relationship between the sputum cells positive for IL-10 and the percentage of sputum eosinophils measured 24 hours after allergen challenge.⁴ Further investigations are needed to elucidate the relationships between IL-10 production, allergen-induced airway eosinophilia, and airway responses.

Several studies have shown that IL-10 production from alveolar macrophages stimulated in vitro is suppressed in asthmatic subjects.^{26–28} It is tempting to speculate that there is a relative shortage of allergen-induced IL-10 production from resident airway T cells of the dual responders. This may necessitate recruitment of IL-10–producing CD4⁺ cells from the circulation, although this might not be sufficient to turn off the ongoing inflammation.

IL-5 has been suggested to be a key proinflammatory cytokine in allergic asthma.²⁹⁻³¹ However, no differences were seen in IL-5-producing T cells between the 2 phenotypes. These results are inconsistent with those of a previous report showing that IL-5-producing lymphocytes in the peripheral blood increased after grass pollen challenge in allergic asthmatic subjects.³² Differences in subjects, in procedures identifying each cell type, or in specifications of anti-IL-5 antibodies used might account for the discrepancy. Alternatively, our sampling for IL-5 might have been done at an earlier time point than 24 hours after allergen challenge because an experimental model of asthma has demonstrated the peak elevation of serum IL-5 at 6 to 12 hours after allergen challenge.³³

In summary, IL-10-producing CD4⁺ cells are increased in the peripheral blood of dual responders compared with in early responders. Allergen challenge decreased IL-10-producing CD4⁺ cells in dual responders but increased IL-10-producing CD4⁺ cells in early responders. These results suggest that this anti-inflammatory cytokine may have a role in attenuating allergen-induced airway responses in atopic subjects.

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