

Decrease of Interleukin-10-Producing T Cells in the Peripheral Blood of Severe Unstable Atopic Asthmatics

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Key Words

Atopic asthma · Flow cytometry · Interleukin-10 · Severity · T cells

Abstract

Background: Although IL-10 is known as an immunoregulatory cytokine produced by various cells including T cells, its basic profile in atopic asthma remains uncertain.

Objective: The profiles of IL-10 production in circulating CD4⁺ T cells of atopic asthmatics were investigated with respect to clinical severity. **Methods:** Forty atopic asthmatics were divided into three groups: mild, and severe but stable and severe unstable asthmatics. Eosinophils were counted in the peripheral blood and sputum, and exhaled nitric oxide was assessed. PBMCs were stimulated with or without anti-CD3 and anti-CD28 antibodies and then processed for detecting IL-10-producing CD4⁺ cells using flow cytometry. **Results:** There was no difference in the eosinophil count in blood or sputum and in nitric oxide level among the three groups. IL-10-producing CD4⁺ cells were mainly detected in a CD45RO⁺ memory population. The frequency of IL-10-producing cells after stimulation was significantly lower in the severe

unstable group compared to the mild group. In addition, the frequency of IL-10-producing cells in the severe unstable group was significantly lower than that in the severe stable group despite the fact that both groups received similar treatments with high-dose inhaled corticosteroids. The IL-10 production of CD4⁺CD45RO⁺ cells in response to dexamethasone did not differ among the three groups. **Conclusions:** IL-10-producing CD4⁺CD45RO⁺ cells in the peripheral blood are decreased in severe unstable asthmatics, which is not explained by the effect of high-dose inhaled corticosteroid medication.

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Introduction

Allergic inflammation in the airways is an important feature of atopic asthma, and the inflammatory process is mediated via various types of cytokines [1, 2]. Th2-type cytokines, interleukin (IL)-4, IL-5, and IL-13, are pro-allergic, while the Th1-type cytokine, interferon (IFN)- γ , has an anti-allergic property. Thus, Th2-skewed cytokine profiles have been a key concept in understanding asth-

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matic inflammation. However, previous studies showed that a passive transfer of antigen-specific Th1-skewed CD4⁺ T cells into antigen-sensitized mice not only failed to attenuate but also exacerbated airway inflammation after an antigen challenge [3, 4]. The results made it difficult to understand the asthmatic inflammation based on the simple counterbalance paradigm of Th1 versus Th2. In this regard, several studies have identified additional populations of T cells showing regulatory properties in the various Th1- and Th2-type inflammations [5–8]. A part of the populations reportedly plays a role through selective production of IL-10 [9–11]. IL-10 markedly inhibits the expression of the MHC class II molecule and costimulatory molecules on antigen-presenting cells, the proliferation of activated T cells by reducing IL-2 production, and the release of inflammatory cytokines and nitric oxide from the activated macrophage/monocyte lineage. Therefore, IL-10 is expected to have strong anti-inflammatory properties. The effects of IL-10 deficiency on eosinophilic airway inflammation and bronchial hyperreactivity in experimental asthma models were quite different among previous studies [12–14]. Although much care should be taken with their interpretation and relevance for human diseases [15], those basic studies may raise the possibility of whether IL-10 is substantially associated with the regulation of atopic asthma.

Clinical studies reported that IL-10 production was impaired in a monocyte/macrophage lineage of adult asthmatics compared with healthy controls [16, 17]. IL-10 mRNA expression by purified T cells of children with asthma was reportedly decreased as compared with that of healthy controls [18]. On the other hand, a study reported an increase of IL-10-producing macrophages in an asthmatic airway [19]. IL-10 concentrations in bronchial lavage fluid were reported to increase in status asthmaticus under mechanical ventilation when compared with stable asthmatics and healthy controls [20]. This inconsistency might be due to a difference in patient characteristics among the studies, including the clinical severity and the content of medications. In addition, many studies have evaluated the potency of IL-10 production from lipopolysaccharide-stimulated macrophages/monocytes. Considering that T cells have an orchestrating role in the pathogenesis of allergy, the profile of IL-10 production in T cells of atopic asthmatics is an important target for investigation.

T cells circulate systemically from the target organs to the bloodstream via the lymphatic system [21, 22]. This property provides a unique window for the detection of cytokine kinetics of T cells in asthmatic subjects using

peripheral blood samples. Indeed, we reported that the kinetics of IL-10-producing CD4⁺ T cells in the peripheral blood after an allergen challenge was different between isolated early and dual responders [23]. Here, we analyzed the profiles of IL-10 production in circulating CD4⁺ cells in atopic asthmatics using flow cytometry [24–26] and then evaluated the relationship between the cytokine profile and the severity of asthma.

