

# Increased Interleukin-4, Interleukin-5, and Interferon- $\gamma$ in Airway CD4<sup>+</sup> and CD8<sup>+</sup> T Cells in Atopic Asthma

Sang-Heon Cho, Luminita A. Stanciu, Stephen T. Holgate, and Sebastian L. Johnston

Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Korea; Infection, Inflammation and Repair Division, University of Southampton, Southampton; and Department of Respiratory Medicine, National Heart and Lung Institute, Imperial College London, London, United Kingdom

**Increased Th2 cytokine production in asthma is widely accepted, but excess production by asthmatic human airway CD4<sup>+</sup> T cells has not been demonstrated, nor has a relationship with disease severity. The importance of airway CD8<sup>+</sup> T cell type 1 and type 2 cytokine production in asthma is unknown. We investigated frequencies of IFN- $\gamma$ , interleukin (IL)-4 and IL-5 producing CD4<sup>+</sup> and CD8<sup>+</sup> blood and sputum T cells from normal subjects and subjects with asthma and compared between cell subsets, subject groups, and body compartments with and without *in vitro* stimulation and investigated relationships between cytokine production and asthma severity. Production of IL-4, IL-5, and IFN- $\gamma$  by unstimulated sputum CD4<sup>+</sup> and CD8<sup>+</sup> T cells was increased in subjects with asthma and related to disease severity, more for CD8<sup>+</sup> than for CD4<sup>+</sup> T cells. Frequencies of sputum CD8<sup>+</sup> T cells producing type 1 and type 2 cytokines were similar to those of CD4<sup>+</sup> T cells. *In vitro* stimulation polarized peripheral blood cytokine production toward IFN- $\gamma$  production, significantly more in subjects with asthma than in normal subjects. These data demonstrate increased type 1 and 2 cytokine production in CD4<sup>+</sup> and CD8<sup>+</sup> T cells in sputum and relate production to disease severity. Findings in blood did not reflect those in airways.**

**Keywords:** asthma; flow cytometry; human; sputum; T lymphocytes

The Th2 hypothesis for asthma argues that alteration in the balance between Th1 and Th2 responses toward Th2 orchestrates the eosinophilia, IgE production, airway hyperresponsiveness, and chronic inflammation that are cardinal features of asthma. However, despite a wealth of animal data supporting the hypothesis, several human studies showing increased Th2 cytokine levels or messenger RNA (mRNA) expression in asthma (1–4) and the wide acceptance of this hypothesis in the respiratory community, there is to date no published evidence demonstrating increased Th2 cytokine protein production in CD4<sup>+</sup> T cells from asthmatic human lung, and no study has reported any relationship of Th2 cytokine production with disease severity.

There are also difficulties interpreting data regarding IFN- $\gamma$  production in relation to the Th2 hypothesis. Data that support this hypothesis include both *in vitro* animal and some human *in vivo* studies (5–11); however, there are conflicting data demonstrating elevated IFN- $\gamma$  levels (12–14) that correlate with disease activity (15). Finally, intracellular cytokine staining of bronchoalveolar lavage (BAL) cells demonstrated no increased interleukin (IL)-4<sup>+</sup>, but markedly increased IFN- $\gamma$ <sup>+</sup> T cells in subjects with allergic asthma compared with healthy subjects (16).

None of these studies has demonstrated excess Th2 cytokine production by asthmatic human lung CD4<sup>+</sup> T cells. Moreover, CD8<sup>+</sup> T cells also produce IL-4 (17), and increased IL-4 production by peripheral blood CD8<sup>+</sup> T cells in asthma has been reported (18), but other type 2 cytokines were not investigated, no comparison was made with CD4<sup>+</sup> T cell IL-4 production, and no relationships with disease severity were reported. We recently reported that production of both type 1 (IFN- $\gamma$ ) and type 2 (IL-4, IL-5, IL-10, and IL-13) cytokines by peripheral blood CD8<sup>+</sup> T cells was comparable with that of CD4<sup>+</sup> T cells (19). However, the frequencies of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells producing IFN- $\gamma$ , but not type 2 cytokines, increased in subjects with asthma compared with normal subjects. We hypothesized that this increased production of IFN- $\gamma$  may result from one or more of: (1) skewing of cytokine production toward type 1 as a result of *in vitro* stimulation with phorbol myristate acetate (PMA) and ionomycin (20), (2) increased cellular activation in allergic asthma leading to increased IFN- $\gamma$  production as a marker of inflammation, and/or (3) preferential recruitment of allergen-specific Th2/Tc2 cells to the lung resulting in reduced type 2 cytokine producing T cells in the peripheral blood.

