Different profile of interleukin-10 production in circulating T cells from atopic asthmatics compared with healthy subjects

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BACKGROUND: Interleukin (IL)-10 is a pleiotropic cytokine released from various cells, including T cells. Although IL-10 is suggested to inhibit allergic responses, its role in asthma remains uncertain. The purpose of the present study was to compare the profile of IL-10 in circulating T cells from stable atopic asthmatics, atopic nonasthmatics and healthy controls.

METHODS: Peripheral blood mononuclear cells were isolated, stained with anti-CD3 and CD4/CD8 antibodies, and then processed for intracellular IL-10 detection by flow cytometry.

RESULTS: A kinetic study in healthy controls showed that stimulation with phorbol 12-myristate 13-acetate and ionomycin significantly increased the frequencies of IL-10-producing $CD3^+$, $CD4^+$ and $CD8^+$ cells. Without stimulation, the frequencies of IL-10-producing $CD3^+$, $CD4^+$ and $CD8^+$cells were significantly higher in asthmatics than in healthy controls, while a similar trend was observed in atopic nonasthmatics. Stimulation for 24 h significantly increased IL-10-producing $CD3^+$, $CD4^+$ and $CD8^+$cells in healthy controls and atopic nonasthmatics, but not in asthmatics.

CONCLUSIONS: The frequency of IL-10-producing T cells is increased in the circulation of stable atopic asthmatics compared with normal controls. The lack of enhancement in their frequency by phorbol 12-myristate 13-acetate and ionomycin in asthmatics suggests that the circulating T cells of asthmatic subjects are maximally stimulated with regards to IL-10 production; alternatively, IL-10 production by T cells from asthmatics may be regulated differently than T cells from other subjects.

Key Words: Asthma; Atopy; Interleukin-10; T cells

Un profil différent de production d'interleukine-10 dans les lymphocytes T circulants d'asthmatiques atopiques par rapport à des sujets en santé

HISTORIQUE : L'interleukine (IL)-10 est une cytokine pléïotropique libérée par diverses cellules, y compris les lymphocytes T. Bien qu'on pense que l'IL-10 inhibe les réponses allergiques, son rôle dans l'asthme demeure incertain. La présente étude visait à comparer le profil de l'IL-10 dans les lymphocytes T circulants chez des asthmatiques atopiques stables, des non-asthmatiques atopiques et des sujets en santé.

MÉTHODOLOGIE : Des cellules sanguines mononucléaires périphériques ont été isolées, colorées par des anticorps anti-CD3 et anti-CD4 et CD8, puis traitées pour déceler l'IL-10 intracellulaire par cytométrie de flux.

RÉSULTATS : Une étude cinétique des sujets en santé a révélé qu'une stimulation par phorbol 12-myristate 13-acétate et par ionomycine accroît de manière considérable la fréquence de cellules $CD3^+$, $CD4^+$ et $CD8^+$ produisant de l'IL-10. Sans stimulation, les cellules $CD3^+$, $CD4^+$ et $CD8^+$ produisaient beaucoup plus souvent de l'IL-10 chez les asthmatiques que chez les sujets en santé, tandis qu'une tendance similaire s'observait chez les non-asthmatiques atopiques. Une stimulation pendant 24 heures accroissait de manière considérable le nombre de cellules $CD3^+$, $CD4^+$ et $CD8^+$ produisant de l'IL-10 chez les sujets en santé et chez les non-asthmatiques atopiques, mais pas chez les asthmatiques.

CONCLUSIONS : La fréquence de lymphocytes T produisant de l'IL-10 s'accroît dans la circulation des asthmatiques atopiques stables par rapport aux sujets en santé. L'absence d'augmentation de la fréquence par phorbol 12-myristate 13-acétate et par ionomycine chez les asthmatiques laisse supposer que les lymphocytes T circulants des asthmatiques sont stimulés au maximum en regard de la production d'IL-10. La production d'IL-10 par les lymphocytes T des asthmatiques pourrait également être régie différemment des lymphocytes T d'autres sujets.