Patients and Methods

Patients and Protocol

Forty nonsmoking patients with atopic asthma were recruited and classified into two groups, 20 mild and 20 severe asthmatics, according to the Global Initiative for Asthma [27]. Atopy was assessed by a radioallergosorbent test to at least two common aeroallergens. Mild intermittent and mild persistent asthmatics were merged into one group identified as the mild group. Eleven of the 20 patients in the mild group were treated with a low dose of an inhaled steroid (400 µg/day corresponding dose of beclomethasone dipropionate; BDP), and the remainder were treated occasionally with an inhaled short-acting β_2 -agonist. All severe asthmatics were treated with a high dose of an inhaled corticosteroid (>1,200 µg/day corresponding dose of BDP) for more than 1 year. They were further classified into a severe stable group (n = 10) and a severe unstable group (n = 10). The severe stable group consisted of patients free from asthmatic symptoms for more than 3 months and showing good pulmonary function [forced expired volume in 1 s (FEV₁) >80% of predicted]. The severe unstable group consisted of patients with frequent symptoms who needed rescue inhalation of a short-acting β_2 -agonist or with lower pulmonary function (FEV₁ <60% of predicted). The profile of the severe unstable group met the typical features of refractory asthma presented by the workshop of the American Thoracic Society [28]. There was no significant difference in the daily dose of inhaled corticosteroids between the severe stable and unstable groups (1,300 ± 245 µg/day vs. 1,356 ± 313 µg/day; corresponding dose of BDP, respectively). Four patients in the severe stable group and 6 patients in the severe unstable group were treated with slow-releasing theophylline in addition to inhaled corticosteroids. None of the patients was treated with oral corticosteroids or a long-acting β_2 -agonist. The project was approved by the institutional ethics committee, and informed consent was obtained from all participants. At the time of enrollment, patient characteristics were assessed according to a detailed questionnaire. The age at onset of asthma was judged as accurately as possible and used to calculate the duration of asthma. All the measurements were performed on the same visit day. Medication with inhaled corticosteroids and/or a short-acting β_2 -agonist was withheld for 12 h before the measurements. Oral theophylline was discontinued 24 h before the measurements. Firstly, a blood sample was taken and immediately processed for white blood cell counts and cell culture. The subjects consecutively underwent measurements of exhaled nitric oxide (NO), spirometry, and sputum induction.

Cell Cultures and Staining

PBMCs were isolated by Lymphoprep (Nycomed, Oslo, Norway) density gradient separation and washed twice with RPMI-1640 con-

taining *L*-glutamine and NaHCO₃ (Sigma, St. Louis, Mo., USA). Cells were suspended in RPMI-1640 with 10% FCS (Sigma) and penicillin/streptomycin (Gibco, Paisley, UK) at a density of 2×10^6 cells/ml. Cells were then cultured in 24-well culture plates (Cellstar; Greiner Labortechnik, Frickenhausen, Germany) in a volume of 1 ml in the presence or absence of stimulation with a soluble anti-CD3 monoclonal antibody (mAb; HIT3a, 1 µg/ml) plus a soluble anti-CD28 mAb (CD28.2, 1 µg/ml) for 24 h at 37°C in 5% CO₂ in an incubator. To inhibit cytokine secretion, monensin (GolgiStop™; BD-PharMingen, San Diego, Calif., USA) at 0.7 µl/ml was added during the last 4 h of incubation. After harvesting, the cells were washed with a washing buffer consisting of PBS with 0.5% bovine serum albumin (Sigma) and 0.1% sodium azide (Sigma). The samples at a density of 1×10^6 cells in 100 µl of washing buffer with 5% normal mouse serum (Cosmo Bio, Tokyo, Japan) were incubated for 10 min. Surface staining was performed using PerCP-anti-CD4 mAb (Leu-3a), FITC-anti- and CD45RO mAb (UCHL1) for 30 min. For intracellular cytokine staining [24–26], the cells were fixed with fixation buffer (Caltag, Burlingame, Calif., USA) for 20 min. After an additional wash, the cells were suspended in a permeabilization buffer (Caltag) with 5% normal rat serum (Cosmo Bio) for 10 min and then added to an allophycocyanin-anti-IL-10 mAb (JES3-19F1) or isotype control IgG2a for 30 min. Paired samples were incubated in the presence of anti-CD3 mAb plus an anti-CD28 mAb, with or without dexamethasone (10^{-7} M), and then processed for intracellular IL-10 staining. All monoclonal antibodies were purchased by BD-PharMingen. The dose of dexamethasone was determined based on the preliminary study, as 10^{-7} M had been almost compatible with IC₅₀ for the IL-10 production in CD4+ cells.

Flow Cytometry

A FACScalibur flow cytometer (Becton-Dickinson, San Jose, Calif., USA) with CellQuest software (Becton-Dickinson) was used. Thirty thousand events were acquired in list mode with debris excluded by the forward scatter threshold. An analysis gate was set on the lymphocyte population based on the forward and side scatter plot. The gated population was analyzed for the identification of CD4+CD45RO+ (memory phenotype) or CD4+CD45RO- (naive phenotype). The frequency of IL-10-producing cells from cultures without stimulation was regarded as the baseline IL-10 production, whilst that from cultures with stimulation was regarded as the potency of IL-10 production in response to polyclonal T-cell receptor ligation.

