

Peripheral blood CD4⁺ and CD8⁺ T cell type 1 and type 2 cytokine production in atopic asthmatic and normal subjects

S.-H. Cho†, L. A. Stanciu, T. Begishivili, P. J. Bates, S. T. Holgate and S. L. Johnston*

University Medicine, Southampton General Hospital, Southampton, and *Department of Respiratory Medicine, National Heart and Lung Institute, St Mary's Imperial College School of Medicine, London, UK. †Currently with Seoul National University College of Medicine, Seoul, Korea

Summary

Background Increased production of IL-4 and IL-5 and decreased production of IFN- γ by CD4⁺ T cells has been implicated in asthma pathogenesis. However, CD8⁺ T cells also produce type 1 and type 2 cytokines and the relative roles of CD4⁺ and CD8⁺ T cell cytokine production in asthma have not been previously studied.

Objective To determine the production of the type 1 and type 2 cytokines by CD4⁺ and CD8⁺ T cell subsets in asthmatic and normal subjects.

Methods Intracellular cytokine staining for IL-4, -5, -10, -13 and IFN- γ was analysed in peripheral blood CD4⁺ and CD8⁺ T cells from 24 atopic asthmatic and 20 normal subjects.

Results Both subsets of T cells produced all cytokines studied and there were no significant differences between CD4⁺ and CD8⁺ T cells in their capacity to produce either type 1 or type 2 cytokines. There were significantly increased frequencies of IFN- γ -positive CD4⁺ ($13.1 \pm 2.4\%$, vs. $7.3 \pm 1.4\%$) and CD8⁺ ($20.0 \pm 2.9\%$, vs. $9.6 \pm 2.1\%$) T cells in asthmatic subjects compared with normal subjects ($P < 0.05$), but not in frequencies of CD4⁺ or CD8⁺ T cells staining positively for IL-4, -5, -10 or -13.

Conclusion The frequencies of peripheral blood CD8⁺ T cells producing type 1 and type 2 cytokines are comparable with the frequencies of CD4⁺ T cells. There was an increased frequency of IFN- γ producing CD4⁺ and CD8⁺ T cells in asthmatic compared with normal subjects. Further studies investigating T cells derived from the airways and investigating various stages within the disease process are required to further elucidate the importance of type 2 and type 1 T cell cytokine production in the pathogenesis of human allergic disease.

Keywords asthma, atopy, flow cytometry, human, Th1/Th2, T lymphocytes

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Introduction

The Th2 hypothesis for asthma argues that an augmented Th2 response, perhaps in concert with a down-regulated Th1 response, orchestrates the airway hyper-responsiveness and chronic inflammation of asthma [1]. However, there have been several studies showing increased levels of IFN- γ in bronchoalveolar lavage from asthmatic subjects [2–4].

Th2 cytokine production has been found in bronchial or nasal biopsies [5,6], but there is debate about the cellular source of Th2 cytokines as IL-4 and IL-5 are also produced from human mast cells, eosinophils and basophils [7,8]. Further, most recent data regarding type 1 and type 2 cytokine expression *in vivo* in asthma have been collected at the mRNA level; however, mRNA transcription is not necessarily followed

by protein synthesis [9]. Immunohistochemistry is an alternative approach; however, recent studies have failed to detect appreciable protein staining for IL-4 and IL-5 in T cells using this approach [7]. An alternative approach to evaluate specific T cell subset-derived cytokines is to generate allergen-specific T cell clones; however, this may represent skewed expansion of highly selected T cells.

In addition, it has recently been demonstrated that purified CD8⁺ T cells possess the capacity to produce a wide range of cytokines, including IL-4 and IL-5 as well as IFN- γ [10,11], and can be classified into a Tc1 subset that secretes predominantly IL-2 and IFN- γ , and a Tc2 subset that secretes IL-4 and IL-5 [12]. The relative roles of CD4⁺ and CD8⁺ T cell type 1 and type 2 cytokine production in asthma pathogenesis have not been investigated.