Allergic airway inflammation is an important characteristic of atopic asthma. Proinflammatory cells, including antigen-presenting cells (APCs), eosinophils, mast cells, basophils and lymphocytes contribute to the development and persistence of the inflammation (1). There is evidence that the T helper (Th)2-type cytokines such as interleukin (IL)-4 or IL-5 have a role in the pathogenenesis of asthma (2). By contrast, the Th1-type cytokine interferon (IFN)-γ has an antiallergic role (3). However, recent studies showing that passive transfer of ovalbumin (OVA)-specific, Th1-skewed, $CD4^+$ T cells into OVA-sensitized mice failed to attenuate, but rather exacerbated, airway inflammation after OVA challenge have made it difficult to understand the asthmatic inflammation based on the simple counterbalance theory of Th1 versus Th2 (4). Thus, both Th1- and Th2-skewed responses in asthmatics may be regulated by more complex mechanisms than previously estimated. These mechanisms likely involve regulatory cytokines, including tranforming growth factor-beta and IL-10 (5,6).

IL-10 is a pleiotrophic cytokine released from various types of cells, including lymphocytes, mast cells, eosinophils and monocytes/macrophages (7). While IL-10 was originally isolated as a cytokine selectively produced from Th2-cells and shown to inhibit Th1-skewed response in mice (8), it is now appreciated that not only Th2 cells, but also Th1 cells, potentially produce IL-10 in humans (9). IL-10 inhibits the production of proinflammatory cytokines from T cells directly and indirectly

through inhibition of the antigen-presenting functions of APCs (10,11). Furthermore, IL-10 downregulates proinflammatory cytokines and chemokine production by granulocytes, and inhibits the survival of eosinophils (12). By contrast, IL-10 enhances B cell maturation into plasma cells (13). Thus, except for this proinflammatory effect on B cells, the major role of IL-10 in atopic diseases is postulated to act as a dampener of inflammation as a feedback mechanism (7).

Several studies reported that IL-10 levels in bronchoalveolar lavage (BAL) fluid and in the supernatant of cultured alveolar macrophages were lower in asthmatics than in healthy subjects (14,15). However, other studies have shown that levels of IL-10 messenger ribonucleic acid (mRNA) in airway tissues and its protein in BAL fluid were higher in asthmatics (16,17). A decreased level of IL-10 protein in the supernatant of peripheral blood mononuclear cells (PBMCs) from asthmatics was reported by some investigators (14), but not by others (15). Thus, the results of previous studies were inconsistent. Among those studies, the levels of IL-10 were evaluated by measuring the bulk amount of cytokine protein in the supernatant, which might not have reflected the amount of IL-10 in each type of producing cells. While alveolar macrophages are known as a major source of IL-10 (15,17), T cells recruited in the lung are the other important source. Considering their orchestrating role in allergic inflammation, the production of IL-10 in T cells needs to be evaluated. T cells circulate systemically from the affected organs to the blood stream via the lymphatic system (18). This unique profile enables detection of the cytokine kinetics of T cells in asthmatics using peripheral blood samples. Thus, the present study investigated the numbers of IL-10-producing, circulating T cells in atopic asthmatics, atopic nonasthmatics and healthy controls at the single cell level using cytokine flow cytometry (19). We hypothesized that asthmatic subjects not on regular treatment would have fewer IL-10-producing T cells than atopic nonasthmatics and normal subjects.

PATIENTS AND METHODS
Patients
Thirty-five subjects were studied, of whom 12 were nonatopic, healthy subjects, 10 were atopic nonasthmatics and 13 were atopic asthmatics. All patients gave signed consent before the study. The study was approved by the Ethics Committee of the McMaster University Health Sciences Centre, Hamilton, Ontario. The asthmatic group consisted of nine male and four female patients with a median age of 35 years (range 22 to 50 years). All of the patients had mild asthma as detailed by the American Thoracic Society criteria (20) and had airway hyperresponsiveness to inhaled methacholine, with a geometric mean provocation concentration of methacholine causing a fall in forced expiratory volume in 1 s of 20% of 3.70 mg/mL (geometric standard error of the mean 1.43 mg/mL). No subject was treated with regular asthma therapy, and all subjects used only an inhaled beta$_2$-agonist as needed, less than daily. Medication was withheld for at least 8 h before the investigation.

The atopic nonasthmatic group consisted of eight male and two female subjects (median age 36 years, range 28 to 43 years) who had a history of allergic rhinitis and/or conjunctivitis but who had never had asthmatic symptoms. Atopy was identified by positive skin prick test responses to one or more of the fol-

lowing allergens: house dust mite, mixed grass pollens, mixed tree pollens, mixed feathers, cat fur and dog hair. They did not take any medications. No asthmatic or atopic nonasthmatic subject suffered from any comorbid condition.