Measurement of Exhaled NO and Spirometry

The exhaled NO was measured using a chemiluminescence NO analyzer (Sievers 280 NOA, Boulder, Colo., USA) according to the official statement of the American Thoracic Society [29]. Exhaled air was sampled via a side port close to the mouth, and the concentration of NO was displayed on the screen of a computer. The endpoint for the measurement was defined when a plateau lasting at least 5 seconds was obtained. Repeated exhalations were recorded to give three values of NO plateaus within 10% of each other. Spirometry data were recorded by a computed spirometer (D-21FX; Chest, Tokyo, Japan) using the standard technique. Measurements were made in triplicate, and the best values of forced vital capacity and FEV₁ were chosen for analysis.

Table 1. Patient characteristics and inflammatory parameters

	Mild (n = 20)	Severe stable (n = 10)	Severe unstable (n = 10)
Males/females	11/9	6/4	5/5
Age, years	42 ± 15	46 ± 13	50 ± 8
Duration, years	11 ± 8	11 ± 7	12 ± 7
Percent FEV ₁	99.3 ± 13.1	77.0 ± 10.8*	51.0 ± 12.4*
Blood eosinophils, %	6.6 ± 5.1	6.5 ± 6.0	6.6 ± 6.4
Sputum eosinophils, %	16 ± 9	20 ± 18	26 ± 17
Exhaled NO, ppb	34.6 ± 17.1	30.0 ± 13.6	25.5 ± 9.8

Means ± SD. * $p < 0.05$ compared with the mild group; no significant differences were found for the other values among the three subject groups.

Sputum Induction and Processing

Sputum induction and its processing were performed by the method described previously [30]. Two hundreds non-squamous cells were examined in a blinded fashion. Eosinophil counts were expressed as percentages of the total non-squamous cell count. The reproducibility of this sputum measurement has been reported previously [30].

Statistical Analysis

Statistical analysis was done with statistical software (Stat View J4.02; Abacus Concepts, Berkeley, Calif., USA). Data with normal distribution were expressed as means ± SD, while nonparametric data were expressed as the median and interquartile range. Comparisons between groups were performed using the Kruskal-Wallis test followed by ad-hoc test with the Mann-Whitney U test. Values of p less than 0.05 were accepted as being statistically significant.

Results

Patient Characteristics and Inflammatory Parameters

Patient characteristics and inflammatory parameters are shown in table 1. There was no significant difference in age, sex or duration of asthma among the three groups. Values of percent FEV₁ in both severe stable and unstable groups were significantly lower than in the mild group. In addition, values of percent FEV₁ in the severe unstable group were significantly lower than that in the severe stable group. Two patients in the mild group and 1 patient from each the severe stable and unstable groups were unable to provide sputum samples for differential cell counts. There was no significant difference in the percentage of blood eosinophils, sputum eosinophils, or exhaled NO level among the three groups.