Regarding the lung, a recent study investigating cytokine production in BAL CD4<sup>+</sup> and CD8<sup>+</sup> cells reported no increase in IFN- $\gamma$  or type 2 cytokine production in either CD4<sup>+</sup> or CD8<sup>+</sup> cells in asthmatic relative to normal subjects (21). Another report has demonstrated increased staining for IL-4 in CD8<sup>+</sup> T cells in subjects with asthma compared with normal subjects (22). However, no relationship with disease severity was reported (22). Studies of T-cell clones reported high frequencies of Th2 clones derived from bronchial biopsies, but these were from only two volunteers with asthma (23).

Published data, therefore, leave many unanswered questions regarding CD4<sup>+</sup> and CD8<sup>+</sup> T cell type 1 and type 2 cytokine production in asthma. We have studied cytokine production by intracellular staining of stimulated and unstimulated peripheral blood mononuclear cells and sputum CD4<sup>+</sup> and CD8<sup>+</sup> T cells, in subjects with asthma and in normal subjects. Correlations between cytokine production and asthma phenotypes were investigated to relate cytokine production to disease severity and to determine the important T-cell sources of the relevant cytokines. We studied airway T cells present in induced sputum because (1) BAL T-cell cytokine production has already been investigated without finding data to support increased type 2 cytokine production by CD4<sup>+</sup> T cells in asthma (16, 21, 22), (2) increased IL-4 and IL-5 mRNA expression in sputum T cells in asthma has been reported (3), (3) induced sputum represents a safe and noninvasive method of sampling airway cells in asthma, and (4) intracellular cytokine staining of sputum airway T cells has not previously been reported.

The hypothesis we wished to address is that there is increased production of type 2 cytokine protein by CD4<sup>+</sup> and CD8<sup>+</sup> T cells in asthmatic human lung and that this excess production is related with disease severity. We also wished to determine which of the T-cell subsets is the more important source of either type

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Correspondence and requests for reprints should be addressed to Sebastian L. Johnston, MB BS, PhD, FRCP, Professor of Respiratory Medicine, Department of Respiratory Medicine, National Heart and Lung Institute, Imperial College London, Norfolk Place, London W2 1PG, UK. E-mail: s.johnston@imperial.ac.uk

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1/2 cytokines in the lung, whether cytokine production is skewed by *in vitro* stimulation with PMA and ionomycin and whether observations made in peripheral blood reflect observations in airway CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

## METHODS

### Subjects

Nine individuals with atopic asthma and seven healthy nonatopic, non-smoking volunteers were recruited (Table 1). Asthma was defined according to American Thoracic Society criteria (24) and was mild to moderate in severity (25), using bronchodilators alone ( $n = 4$ ) or with inhaled steroid 200–1,000  $\mu\text{g}/\text{day}$  ( $n = 5$ , median dose 400  $\mu\text{g}/\text{day}$ ). None had oral corticosteroids or symptomatic respiratory infection in the 6 weeks preceding the study. Atopy was defined by a positive skin prick test response to one or more of *Dermatophagoides pteronyssinus*, mixed grass, tree pollen, cat, or dog (ALK, Horsholm, Denmark). Bronchial responsiveness was expressed as log PC<sub>20</sub>-histamine (26). Control subjects had no history of asthma or allergic disease, no respiratory symptoms, and were negative to skin tests.

Subjects gave informed written consent and the study was approved by the local Ethics Committee.

### Preparation of Sputum and Peripheral Blood Mononuclear Cells

Sputum was induced with 4.5% saline (27), saliva removed, and sputum weighed, diluted with 0.1% dithiothreitol, mixed for 30 minutes, filtered, centrifuged, and supernatant removed (28). Cells were resuspended in 1 ml phosphate-buffered saline (PBS), counted, and adjusted to  $5 \times 10^6/\text{ml}$ . Squamous cells were less than 30% and viability more than 90%.

Peripheral blood mononuclear cells were separated from 15 ml heparinized blood using Lymphoprep (Nycomed, Oslo, Norway), counted, and adjusted to  $3 \times 10^6/\text{ml}$  in RPMI 1640–2 mM glutamine (Gibco, Uxbridge, UK).