The healthy control group consisted of six male and six female subjects (median age 35 years, range 27 to 49 years) who had no history of allergic diseases and negative skin tests. All subjects were nonsmokers, were not exposed to allergens and had not had exacerbations of symptoms or respiratory tract infections for at least two weeks before the investigation.

Monoclonal antibodies
Phycoerythrin (PE)-conjugated antihuman IL-10 monoclonal antibody (mAb), JES3-19F1 (rat immunoglobulin [Ig]G$_{2a}$) and PE-isotype control rat IgG$_{2a}$ were purchased from PharMingen (USA). Positive staining could be inhibited by preincubation with 25 μg/mL recombinant human IL-10 (PharMingen, USA) or with excessive concentrations (50 μg/mL) of fluorescent-unlabelled antihuman IL-10 mAb. In nonpermeabilized cells, positive staining for IL-10 decreased by 31.5% of the paired sample with permeabilization, showing that approximately 70% of the signal may be the intracellular origin. Fluorescent isothiocyanate-conjugated anti-CD4 mAb, RPA-T4 (mouse IgG$_1$, kappa) or anti-CD8 mAb, RPA-T8 (mouse IgG$_1$, kappa) and CyChrome (PharMingen, USA)-conjugated anti-CD3 mAb, HIT3a (mouse IgG$_{2a}$, kappa) were used for identifying T cell subsets.

Cell cultures
PBMCs were isolated by Lymphoprep (Nycomed, Norway) density gradient separation and washed with RPMI-1640 (Gibco BRL, USA). Cells were suspended in RPMI-1640 with 10% fetal calf serum (Gibco BRL, USA) and 25 mM of Hepes (Gibco BRL) at a density of 2×10^6 cells/mL. Cells were stimulated in six-well culture plates (Becton Dickinson Labware, USA) in a volume of 5 mL with phorbol 12-myristate 13-acetate (PMA) (Sigma, USA) at a concentration of 10 ng/mL plus ionomycin (Sigma, USA) at a concentration of 2 μM for 4 h, 8 h and 24 h at 37°C in 5% carbon dioxide in an incubator. Cells cultured for 4 h without stimulation served as a control because, in a preliminary study of unstimulated cells, a longer incubation of up to 24 h showed no difference in the frequency of IL-10-producing T cells. Brefeldin A (Sigma, USA) at a concentration of 10 μg/mL was added during the last 2 h of incubation to inhibit cytokine secretion. After harvest, the cell viability was assessed by the trypan blue dye (Gibco BRL, USA) method. The cells were then centrifuged at 500 xg for 10 min and washed in phosphate buffered saline (PBS) with 0.2% fetal calf serum and 0.1% sodium azide (BDH Inc, Canada), referred to as washing buffer. The cells were transferred to 5 mL polystyrene tubes (Becton Dickinson Labware, USA) with 1×10^6 cells/tube and were suspended in washing buffer.

Staining
Cells were washed and suspended at a density of 1×10^6 cells in 100 μL of PBS with 0.1% sodium azide and 5% normal mouse serum (Sigma, USA), and incubated for 10 min on ice; this was followed by surface staining with anti-CD3 mAb and anti-

TABLE 1
Time course of interleukin-10 production (median count) in six healthy controls after stimulation with phorbol 12-myristate 13-acetate and ionomycin

	Control	4 h after stimulation	8 h after stimulation	24 h after stimulation
CD3+ (%)	0.00 (0.90)	3.81 (4.82)*	3.51 (2.54)*	3.41 (4.11)*
CD4+ (%)	0.02 (0.54)	3.13 (7.34)*	2.74 (4.13)*	5.20 (7.83)*
CD8+ (%)	0.03 (0.67)	1.10 (1.55)*	1.05 (2.41)	2.29 (4.07)*

Values are presented as medians with interquartile ranges in parenthesis.
*P<0.05 compared with control

CD4 mAb/anti-CD8 mAb for 20 min on ice in the dark. For intracellular cytokine staining, the cells were washed and fixed in 100 μL of fixation buffer (Caltag, USA) containing 4% paraformaldehyde for 20 min on ice. After a wash, cells were suspended in 100 mL of permeabilization buffer containing 0.1% saponin (Caltag, USA) with 5% normal rat serum (Sigma, USA) for 10 min at room temperature and incubated with anti-IL-10 mAb or isotype control IgG_{2a} for 30 min at room temperature in the dark. After a final wash, cells were suspended in PBS with 1% paraformaldehyde (BDH Inc, Canada) and kept in the dark at 4°C until analysis.