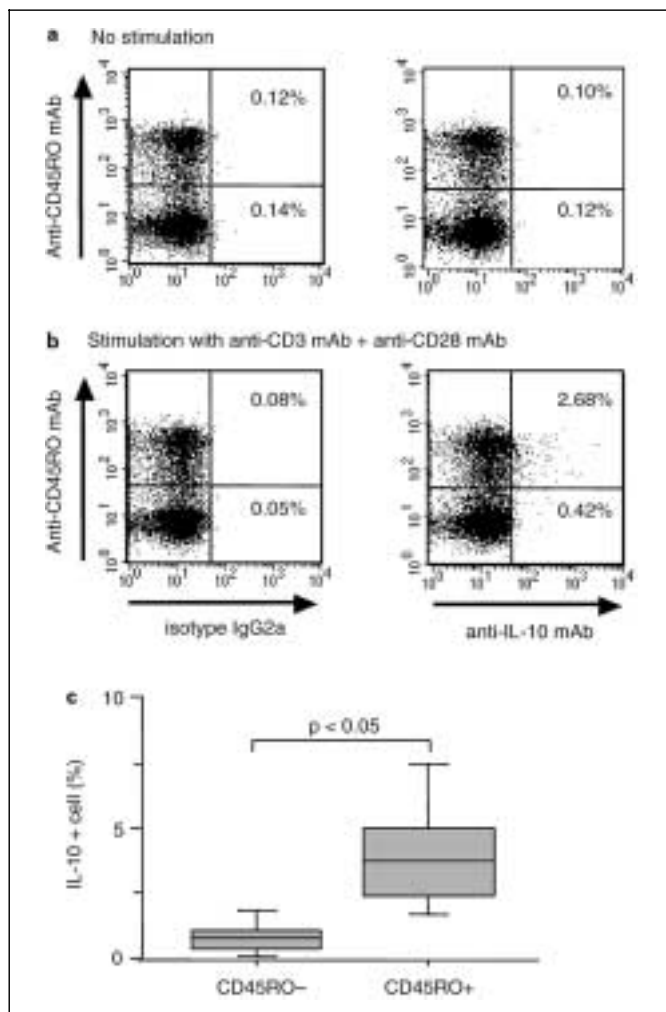


Fig. 1. IL-10-producing cells in cultured PBMCs. **a, b** Typical dot plots are shown from samples of a subject with mild asthma. PBMCs were cultured for 24 h in the presence (**b**) or absence (**a**) of anti-CD3 plus anti-CD28 antibodies and processed for flow cytometry. IL-10-producing cells were assessed for CD4+CD45RO– and CD4+CD45RO+ subsets. Samples with isotype IgG2a served as controls. **c** Frequencies of IL-10-producing cells in the CD4+CD45RO– and CD4+CD45RO+ subsets are compared using total samples of asthmatics ($n = 40$). The boxes span the 25th and 75th percentiles, with a median noted by the inside bar. The length of the whisker is based on the 10th and 90th percentiles.

Table 2. Lymphocyte subsets (%) among the groups

	Mild ($n = 20$)	Severe stable ($n = 10$)	Severe unstable ($n = 10$)
CD4+ cells (in PBMCs)	31.6 (9.4)	37.5 (14.6)	36.0 (12.4)
CD4+CD45RO+ cells (in PBMCs)	11.8 (4.6)	12.0 (4.4)	11.8 (6.4)

Significant differences were not found among the three study groups. Medians with inter-quartile ranges (in parentheses).

Intracellular IL-10 in CD4+ Cells with Reference to Asthma Severity

There was no significant difference in the frequencies of the CD4+ population, CD4+CD45RO+ population or CD4+CD45RO– population in PBMCs among the three groups (table 2). The frequency of IL-10-producing CD4+ cells in cultured PBMCs were assessed for CD4+CD45RO+ (memory) and CD4+CD45RO– (naive) subsets (fig. 1). At the unstimulated baseline, the IL-10 production level was quite limited, typically below 1% of the total CD4+ cells. When activated with anti-CD3 mAb and anti-CD28 mAb, the frequency of IL-10-producing CD4+ cells was significantly increased both in the CD45RO+ subset and in the CD45RO– subset, while the CD45RO+ subset significantly dominated. Therefore, the analysis of IL-10-producing cells in the following study was focused on the CD4+CD45RO+ subset (fig. 2).

The frequency of IL-10-producing CD4+CD45RO+ cells in the severe unstable group, but not in the severe stable group, was significantly lower than that in the mild group in the absence of stimulation. Even after stimulation, the frequency of IL-10-producing CD4+CD45RO+ cells in the severe unstable group, but not in the severe stable group, was significantly lower than that in the mild group. Furthermore, the frequency of IL-10-producing CD4+CD45RO+ cells in the severe unstable group was significantly lower than that in the severe stable group.

In vitro Responsiveness of IL-10-Producing Cells to Dexamethasone

To elucidate whether T-cell responsiveness to corticosteroids might be different among the patient groups, the effect of dexamethasone was examined for the frequency of IL-10-producing CD4+CD45RO+ cells in vitro (fig. 3). Although the stimulation of PBMCs in the presence of dexamethasone (10^{-7} M) significantly decreased the frequency of IL-10-producing CD4+CD45RO+ cells, the inhibitory effect of dexamethasone was not different among the three groups.