To determine cytokines derived from single cell subtypes such as CD4⁺ T cells or CD8⁺ T cells, T cell surface marker expression and cytokine production must be assessed simultaneously. Recent intracellular cytokine staining methods using flow cytometric analysis were found to have a high concordance to ELISA analysis [13] and have enabled assessment of

Correspondence: Sebastian L. Johnston, Department of Respiratory Medicine, National Heart and Lung Institute, St Mary's Imperial College School of Medicine, Norfolk Place, London W2 1PG, UK. E-mail: s.johnston@ic.ac.uk

production of type 1 and type 2 cytokines at the single cell level [4,14–17].

This study was performed to investigate human CD4 and CD8 T cell type 1 and type 2 cytokine production, and to investigate the type 2 hypothesis for asthma that asthmatic subjects have increased production of Th2 and/or Tc2 cytokines relative to normal subjects (rather than only Th2).

Methods

Subjects

A total of 24 atopic asthmatic and 20 healthy non-atopic volunteers were recruited into the study. Their clinical and demographic data are shown in Table 1. All were non-smokers. Subjects with asthma were selected according to the American Thoracic Society criteria [18]. They all showed positive responses to skin prick test and were mild to moderate asthmatic patients according to NHBLI/WHO international guidelines for asthma management [19], using bronchodilators as required alone (nine subjects) or with inhaled steroid 200–1000 µg/day (15 subjects, median dose: 400 µg/day). None had had oral corticosteroid treatment or symptoms of respiratory tract infection in the 6 weeks preceding the study. Atopy was defined by a positive weal response (>3 mm) on skin prick test to one or more of five common inhalant allergens, including *Dermaphagoides pteronyssinus*, mixed grass pollen, mixed tree pollen, cat fur or dog hair (ALK, Hørsholm, Denmark). Histamine challenge was performed by Chai's method [20]. The healthy non-atopic control subjects had no history of asthma or other allergic disease, had no respiratory symptoms and they were all negative to skin tests.

Subjects gave written informed consent and the study was approved by the Southampton Joint University and Hospital Ethics Committee.

Intracellular cytokine staining

One asthmatic and one normal control subject were matched in all experiments except four where only one asthmatic subject was studied. Heparinized blood was withdrawn from the subjects and mononuclear cells were isolated. Cells were counted, and adjusted to 3×10^6 /mL in RPMI 1640 supplemented with 2 mM glutamine (Gibco BRL, Life Technologies, Uxbridge, UK). One-millilitre aliquots were then cultured for 4 h at 37°C in the presence of 10 µg/mL of brefeldin-A (Sigma, Poole, UK) alone to assess spontaneous production, or with 25 ng/mL of phorbol myristate acetate (PMA) and 1 µg/mL of ionomycin to

assess production after stimulation. Cultured cells were washed in phosphate buffer saline (PBS) and resuspended in 2 mL of RPMI 1640–2 mM glutamine. Cell suspension (100 µL) was incubated for 30 min at 4°C with 20 µL of monoclonal antibodies for surface markers: anti-CD3-PerCP (Becton Dickinson, San Jose, CA, USA), anti-CD4-FITC or anti-CD8-FITC (both 1:10, Becton Dickinson). After washing with stain buffer (filtered PBS supplemented with 1% fetal calf serum and 0.1% NaN₃), 500 µL of permeabilizing solution (Becton Dickinson) was added to each tube and incubated for 10 min in the dark. After washing, 20 µL of anti-cytokine-PE (anti-IL-4, clone 8D4-8; anti-IL-5, clone TRFK5; anti-IL-10, clone JES3-9D7; anti-IL-13, clone JES10-5A2; anti-IFN-γ, clone B27; Pharmingen, San Diego, CA, USA) were added to each tube and incubated for 30 min at room temperature (15°C). PE-conjugated isotypic IgG1 antibodies were used as a negative control. After a final wash with stain buffer, the cells were fixed with 1% paraformaldehyde and analysed by flow cytometry. A FACScan flow cytometer (Becton Dickinson) equipped with 15-mV argon laser and appropriate filters for fluorescein isothiocyanate (FITC) (530 nm), PE (585 nm) and PerCP (>650 nm) was used. An electronic gate was set on the lymphocytes on the forward and side scatter plot, 10000 cells were computed in this gate and analysed with appropriate software (Lysis, Becton Dickinson). CD3-PerCP positive cells falling in this gate were identified and selected into a second gate according to positivity for anti-CD4-FITC or anti-CD8-FITC. Cytokine production was analysed by detection of PE staining in the gate of CD3⁺CD4⁺ or CD3⁺CD8⁺ lymphocytes after subtracting the negative control staining. The number of cells staining for each cytokine was expressed as a percentage of CD3⁺CD4⁺ T cells or CD3⁺CD8⁺ T cells.