Flow cytometric analysis
A FACScan flow cytometer (Becton Dickinson, USA) was used for analysis. Ten thousand events were acquired in list mode, with debris excluded by the forward scatter threshold. The data were analyzed using CELLQuest software (Becton Dickinson, USA). An analysis gate was set on the lymphocyte population based on the forward and side scatter plot. Firstly, CD3+-stained cells falling within the gated area were identified. The gated population was further analyzed for the identification of CD3+CD4+ (CD4+) or CD3+CD8+ (CD8+). Previous studies have shown that the stimulation with PMA and calcium ionophore downregulates surface CD4 expression on T cells, which may make it difficult to identify CD4+ T cells (21). Thus, the CD4+ population was also analyzed by gating on the CD3+CD8− population. Cells positive for IL-10 were expressed as a percentage of each subset. The cut-off level for definition of positive cells was set so that less than 2% of isotype antibody-stained cells were positive. Then, the frequency of true-positive cells was obtained by subtracting the value of isotype control from the value of sample stained with anti-IL-10 mAb.

Statistical analyis
Statistical analysis was completed using StatView J4.02 (Abacus Concepts Inc, USA). Data were expressed as medians and interquartile ranges. When multiple comparisons were made among groups, significant differences were assessed using the Kruskal-Wallis test, followed by the Mann-Whitney U test. Wilcoxon signed rank test was used for within-group comparisons. Values of P less than 0.05 were accepted as statistically significant.

RESULTS

Time course of IL-10 production
PBMCs from six healthy controls were examined for the relative frequencies of IL-10-producing cells after stimulation
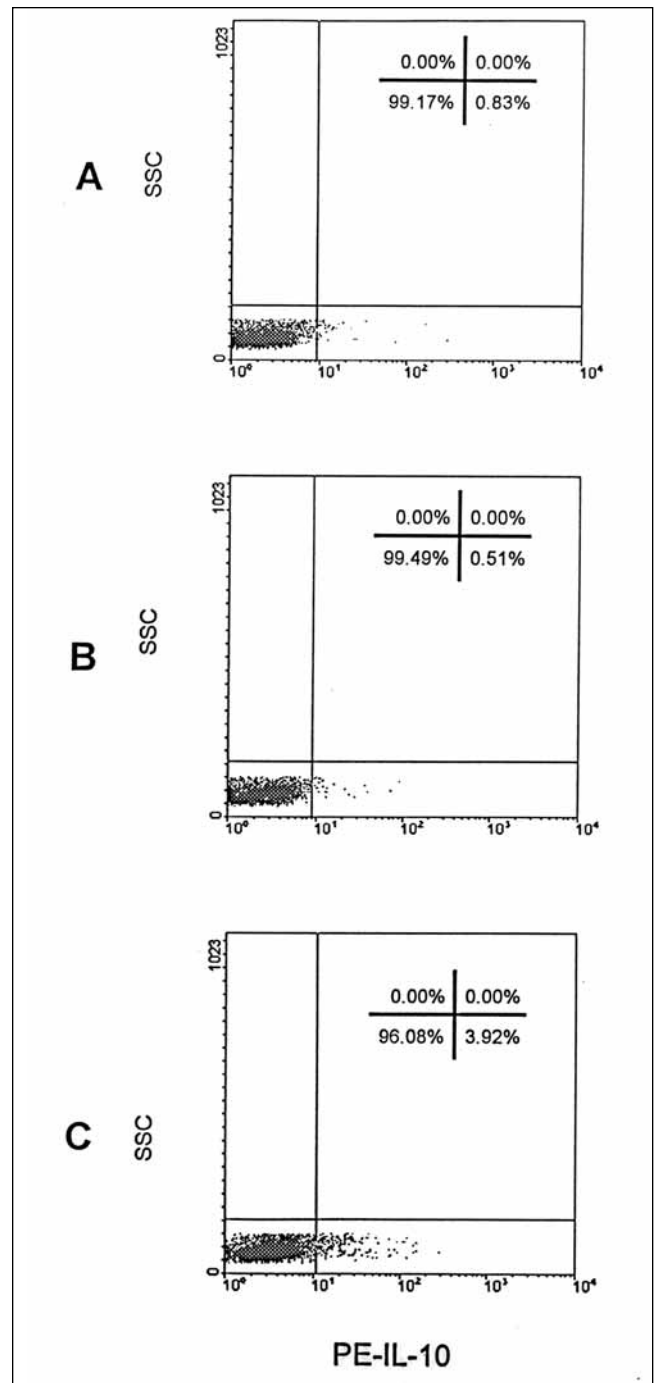