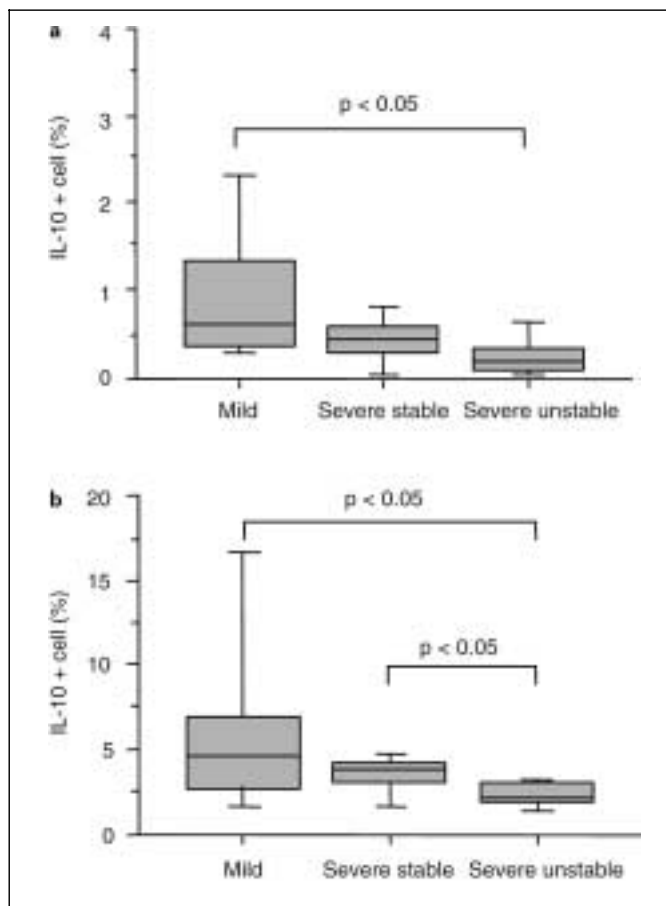


Fig. 2. Frequencies of IL-10-producing cells in the CD4+CD45RO+ subset among subject groups. PBMCs were cultured for 24 h in the presence (**b**) or absence (**a**) of anti-CD3 plus anti-CD28 antibodies and processed for flow cytometry. IL-10-producing cells were assessed for the CD4+CD45RO+ subset. The boxes span the 25th and 75th percentiles, with the median noted by the inside bar. The length of the whisker is based on the 10th and 90th percentiles.

Discussion

In the present study, IL-10-producing cells were mainly detected in the CD4+CD45RO+ subset, the so-called memory subset of helper T cells. Intriguingly, the frequency of IL-10-producing CD4+CD45RO+ cells was significantly decreased in the peripheral blood of the severe unstable group compared with the mild and severe stable groups. The decreased frequency likely reflects the decrease in the absolute number of this subset in the peripheral blood of the severe unstable group because there was no difference in the frequency of CD4+CD45RO+ cells in PBMCs among the three groups. This impairment may not be allergen specific, as the decreased frequency of IL-

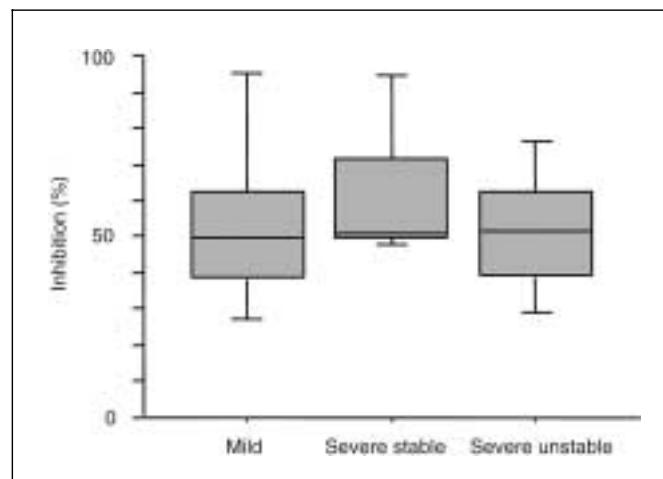


Fig. 3. Effects of dexamethasone on the production of IL-10 among subject groups. PBMCs were stimulated with anti-CD3 plus anti-CD28 antibodies for 24 h in the presence or absence of dexamethasone (10^{-7} M) and processed for the detection of IL-10-producing CD4+CD45RO+ cells. Values are expressed as a percent inhibition of the paired control obtained from a culture in the absence of dexamethasone. The boxes span the 25th and 75th percentiles, with the median noted by the inside bar. The length of the whisker is based on the 10th and 90th percentiles.

10-producing cells was shown using specimens after polyclonal T cell receptor activation.

The arising question is whether the decrease in IL-10-producing cells is substantially associated with the pathogenesis of severe unstable asthma. In this regard, the effect of inhaled corticosteroids should be carefully considered. It is known that the production of cytokines is generally inhibited by the high concentration of corticosteroids [31–34]. Because severe asthmatics in this study had been treated with high-dose inhaled corticosteroids for at least 1 year, the lower frequency of IL-10-producing cells might merely reflect a dose-dependent inhibition by corticosteroids. However, the frequency of IL-10-producing CD4+CD45RO+ cells in the severe unstable group was significantly lower than that in the severe stable group despite the fact that both groups had taken a similar daily dose of inhaled corticosteroids. In addition, the *in vitro* responsiveness of CD4+CD45RO+ cells to dexamethasone was not different among three groups. The remaining concern is that the concentration of dexamethasone in the present experiment might be higher than the actual concentration of corticosteroids attained in usual medications for asthmatic airways. A previous study reported that preincubation of peripheral blood T cells in the presence of a relatively low concentration of dexamethasone enhanced IL-10

production from T cells after a secondary T cell receptor ligation in the absence of dexamethasone [35]. According to the report, IL-10-producing cells are expected to increase during treatment with inhaled corticosteroids.