### Intracellular Cytokine Staining

One-milliliter aliquots of cells were cultured with 10  $\mu\text{g}/\text{ml}$  Brefeldin-A (Sigma, Poole, UK) for 4 hours at  $37^\circ\text{C} \pm 25$  ng/ml PMA and 1  $\mu\text{g}/\text{ml}$  ionomycin, washed in PBS, and resuspended in 2 ml RPMI 1640 and 2 mM glutamine. Sputum cells were incubated with RPMI/10% human AB serum for 30 minutes at  $4^\circ\text{C}$  to reduce nonspecific Fc binding. Cell suspensions (100  $\mu\text{l}$ ) were incubated for 30 minutes at  $4^\circ\text{C}$  with 20  $\mu\text{l}$  of peridin chlorophyll–protein-conjugated anti-CD3 antibodies (Becton Dickinson, San Jose, CA), diluted 1:5 in stain buffer (filtered PBS with 1% fetal calf serum and 0.1% sodium azide) and fluorescein isothiocyanate-conjugated anti-CD4 or anti-CD8 antibodies (both diluted 1:10). After washing with stain buffer, 500  $\mu\text{l}$  of permeabilizing solution (Becton Dickinson) was added and incubated for 10 minutes in the dark. After washing 20  $\mu\text{l}$  of anticytokine-phycoerythrin-conjugated or isotype control IgG1-phycoerythrin-conjugated (1:10 dilution in permeabilizing solution; Pharmingen, San Diego, CA) were added and incubated for 30 minutes at room temperature. Cells were washed, fixed with 1% paraformaldehyde, and analyzed by flow cytometry (FACScan [Becton Dickinson]). Lymphocytes (10,000) were gated, and CD3-PerCP-positive cells in this gate identified and selected into a second

gate according to positivity for fluorescein isothiocyanate-conjugated anti-CD4 or anti-CD8. Cytokine production was analyzed by detection of phycoerythrin staining in the gate of CD3<sup>+</sup>CD4<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup> lymphocytes. Frequencies of cells staining for each cytokine were expressed as percentage CD3<sup>+</sup>CD4<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup> T cells. There was no significant effect of dithiothreitol on cytokine staining for IFN- $\gamma$ , IL-4, and IL-5 in peripheral blood mononuclear cells (Figure 1). Specificity of cytokine staining was also confirmed by abolition of staining by preincubation of sputum cells with 100 $\times$  excess of each of these cytokines.

### Statistical Analysis

Statistical analyses were performed using SPSS for Windows and data presented as mean  $\pm$  SEM. Mann-Whitney U tests were used for comparisons between groups and Wilcoxon signed-rank test for within-group comparisons. Spearman correlation tests were used to investigate relationships between asthma severity and frequencies of CD4<sup>+</sup> or CD8<sup>+</sup> T cells producing cytokines. Values of  $p < 0.05$  were considered statistically significant.

## RESULTS

### Frequencies of Airway CD4<sup>+</sup> and CD8<sup>+</sup> T Cells Spontaneously Producing IL-4, IL-5, and IFN- $\gamma$ Are Significantly Higher in Subjects with Atopic Asthma than in Normal Subjects

Confirming our previous study (19), we found very low frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T cells staining positively for IL-4, IL-5, or IFN- $\gamma$  in unstimulated peripheral blood mononuclear cells ( $< 2\%$ ), and no difference between the two subject groups ( $p = \text{NS}$ ).

Frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T cells spontaneously producing IL-4 or IL-5 were significantly increased in the airway of subjects with asthma compared with normal subjects ( $p \leq 0.03$  and  $p \leq 0.01$  for CD4<sup>+</sup> and CD8<sup>+</sup> T cells, respectively, Figure 2). Frequencies of airway CD4<sup>+</sup> and CD8<sup>+</sup> T cells producing IFN- $\gamma$  were also increased in asthmatic subjects ( $p = 0.05$  and  $p < 0.01$  respectively, Figure 2).

### CD8<sup>+</sup> T Cells Produce Type 1 and Type 2 Cytokines at Similar Frequencies to CD4<sup>+</sup> T Cells in both Normal Subjects and Subjects with Asthma

We compared production of IL-4, IL-5, and IFN- $\gamma$  between the two T-cell subsets, in blood and airway T cells, both with and without *in vitro* stimulation, in atopic subjects with asthma and normal subjects. Our previous observation of similar type 1 and type 2 cytokine production in unstimulated or stimulated blood CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets in both groups was confirmed (19) ( $p = \text{NS}$  in all cases, Figure 3). Frequencies of airway CD8<sup>+</sup> T cells staining positively for each of IL-4, IL-5, and IFN- $\gamma$  were also very similar to those of airway CD4<sup>+</sup> T cells, in either atopic asthmatic or normal subjects, for both stimulated and unstimulated cells ( $p = \text{NS}$  in all cases, Figure 3).

### Frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T Cells Spontaneously Producing IL-4, IL-5, and IFN- $\gamma$ Increased in the Airway Compared with Peripheral Blood

We next compared spontaneous production of IL-4, IL-5, and IFN- $\gamma$  by CD4<sup>+</sup> and CD8<sup>+</sup> T cells from blood and airway. Frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T cells spontaneously producing each of IL-4, IL-5, and IFN- $\gamma$  were significantly increased in airway T cells compared with peripheral blood T cells in both atopic asthmatic and normal subjects ( $p \leq 0.05$  in all cases, except IL-4<sup>+</sup>CD8<sup>+</sup> T cells in normal subjects [ $p = 0.12$ ], Figures 3A and 3B, respectively).

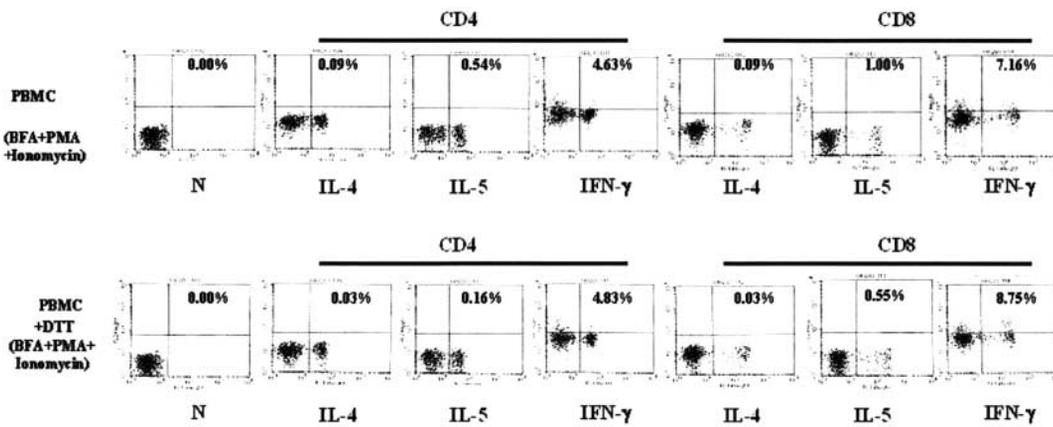
TABLE 1. SUBJECT DEMOGRAPHICS

	Asthma ( $n = 9$ )	Normal ( $n = 7$ )
Age, yr	41.7 $\pm$ 4.7	31.7 $\pm$ 3.9
FEV <sub>1</sub> , % predicted	88.3 $\pm$ 4.2	101.2 $\pm$ 4.2
Blood eosinophils, per mm <sup>3</sup>	233.3 $\pm$ 55.3	85.7 $\pm$ 14.2
Serum IgE, IU/ml	175.0 $\pm$ 101.5	37.8 $\pm$ 13.0
PC <sub>20</sub> histamine, mg/ml*	1.9	> 16

Definitions of abbreviations: FEV<sub>1</sub> = forced expiratory volume in 1 second; PC<sub>20</sub> = concentration of histamine producing a 20% fall in FEV<sub>1</sub>.

Data expressed as mean  $\pm$  SEM.

\* Geometric mean.



**Figure 1.** Effects of dithiothreitol on intracellular cytokine staining of peripheral blood T cells from a subject with atopic asthma. Experiments were performed in peripheral blood mononuclear cells from two subjects with atopic asthma to investigate whether dithiothreitol treatment influences intracellular cytokine staining. There was no significant effect of dithiothreitol on cytokine staining for interleukin (IL)-4, IL-5, or IFN- $\gamma$  in T cells. These data were similar in another independent experiment.

**In Vitro Stimulation Increases IL-4-, IL-5-, and IFN- $\gamma$ -Producing Peripheral Blood, but not Airway CD4+ and CD8+ T cells**

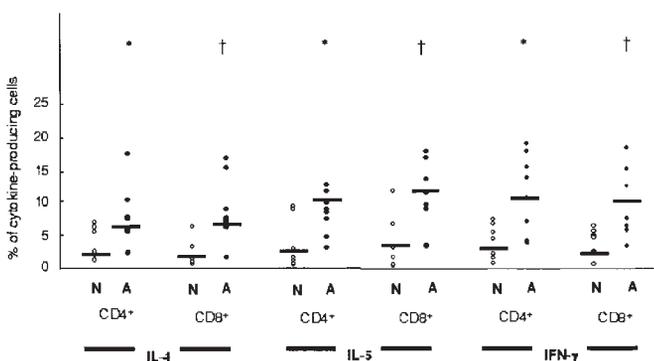
As previously reported (19), in peripheral blood, frequencies of IL-4-, IL-5-, and IFN- $\gamma$ -positive CD4+ and CD8+ T cells increased significantly on *in vitro* stimulation with PMA and ionomycin in both atopic subjects with asthma and normal subjects ( $p < 0.05$  in all cases, data not shown). In contrast, frequencies of IL-4-, IL-5-, and IFN- $\gamma$ -producing airway sputum CD4+ and CD8+ T cells was not further increased by *in vitro* stimulation with PMA and ionomycin in either atopic asthmatic or normal subjects ( $p = NS$  in all cases, data not shown).