Figure 1) *Typical dot plots of interleukin (IL)-10-producing CD3+ cells in a healthy control subject. Panel A and B show unstimulated samples stained with isotype control immunoglobulin (Ig)G_{2a} and anti-IL-10 antibody, respectively. Panel C shows a sample of the same subject stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 24 h, and then stained with anti-IL-10 antibody. PE-IL-10 Phycoerythrin interleukin-10; SSC Side scatter*

with PMA and ionomycin (Table 1). The frequencies of IL-10-producing CD3+ cells after 4 h, 8 h and 24 h of stimulation were all significantly higher than that of the control samples (P<0.05). A typical dot plot is shown in Figure 1. Similarly, the frequencies of IL-10-producing CD4+ cells after 4 h, 8 h and 24 h of stimulation were significantly higher than

TABLE 2
Frequencies (median count) of interleukin (IL)-10-producing cells among subject groups

	Healthy controls (n=12)	Atopic nonasthmatics (n=10)	Atopic asthmatics (n=13)
Mononuclear cells (%)			
CD3+	64.7 (11.6)	66.6 (15.4)	62.8 (11.9)
CD4+	37.3 (10.4)	36.6 (16.6)	38.2 (13.8)
CD8+	19.6 (8.0)	21.2 (8.8)	20.1 (15.9)
Baseline IL-10 (+) cells (%)			
CD3+	0.00 (0.90)	0.40 (1.54)	1.61 (2.37)*
CD4+	0.02 (0.54)	1.39 (3.07)*	1.48 (2.45)*
CD8+	0.03 (0.67)	0.12 (1.80)	1.04 (2.45)*
Stimulated IL-10 (+) cells (%)			
CD3+	3.41 (4.11)†	2.76 (2.64)†	2.10 (3.54)
CD4+	5.20 (7.42)†	1.88 (3.22)	2.01 (2.26)
CD8+	2.29 (4.07)†	2.01 (2.77)	1.62 (2.99)

*Values are presented as medians with interquartile ranges in parenthesis. *P<0.05 compared with normal controls; †P<0.05 compared with baseline IL-10 (+) cells*

that of the control samples (P<0.05). The other method for enumerating CD4+ cells, gated as CD3+CD8– cells, also showed similar kinetics (data not shown). The frequencies of IL-10-producing CD8+ cells after 4 h and 24 h were significantly higher than that of the control samples (P<0.05). These results suggest that the frequencies of IL-10-producing cells reached a maximum after 24 h of stimulation. Therefore, we examined the frequencies of IL-10-producing cells at 4 h without stimulation (prestimulation) and 24 h with stimulation (poststimulation).

Proportions of CD3+, CD4+ and CD8+ cells among subject groups
Cell viability at the beginning of incubation ranged from 97% to 100%, and no significant difference was observed among subject groups. There was no difference in the proportions of CD3+, CD4+ or CD8+ cells among subject groups (Table 2).

Frequency of IL-10-producing T cells at baseline
To evaluate the spontaneous production of IL-10, the frequencies of IL-10-producing cells, cultured for 4h without stimulation, were compared among groups. The frequency of IL-10-producing CD3+ cells in asthmatics was significantly higher than in healthy controls (P=0.008) (Figure 2, Top). The values from atopic nonasthmatics showed an intermediate distribution between healthy controls and asthmatics. The frequencies of IL-10-producing CD4+ cells in both atopic nonasthmatics and asthmatics were significantly higher than in healthy controls (P=0.029 and P=0.011, respectively) (Figure 2, Middle). The frequency of IL-10-producing CD8+ cells in asthmatics was also significantly higher than in healthy controls (P=0.015) (Figure 2, Bottom).