Although several patients had been treated with slow-release theophylline in combination with inhaled corticosteroids, the number of such patients in each group did not significantly differ between the stable and unstable groups. The notable difference in medical history was that the patients with severe unstable asthma had been forced to inhale short-acting β_2 -agonists more frequently. Previous studies showed that cyclic AMP-elevating compounds, such as xanthine derivatives and β_2 -agonists had a stimulatory, rather than an inhibitory, effect on IL-10 production from inflammatory cells [36–39], which makes it unlikely that the frequent use of β_2 -agonists might lead to the decrease in IL-10-producing cells in PBMCs of the severe unstable asthmatics.

The use of inhaled corticosteroids has long been the mainstay of asthma medication, and this therapeutic strategy was derived from a significant amount of evidence indicating that inhaled corticosteroids have a strong anti-inflammatory effect on asthmatic airways with limited adverse effects [27]. Most patients are relieved from symptoms and able to improve their quality of life through the use of inhaled corticosteroids. However, 5–10% of patients remain symptomatic or have persistent airflow limitations despite treatment with a high-dose inhaled corticosteroids. These patients have been an intensive target of clinical investigations and have recently been characterized as a clinical phenotype of refractory asthma or difficult/therapy-resistant asthma [28, 40]. Although many factors contribute to the development of such a clinical phenotype, the profile of airway inflammation in those patients is an important issue to be addressed. In the present study, the percentage of sputum eosinophils and the exhaled NO level, both known to be surrogate markers of asthmatic airway inflammation, were not different among the mild, severe stable, and severe unstable groups. This suggests that the severity and stability of asthma might not be simply explained by the extent of an eosinophilic airway inflammation or NO production. Indeed, previous studies reported that exhaled NO level was well associated with clinical parameters in steroid-naïve patients but not in steroid-treated patients [41, 42].

Previous studies have reported that IL-10 production in response to lipopolysaccharides was impaired in a monocyte/macrophage lineage of asthmatics [16, 17]. On the contrary, other studies reported an increase in IL-10-producing T cells and macrophages in asthmatic airways and that

those cells were further increased after an allergen challenge [19, 43, 44]. This inconsistency among previous studies may partly be explained by the difference in patient history or the difference in experimental protocols through which specimens were obtained. The former difference is supported by our present investigation that lower potency of IL-10 production is associated with the phenotype of severe unstable asthma. This lower potency is not restricted to T cells because a recent study reported that the production of IL-10 in monocytes was also impaired in severe asthmatics [45]. The mechanism of the impairment might be explained by genetic background, since IL-10 production is partly determined via single nucleotide polymorphisms on the promoter regions [46, 47]. One study has already reported a possible association of single nucleotide polymorphisms of IL-10 with asthma severity [48]. Accumulating evidence suggests that the impaired production of IL-10 may be a contributing factor of severe unstable asthma.

Although cytokine flow cytometry is a useful procedure for investigating the cytokine profile and kinetics in clinical specimens consisting of heterogeneous cell populations, there must be several points to limit its relevance. The population of peripheral CD4+CD45RO+ T cells analyzed in the present study is polyclonal and had been polyclonally stimulated *in vitro* for the analysis of cytokine production. The population of IL-10-producing cells that migrate to sites of airway inflammation is likely to be present among peripheral blood T cells, but it undoubtedly constitutes a minority. This issue may be more critical when comparing the results obtained here to those in previous studies using specimens obtained from airways for ELISA. Therefore, it remains to be elucidated whether the decreased IL-10-producing cells found in peripheral blood actually reflect the profile of T-cell subpopulations in the airways of severe unstable asthmatics.

In summary, IL-10-producing memory CD4+ cells in the peripheral blood decreased more in severe unstable asthmatics than in the mild and severe stable asthmatics, which is not explained by the treatment with high-dose inhaled corticosteroids. Although the relatively small sample size might limit the interpretation of results, the present study suggests that the profile of IL-10 production is an attractive target of further investigation in order to understand the pathogenesis of severe unstable asthma.