Statistical analysis

Statistical analyses were performed using SPSS for Windows. Student's unpaired *t*-tests were used to compare cytokine staining frequencies between groups and paired *t*-tests were used to compare paired data within groups. Spearman correlation tests were used to evaluate the relationship between serum total IgE, blood eosinophil counts and the frequencies of CD3⁺CD4⁺ or CD3⁺CD8⁺ T cells expressing cytokines. Values of *P* < 0.05 were accepted as statistically significant. Data were presented as mean ± SEM.

Results

Human CD8⁺ and CD4⁺ peripheral blood T cell subsets both produce IL-4, -5, -10, -13 and IFN-γ, and frequencies of type 1 and type 2 cytokine staining are comparable between T cell subsets

To examine the capacity of human CD4⁺ and CD8⁺ peripheral blood T cells to produce type 1 and type 2 cytokines, intracellular cytokine staining of CD3⁺CD4⁺ T cells and CD3⁺CD8⁺ T cells for IL-4, -5, -10, -13 and IFN-γ was investigated after incubation with brefeldin-A alone for 4 h (spontaneous) and after incubation with brefeldin-A and stimulation with PMA and ionomycin for 4 h (stimulated).

Frequencies of spontaneous cytokine staining were uniformly low and not different between CD4⁺ and CD8⁺ T cell subsets (*P*=NS), with mean frequencies of positively staining cells being 2% or less for all cytokines studied. After stimulation

Table 1. Demographic data of the subjects with asthma and normal control subjects

	Asthmatic subjects (<i>n</i> =24)	Control subjects (<i>n</i> =20)
Age (yr)	41.5 ± 2.4	32.3 ± 1.9
FEV ₁ (% predicted)	90.1 ± 2.7	100.8 ± 1.7
Blood eosinophil count (/mm ³)	281.8 ± 35.8	65.0 ± 10.9
Serum IgE (IU/mL)	166.4 ± 71.6	29.4 ± 8.9
PC ₂₀ -histamine* (mg/mL)	1.6 (0.007–16)	> 16

Data are mean ± SEM. *Geometric mean (range).

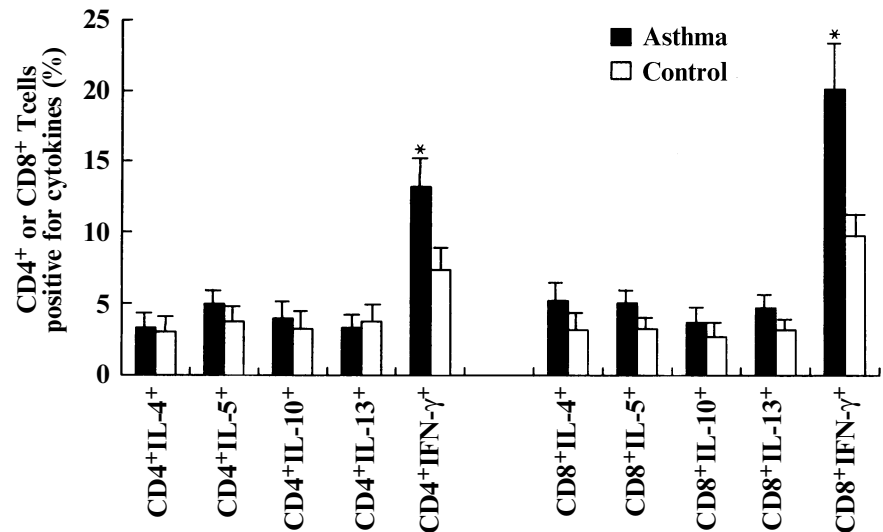


Fig. 1. Comparison between asthmatic and normal subjects of intracellular cytokine staining from peripheral CD3⁺CD4⁺ T cells and CD3⁺CD8⁺ T cells after stimulation with PMA and ionomycin. * $P < 0.05$.