The effect of stimulation on IL-10 production
To investigate the effect of PMA and ionomycin on IL-10 production, the frequencies of IL-10-producing cells pre- and poststimulation were compared (Table 2). The stimulation
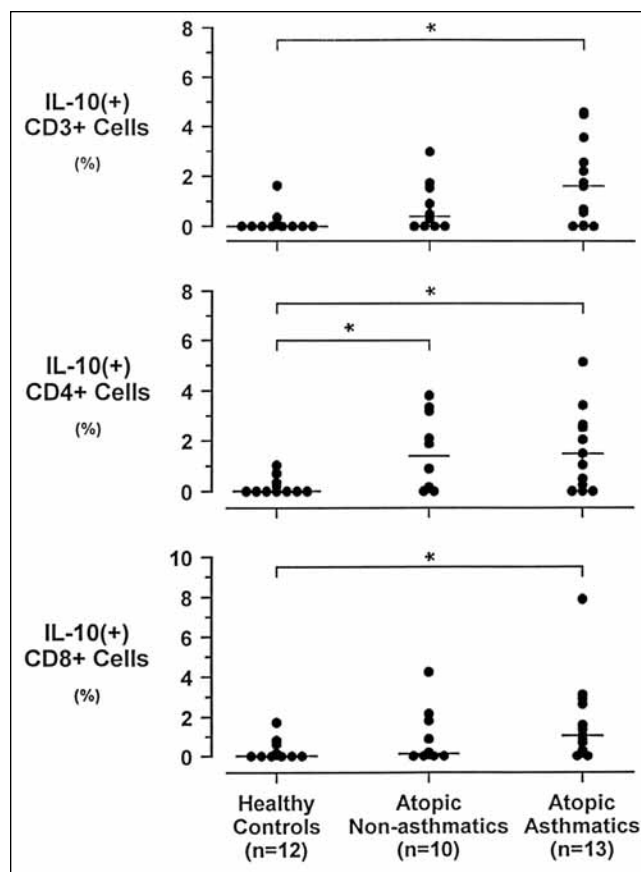

Figure 2) *The frequency of interleukin (IL)-10-producing CD3+ cells in atopic asthmatics was significantly higher than in healthy controls. The frequencies of IL-10-producing CD4+ cells in both atopic nonasthmatics and asthmatics were significantly higher than in healthy controls. The frequency of IL-10-producing CD8+ cells in asthmatics was also significantly higher than in healthy controls. *P<0.05*

significantly increased the frequencies of IL-10-producing CD3+, CD4+ and CD8+ cells in healthy controls (P=0.003, P=0.007 and P=0.005, respectively). In atopic nonasthmatics, the stimulation significantly increased the frequency of IL-10-producing CD3+ cells (P=0.008), whereas for CD4+ and CD8+ cells, the effect did not reach statistical significance (P=0.066 and P=0.051, respectively). In asthmatics, however, the stimulation did not show any effect on the frequencies of IL-10-producing CD3+, CD4+ and CD8+ cells. There was no difference in the frequencies of IL-10-producing CD3+, CD4+ and CD8+ cells after stimulation among groups.

DISCUSSION
This study has demonstrated that the frequencies of IL-10-producing CD4+ and CD8+ T cells were higher in atopic asthmatics than in healthy controls. In atopic nonasthmatics, the frequency of IL-10-producing CD4+, but not CD8+, T cells was higher than in healthy controls. Furthermore, stimulation with PMA and ionomycin increased the frequencies of IL-10-producing CD4+ and CD8+ T cells in healthy controls and atopic nonasthmatics, but not in asthmatics.

The time course study using healthy control samples showed that the stimulation with PMA and ionomycin

increased the frequencies of IL-10-producing CD4$^+$ and CD8$^+$ T cells after 4 h of stimulation. Their frequencies reached a maximum after 24 h of stimulation. A previous study demonstrated that IL-10 mRNA from Th1- and Th2-like clones was first detected after 8 h of stimulation with anti-CD3 mAb plus phorbol acetate and reached a maximum after 24 h (22). The production of IL-10 protein, detected by ELISA, appeared after 12 h of stimulation and continued to increase thereafter. The earlier appearance of IL-10-producing cells in the current study seems inconsistent with the previous study. A small accumulation of IL-10 protein in the supernatant of cultured T cells at the earlier time points may not be detected by ELISA due to its limitation of sensitivity. Cytokine flow cytometry is expected to determine more detailed kinetics of IL-10 production, particularly in the early phase of activation in each subset of T cells.