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References

- Chung KF, Barnes PJ: Cytokines in asthma. *Thorax* 1999;54:825–857.
- Umetsu DT, DeKruyff RH: TH1 and TH2 CD4+ cells in human allergic diseases. *J Allergy Clin Immunol* 1997;100:1–6.
- Hansen G, Berry G, DeKruyff RH, Umetsu DT: Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J Clin Invest* 1999;103:175–183.
- Randolph DA, Stephens R, Carruthers CJ, Chaplin DD: Cooperation between Th1 and Th2 cells in a murine model of eosinophilic airway inflammation. *J Clin Invest* 1999;104:1021–1029.
- Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL: Regulatory T cell clones induced by oral tolerance: Suppression of autoimmune encephalomyelitis. *Science* 1994;265:1237–1240.
- Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG: A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997;389:737–742.
- Yssel H, Groux H: Characterization of T cell subpopulations involved in the pathogenesis of asthma and allergic diseases. *Int Arch Allergy Immunol* 2000;121:10–18.
- Shevach EM: Certified professionals: CD4(+)CD25(+) suppressor T cells. *J Exp Med* 2001;193:F41–F46.
- Borish L: IL-10: Evolving concepts. *J Allergy Clin Immunol* 1998;101:293–297.
- Pretolani M: Interleukin-10: An anti-inflammatory cytokine with therapeutic potential. *Clin Exp Allergy* 1999;29:1164–1171.
- Umetsu DT, DeKruyff RH: Interleukin-10: The missing link in asthma regulation? *Am J Respir Cell Mol Biol* 1999;21:562–563.
- Justice JP, Shibata Y, Sur S, Mustafa J, Fan M, Van Scott MR: IL-10 gene knockout attenuates allergen-induced airway hyperresponsiveness in C57BL/6 mice. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L363–368.
- Makela MJ, Kanehiro A, Borish L, Dakhama A, Loader J, Joetham A, Xing Z, Jordana M, Larsen GL, Gelfand EW: IL-10 is necessary for the expression of airway hyperresponsiveness but not pulmonary inflammation after allergic sensitization. *Proc Natl Acad Sci USA* 2000;97:6007–6012.
- Tournoy KG, Kips JC, Pauwels RA: Endogenous interleukin-10 suppresses allergen-induced airway inflammation and nonspecific airway responsiveness. *Clin Exp Allergy* 2000;30:775–783.
- Epstein MM: Do mouse models of allergic asthma mimic clinical disease? *Int Arch Allergy Immunol* 2004;133:84–100.
- Borish L, Aarons A, Rumblyrt J, Cvietusa P, Negri J, Wenzel S: Interleukin-10 regulation in normal subjects and patients with asthma. *J Allergy Clin Immunol* 1996;97:1288–1296.
- John M, Lim S, Seybold J, Jose P, Robichaud A, O'Connor B, Barnes PJ, Chung KF: Inhaled corticosteroids increase interleukin-10 but reduce macrophage inflammatory protein-1 α , granulocyte-macrophage colony-stimulating factor, and interferon-gamma release from alveolar macrophages in asthma. *Am J Respir Crit Care Med* 1997;157:256–262.
- Koning H, Neijens HJ, Baert MR, Oranje AP, Savelkoul HF: T cells subsets and cytokines in allergic and non-allergic children. II. Analysis and IL-5 and IL-10 mRNA expression and protein production. *Cytokine* 1997;9:427–436.
- Magnan A, van Pee D, Bongrand P, Vervloet D: Alveolar macrophage interleukin (IL)-10 and IL-12 production in atopic asthma. *Allergy* 1998;53:1092–1095.
- Tillie-Leblond I, Pugin J, Marquette CH, Lamblin C, Saulnier F, Brichet A, Wallaert B, Tonnel AB, Gosset P: Balance between proinflammatory cytokines and their inhibitors in bronchial lavage from patients with status asthmaticus. *Am J Respir Crit Care Med* 1999;159:487–494.
- Krug N, Tschernig T, Holgate S, Pabst R: How do lymphocytes get into the asthmatic airways? Lymphocyte traffic into and within the lung in asthma. *Clin Exp Allergy* 1998;28:10–18.
- Westermann J, Engelhardt B, Hoffmann JC: Migration of T cells in vivo: Molecular mechanisms and clinical implications. *Ann Intern Med* 2001;135:279–295.
- Matsumoto K, Gauvreau GM, Rerecich T, Watson RM, Wood LJ, O'Byrne PM: IL-10 production in circulating T cells differs between allergen-induced isolated early and dual asthmatic responders. *J Allergy Clin Immunol* 2002;109:281–286.
- Krug N, Jung T, Napp U, Wagner K, Schultze-Werninghaus G, Heusser C, Rieger CH, Schauer U, Fabel H: Frequencies of T cells expressing interleukin-4 and interleukin-5 in atopic asthmatic children. Comparison with atopic asthmatic adults. *Am J Respir Crit Care Med* 1998;158:754–759.
- Prussin C: Cytokine flow cytometry: Understanding cytokine biology at the single-cell level. *J Clin Immunol* 1997;17:195–204.
- Magnan AO, Mely LG, Camilla CA, Badier MM, Montero-Julian FA, Guillot CM, Casano BB, Prato SJ, Fert V, Bongrand P, et al: Assessment of the Th1/Th2 paradigm in whole blood in atopy and asthma. Increased IFN-gamma-producing CD8(+) T cells in asthma. *Am J Respir Crit Care Med* 2000;161:1790–1796.
- Anonymous: Asthma management and prevention. Global Initiative for Asthma. *Ir Med J* 2000;suppl-vi:1–39.
- Anonymous: Proceedings of the ATS workshop on refractory asthma: Current understanding, recommendations, and unanswered questions. American Thoracic Society. *Am J Respir Crit Care Med* 2000;162:2341–2351.
- Anonymous: Recommendations for standardized procedures for the on-line and off-line measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide in adults and children – 1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. *Am J Respir Crit Care Med* 1999;160:2104–2117.
- Inoue H, Aizawa H, Fukuyama S, Takata S, Matsumoto K, Shigyo M, Koto H, Hara N: Effect of inhaled glucocorticoid on the cellular profile and cytokine levels in induced sputum from asthmatic patients. *Lung* 1999;177:53–62.
- Brinkmann V, Kristofic C: Regulation by corticosteroids of Th1 and Th2 cytokine production in human CD4+ effector T cells generated from CD45RO– and CD45RO+ subsets. *J Immunol* 1995;155:3322–3328.
- Fushimi T, Okayama H, Seki T, Shimura S, Shirato K: Dexamethasone suppressed gene expression and production of interleukin-10 by human peripheral blood mononuclear cells and monocytes. *Int Arch Allergy Immunol* 1997;112:13–18.
- Hodge S, Hodge G, Flower R, Han P: Methylprednisolone up-regulates monocyte interleukin-10 production in stimulated whole blood. *Scand J Immunol* 1999;49:548–553.
- Visser J, van Boxel-Dezaire A, Methorst D, Brunt T, de Kloet ER, Nagelkerken L: Differential regulation of interleukin-10 (IL-10) and IL-12 by glucocorticoids in vitro. *Blood* 1998;91:4255–4264.
- Hawrylowicz C, Richards D, Loke TK, Corrigan C, Lee T: A defect in corticosteroid-induced IL-10 production in T lymphocytes from corticosteroid-resistant asthmatic patients. *J Allergy Clin Immunol* 2002;109:369–370.
- Nemeth ZH, Hasko G, Szabo C, Vizi ES: Amrinone and theophylline differentially regulate cytokine and nitric oxide production in endotoxemic mice. *Shock* 1997;7:371–375.
- Szelenyi J, Kiss JP, Puskas E, Selmecey Z, Szelenyi M, Vizi ES: Opposite role of α_2 - and β -adrenoceptors in the modulation of interleukin-10 production in endotoxaemic mice. *Neuroreport* 2000;11:3565–3568.
- Kambayashi T, Wallin RP, Ljunggren HG: cAMP-elevating agents suppress dendritic cell function. *J Leukoc Biol* 2001;70:903–910.
- Suberville S, Bellocq A, Fouqueray B, Philippe C, Lantz O, Perez J, Baud L: Regulation of interleukin-10 production by beta-adrenergic agonists. *Eur J Immunol* 1996;26:2601–2605.
- Chung KF, Godard P, Adelroth E, Ayres J, Barnes N, Barnes P, Bel E, Burney P, Chaney P, Connett G, et al: Difficult/therapy-resistant asthma: The need for an integrated approach to define clinical phenotypes, evaluate risk factors, understand pathophysiology and find novel therapies. ERS Task Force on Difficult/Therapy-Resistant Asthma. European Respiratory Society. *Eur Respir J* 1999;13:1198–1208.