the frequencies of CD3⁺CD8⁺ T cells staining for each of IL-4, -5, -10, -13 and IFN- γ were similar to those of CD3⁺CD4⁺ T cells ($P = \text{NS}$, Fig. 1). Individual spontaneous staining frequencies and those with stimulation are depicted in Figs 2 and 3. Stimulation induced significant increases in the frequencies of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells staining positively for each of IL-4, -5, -10, -13 and IFN- γ in both asthmatic and normal subjects ($P < 0.05$, Figs 2 and 3). These data confirmed that both type 1 and type 2 cytokines are similarly inducible in both T cell subsets.

Type 1 and type 2 cytokine production by human CD4⁺ and CD8⁺ peripheral blood T cell subsets within individual subjects are closely correlated

Correlations within individual subjects between staining frequencies of each T cell subset for type 1 and type 2 cytokines were examined. There were significant correlations within individual subjects between CD4⁺ T cells and CD8⁺ T cells in terms of cytokine staining with stimulation for IL-4, -5, -10, -13 and IFN- γ both in atopic asthmatic and normal control subjects ($P < 0.001$ for each cytokine). These data suggest that type 1 or type 2 cytokine production by the two T cell subsets go in tandem within individuals, rather than in opposing directions.

Frequencies of CD3⁺CD4⁺ and CD3⁺CD8⁺ peripheral blood T cells positive for type 1 but not type 2 cytokines are increased in asthmatic subjects compared with normal subjects

As reported above, spontaneous staining frequencies were extremely low and there were no statistical differences between the subject groups for any of the cytokines studied ($P = \text{NS}$). With stimulation, there was a significantly increased frequency of CD3⁺CD4⁺ peripheral blood T cells staining positively for IFN- γ in atopic asthmatic subjects ($13.1 \pm 2.4\%$) compared with normal control subjects ($7.3 \pm 1.4\%$, $P < 0.05$, Fig. 1). There was a similar increased frequency of CD3⁺CD8⁺ T cells staining positively for IFN- γ in atopic asthmatic subjects ($20.0 \pm 3.3\%$) compared with control subjects ($9.6 \pm 2.1\%$, $P < 0.05$, Fig. 1). There were no significant differences between the two patient groups in the frequencies of CD3⁺CD4⁺ or

CD3⁺CD8⁺ peripheral blood T cells staining positively for any of the type 2 cytokines, IL-4, -5, -10 or -13. ($P = \text{NS}$, Fig. 1).

Peripheral blood T cell cytokine staining frequencies are not correlated with clinical parameters of asthma severity

We investigated whether there were any relationships between clinical markers of asthma severity or atopy (% predicted FEV₁, blood eosinophil counts, immunoglobulin (Ig)E levels or bronchial hyper-responsiveness) and the frequencies of CD3⁺CD4⁺ or CD3⁺CD8⁺ T cells staining positively for cytokines with biologically relevant functions. There were no significant relationships between hyper-responsiveness or percentage predicted FEV₁ and the frequencies of T cells staining positively for any cytokine ($P = \text{NS}$), between peripheral blood eosinophil counts and the frequencies of T cells staining positively for IL-5 ($P = \text{NS}$) or between serum total IgE level and the frequencies of T cells staining positively for IL-4, IL-13, or IFN- γ ($P = \text{NS}$).

Discussion

Recent studies have shown that CD8⁺ T cells can be classified into Tc1 which secrete predominantly IL-2 and IFN- γ , and Tc2 which secrete IL-4 and -5 [11,12]. In the recent study, using flow cytometric single cell staining methods, we have confirmed that adult human peripheral blood CD8⁺ T cells produce the same range of type 1 and type 2 cytokines as CD4⁺ T cells, including each of IL-4, -5, -10, -13 and IFN- γ , and that the frequencies staining positively occur at similar rates in the two T cell subsets.