At unstimulated baseline, the frequencies of IL-10-producing CD4$^+$ and CD8$^+$ T cells were significantly higher in atopic asthmatics than in healthy controls. A similar difference was noted in CD4$^+$ T cells between atopic nonasthmatics and healthy controls. T cells circulate systemically from the affected organs to the blood stream via the lymphatic system (18). Our findings in the peripheral blood may reflect the increased numbers of IL-10-producing T cells in the airways of atopic asthmatics. This speculation is supported by a report showing that IL-10 mRNA-positive T cells are increased in the airways of atopic asthmatics compared with those of healthy controls (16). Persistence of airway inflammation is frequently observed in biopsy specimens of asthmatics, even when they are symptom free (23). Based on the concept that a major role of T cell-derived IL-10 may be anti-inflammatory, the increase in IL-10-producing T cells may be explained as chronic activation of feedback inhibitory mechanisms (7). Alternatively, the increase in T cell-derived IL-10 may contribute to the persistence of inflammation through its proallergic ability as an inducer of Igs – in particular, IgE from B cells (13). Indeed, IL-10 is regarded as one of the pathogenic cytokines in some autoimmune diseases, in which the overproduction of autoimmune antibodies plays a role (24).

Another interesting finding is the increased frequency of IL-10-producing CD8$^+$ T cells in asthmatics, which was not observed in atopic nonasthmatics. While the contribution of activated/memory CD4$^+$ T cells in asthma is widely appreciated, recent investigations have suggested that CD8$^+$ T cells may also be involved in the development of this disease. CD8$^+$ T cells, skewed toward cytotoxic phenotype, similar to Th2-phenotype in CD4$^+$ T cells, acquired a capability for IL-5 production and contributed to virus-induced eosinophilia in mice

(25). In asthmatics, increased numbers of IL-4 or IL-5 mRNA-expressing CD8$^+$ T cells have been shown in airway biopsies, as well as an increased level of IL-4 protein in peripheral blood CD8$^+$ T cells (26,27).

Stimulation with PMA and ionomycin increased the frequencies of IL-10-producing CD4$^+$ and CD8$^+$ T cells in healthy controls and modestly in atopic nonasthmatics, but not in asthmatics. These results were surprising because we had expected that additional stimulus would enhance T cell IL-10 production in asthmatics, in whom the frequency of IL-10-producing T cells was increased at baseline. Although those compounds have been widely used to induce cytokine production from T cells in vitro, it does not seem to be the case with the induction of IL-10 in asthmatic subjects. An important caveat is that a full time course for the stimulation was not established, and time points later than 24 h may have given a greater response in asthmatics. However, at the 24 h time point, differences were noted between asthmatics and the other subjects. This raises the possibility that circulating T cells of asthmatic subjects are maximally stimulated with regard to IL-10 production; alternatively, IL-10 production T cells in asthmatics may be regulated differently than T cells in other subjects. A further possibility is that spontaneous production of IL-10 from T cells in vitro suppresses the PMA- or ionomycin-induced responses. Of note is a recent study that demonstrated that altering the balance between the protein kinase C pathway and calcium signalling redirected Th2 to Th1 and vice versa, although both signals were required for T cell activation (28), and stimulation for calcium signalling inhibited the production of IL-4, even in Th2 clones. Given that IL-10 has been regarded as a Th2-type cytokine and that atopic asthmatics are expected to have a Th2-skewed background, it is important to know whether a similar mechanism is involved for regulating IL-10 production.

CONCLUSIONS

The present study demonstrated that the frequency of IL-10-producing T cells is increased in the circulation of atopic asthmatics. Additionally, the lack of enhancement in the frequency of these T cells by PMA and ionomycin in asthmatics raises the possibility that circulating T cells of asthmatic subjects are maximally stimulated with regard to IL-10 production, or that IL-10 production by T cells in asthmatic subjects may be regulated differently than in other subjects.

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