- 41 Jatakanon A, Lim S, Kharitonov SA, Chung KF, Barnes PJ: Correlation between exhaled nitric oxide, sputum eosinophils, and methacholine responsiveness in patients with mild asthma. *Thorax* 1998;53:91–95.
- 42 Lim S, Jatakanon A, Meah S, Oates T, Chung KF, Barnes PJ: Relationship between exhaled nitric oxide and mucosal eosinophilic inflammation in mild to moderately severe asthma. *Thorax* 000;55:184–188.
- 43 Colavita AM, Hastie AT, Musani AI, Pascual RM, Reinach AJ, Lustine HT, Galati SA, Zangrilli JG, Fish JE, Peters SP: Kinetics of IL-10 production after segmental antigen challenge of atopic asthmatic subjects. *J Allergy Clin Immunol* 2000;106:880–886.
- 44 Robinson DS, Tsicopoulos A, Meng Q, Durham S, Kay AB, Hamid Q: Increased interleukin-10 messenger RNA expression in atopic allergy and asthma. *Am J Respir Cell Mol Biol* 1996;14:113–117.
- 45 Tomita K, Lim S, Hanazawa T, Usmani O, Stirling R, Chung KF, Barnes PJ, Adcock IM: Attenuated production of intracellular IL-10 and IL-12 in monocytes from patients with severe asthma. *Clin Immunol* 2002;102:258–266.
- 46 Turner DM, Williams DM, Sankaran D, Lazarus M, Sinnott PJ, Hutchinson IV: An investigation of polymorphism in the interleukin-10 gene promoter. *Eur J Immunogenet* 1997;24:1–8.
- 47 Hobbs K, Negri J, Klennert M, Rosenwasser LJ, Borish L: Interleukin-10 and transforming growth factor-beta promoter polymorphisms in allergies and asthma. *Am J Respir Crit Care Med* 1998;158:1958–1962.
- 48 Lim S, Crawley E, Woo P, Barnes PJ: Haplotype associated with low interleukin-10 production in patients with severe asthma. *Lancet* 1998;352:113.