One facet of 'Th2 hypothesis for asthma' is that asthmatic subjects may have a relative deficiency in IFN- γ production. Data that support this facet of the hypothesis include the fact that IFN- γ inhibits IgE synthesis and Th2 commitment *in vitro* [21,22]. Jung et al. [23] showed decreased proportion of IFN- γ -positive CD4⁺ T cells in atopic subjects (a mixed group of asthma and/or atopic dermatitis) compared with normal controls. However, it is not stated whether these subjects were asthmatic or not, and the median age was 12.3 years. In this study we have observed an increased frequency of peripheral

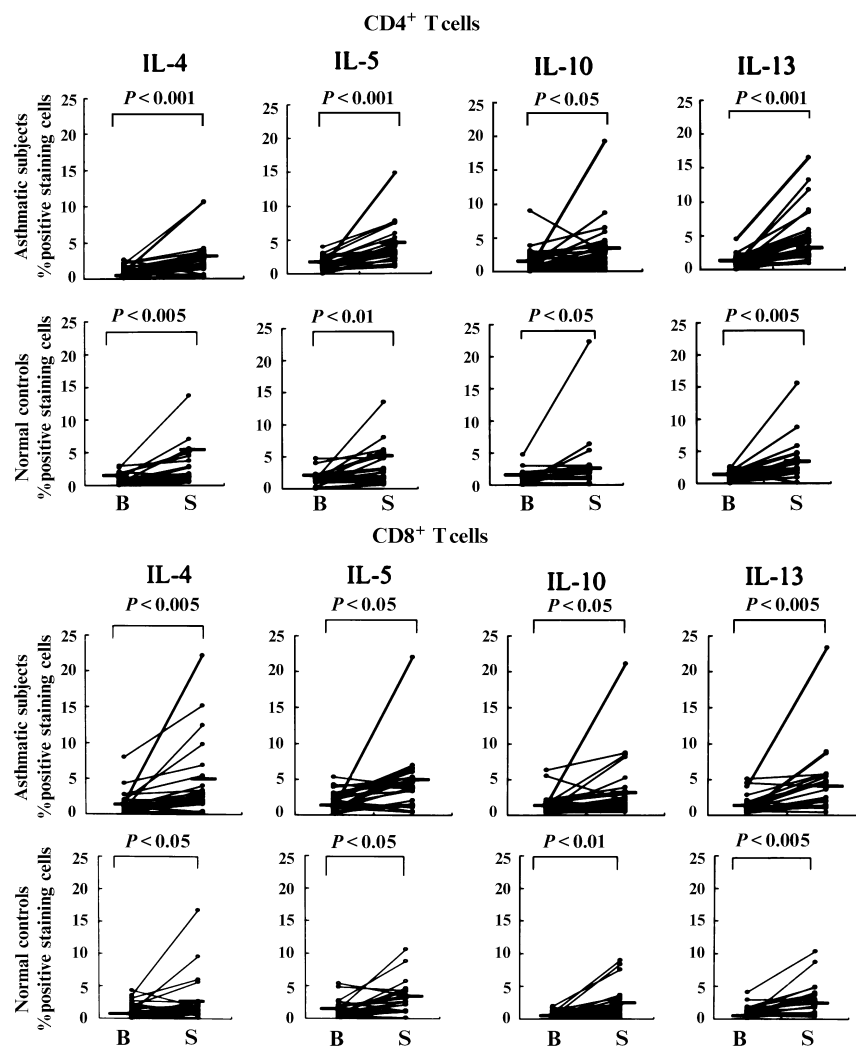


Fig. 2. Frequencies and inducibility of intracellular type 2 cytokine staining by stimulation with PMA and ionomycin in peripheral CD3⁺CD4⁺ T cells and CD3⁺CD8⁺ T cells. B=baseline, spontaneous cytokine staining without stimulation; S=cytokine staining with stimulation.

blood CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells staining positively for IFN- γ after stimulation with PMA and ionomycin in adult asthmatic subjects compared with healthy control subjects. These data suggest that in adult-established asthma (the mean age of our patients was 41 years), frequencies of IFN- γ -producing peripheral blood T cells of both CD3⁺CD4⁺ and CD3⁺CD8⁺ subsets were higher than those from normal subjects. These data are in accordance with previous work demonstrating that IFN- γ is elevated in serum during acute severe asthma [24], and staining is increased in peripheral blood and bronchoalveolar lavage (BAL) T cells in asthmatic subjects [4]. Serum IFN- γ has also been reported to correlate with measures of disease activity in asthmatic subjects [25]. IFN- γ has a number of pro-inflammatory actions relevant to the pathogenesis of asthma [26–28] and systemic use of IFN- γ was ineffective in the treatment of allergic disease [29]. These data suggest the fact that the role of IFN- γ in asthma and atopic disease is complex.

Our data demonstrating increased CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells staining positively for IFN- γ in peripheral blood in asthma is not incompatible with the Th2 hypothesis for asthma, as this finding may relate to increased T cell activation in peripheral blood in asthma, to the non-specific method of stimulation, or to recruitment of allergen-specific

type 2 T cells to the airway. To clarify the pathophysiological role of CD3⁺CD4⁺ and CD3⁺CD8⁺ type 1 and type 2 cytokine production in the development of atopic asthma, further studies preferably in the lung and investigating various stages of the disease are required.

The other facet of the 'Th2 hypothesis for asthma' relates to increased production of Th2 cytokines in allergic disease and asthma [1,30–32]. Most data investigating the Th2 hypothesis in asthma have reported the roles of IL-4 and IL-5. However, these studies all have drawbacks in that they have investigated cloned T cells (which may represent a skewed population), mRNA expression rather than protein production, or the cellular source of the cytokines has not been clearly established. In addition, Krug et al. [4] reported no significant difference in IL-4 production between adult asthmatic and normal subjects at the single cell level. However, this study did not differentiate between CD4⁺ and CD8⁺ T cell subsets. Furthermore, IL-10 and IL-13 are both also important regulators of T cell type 1 and type 2 functions. IL-13 has been shown to induce IgE switching in B cells independently from IL-4 [33] and to selectively induce VCAM-1 expression on endothelial cells [34]. IL-13 mRNA and protein were elevated in BAL cells and fluid in asthmatic patients after allergen challenge [35]. IL-10 may down-regulate

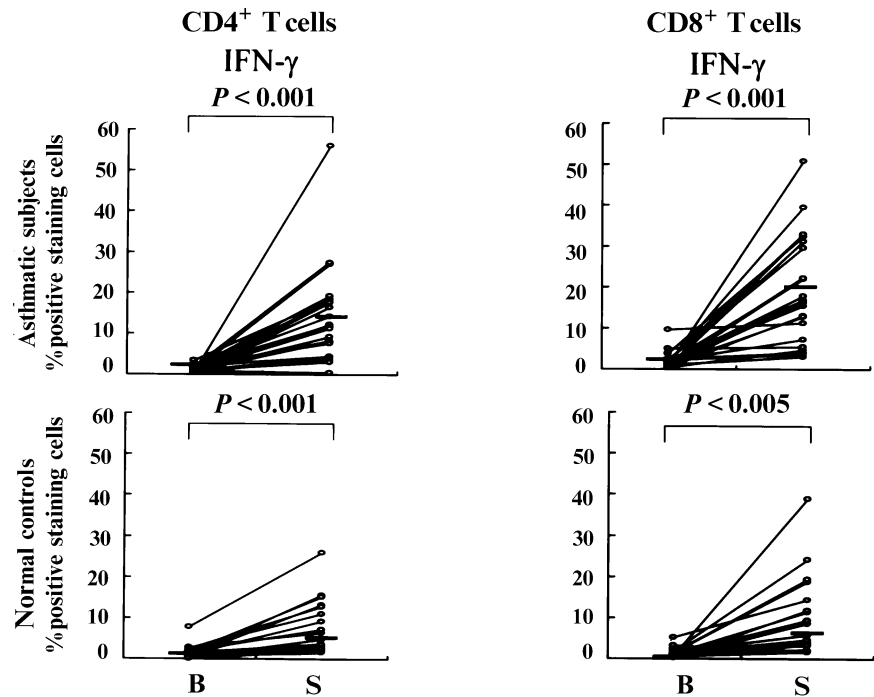


Fig. 3. Frequencies and inducibility of intracellular type 1 cytokine staining by stimulation with PMA and ionomycin in peripheral CD3⁺CD4⁺ T cells and CD3⁺CD8⁺ T cells. B=baseline, spontaneous cytokine staining without stimulation; S=cytokine staining with stimulation. *Significant difference compared with the control subjects, $P < 0.05$.

allergic inflammation through its influence on eosinophils, mast cells and IgE synthesis, as well as by direct action on T cells [36]. Borish et al. [37] reported that IL-10 mRNA of BAL cells and IL-10 in BAL fluid were significantly reduced in the asthmatic subjects, while Robinson et al. [38] reported that IL-10 mRNA expression was increased in BAL T cells and macrophages in atopic asthmatic patients. Further investigation of the role of IL-10 in asthma development is clearly required.

We therefore compared the frequencies of CD3⁺CD4⁺ and CD3⁺CD8⁺ peripheral blood T cells staining positively for IL-4, -5, -10 and -13 between asthmatic and normal control subjects. We could find no significant differences between groups in staining frequencies for any of the cytokines investigated in either T cell subset. We therefore failed to demonstrate any evidence of an imbalance towards type 2 cytokine production in these adult asthmatic subjects. There are several possible interpretations of these data. Firstly, cytokines can be produced from several cell types. IL-4 and IL-5 can be secreted from mast cells, eosinophils and basophils as well as T cells [7,8]. In previous studies, the cellular sources of cytokines are not well defined. Another possible interpretation of our data is that type 2 cytokine predominance may only be present in peripheral blood at certain times during the disease process, for example at birth, in childhood or early in the disease process. This interpretation is supported by the study of Krug et al. [39] who reported increased IL-4 and -5 staining frequencies in peripheral blood CD3⁺ T cells in asthmatic compared with normal children, but not in adults, and by Magnan et al. who observed increased whole blood IL-4 and decreased T cell IFN- γ production in atopy, but increased whole blood IL-4 and increased CD8⁺ IFN- γ production in atopic asthma [40]. This suggests an evolution of immune responses towards type 1 as the subject's age or the disease process advances. Nonetheless, there may well still be evidence of type 2 immune responses in the local environment (airway), as suggested by the previous studies investigat-

ing mRNA expression. Corrigan et al. [41] found that percentages of peripheral blood CD4⁺ but not CD8⁺ T cells expressing type 2 cytokine mRNA are elevated in patients with exacerbations of asthma. The difference to our data might be related to the different stage of the disease (exacerbated stage vs. stable state), differences in subjects studied, or to differences in methods. Further studies investigating type 1 and type 2 responses in the airway, and in which the cellular source of the cytokines is clearly defined, are now required.

Our failure to find evidence of increased type 2 cytokine production from peripheral blood T cell subsets may relate to the polyclonal rather than specific nature of the stimulus used, or to the fact that allergen-specific type 2 T cells may have been recruited in the airway in the asthmatic subjects, as previously discussed. Evidence against the latter possibility is provided by Krug et al., who observed similar patterns of CD3⁺ T cell cytokine production in peripheral blood and in BAL [4]. PMA plus ionomycin broadly activates lymphocytes, and is therefore a useful stimulus to assess the overall potential of various subpopulations to express selected cytokines [17]. Furthermore, there are many published papers using PMA and ionomycin for assessing intracellular Th1/Th2 cytokine production [23,39,40] and no differences have been observed between this method and anti-CD3 stimulation [23]. Flow cytometry has been used to assess antigen-specific T cells against virus-specific antigens [42,43], but not allergen-specific responses. Devouassoux et al. [44] could detect allergen-specific basophils, but not T cells at the single cell level.

In conclusion, we have demonstrated that human peripheral blood CD3⁺CD8⁺ T cells produce both type 1 and type 2 cytokines, including each of IFN- γ , IL-4, -5, -10 and -13. We have further shown that frequencies of CD3⁺CD8⁺ T cells staining positively for type 1 and type 2 cytokines are as high as those of CD3⁺CD4⁺ T cells, and that staining frequencies in the two T cell subsets within individual subjects are well correlated. We

found evidence of increased frequencies of IFN- γ -positive CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells amongst asthmatic subjects compared with normal subjects, but identical frequencies of type 2 cytokine staining frequencies between the groups. Further studies investigating T cells derived from the airways, employing other stimuli such as allergens and investigating various stages within the disease process, are required to further elucidate the importance of type 2 and type 1 T cell cytokine production in the pathogenesis of human allergic disease.

Acknowledgement

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