**PMA and Ionomycin Stimulation of Peripheral Blood Mononuclear Cells Preferentially Increases IFN- $\gamma$  Production in CD4+ and CD8+ T Cells More in Atopic Asthma than in Normal Subjects**

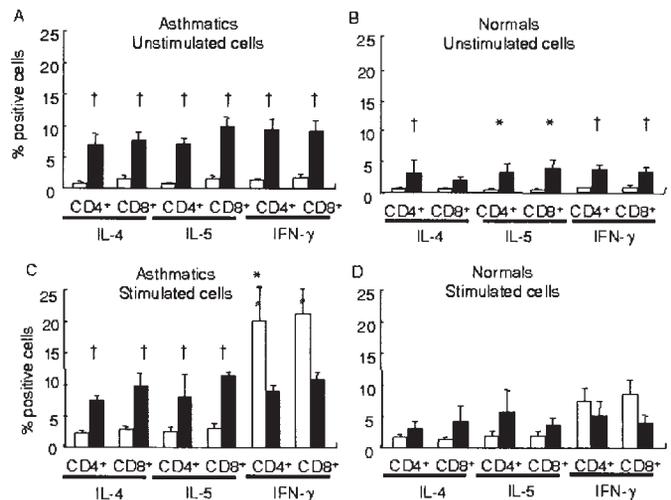
To determine whether stimulation induced skewing of the cytokine repertoire and whether this was different between T cell

subsets or between subject groups, the magnitudes of induction were assessed for each blood T cell subset and for each subject group.

The increase in frequency of both IL-4- and IL-5-positive cells after *in vitro* stimulation with PMA and ionomycin was modest in both CD4+ and CD8+ T cells (between twofold and fourfold) in both atopic asthmatic and normal subjects for each T-cell subset. In contrast, the increase in frequency of IFN- $\gamma$ -positive T cells after *in vitro* stimulation with PMA and ionomycin for both CD4+ and CD8+ blood T cells was between 9-fold and 15-fold. The induction in IFN- $\gamma$  in response to *in vitro* stimulation was significantly greater than the inductions observed for both IL-4 and IL-5 in both T-cell subsets ( $p < 0.05$  in each case).



**Figure 2.** Increased in frequencies of airway CD4+ or CD8+ T cells spontaneously producing IL-4, IL-5, and IFN- $\gamma$  in subjects with atopic asthma compared with normal subjects. Airway CD4+ and CD8+ T cell cytokine production was assessed by intracellular staining and flow cytometry of sputum cells after 4 hours of incubation with Brefeldin without *in vitro* stimulation. (N: open circles = subjects without atopy or asthma, A: solid circles = subjects with atopic asthma). Bars are the means. Statistical analysis was performed using Mann-Whitney U tests to compare percentage of cytokine-producing CD4+ and CD8+ T cells between subjects with atopic asthma and normal subjects. \* $p \leq 0.05$ , † $p \leq 0.01$ .



**Figure 3.** Comparison of airway versus peripheral blood CD4+ and CD8+ T cells in their relative capacities to produce type 1 and type 2 cytokines and their response to *in vitro* stimulation. The percentage of cytokine-producing CD4+ or CD8+ T cells was assessed by intracellular staining after 4 hours incubation with Brefeldin alone (unstimulated, A and B) or with *in vitro* stimulation with PMA and ionomycin in the presence of Brefeldin (stimulated, C and D). Frequencies of stimulated and unstimulated CD4+ or CD8+ T cells producing each of IL-4, IL-5, and IFN- $\gamma$  were compared between peripheral blood (open bars) and airway (filled bars) T cells in subjects with asthma (A and C), and control (B and D) subjects. Values are means  $\pm$  SEM. \* $p \leq 0.05$ , † $p < 0.01$ .

Finally, to determine whether induction of IFN- $\gamma$  by PMA/ionomycin was greater in subjects with asthma than in normal subjects, the fold induction was compared between subject groups. There were no differences in induction in IL-4 or IL-5 between subjects with asthma and normal subjects ( $p = \text{NS}$  for each cytokine). However, the fold increase in IFN- $\gamma$ -positive cells was greater in magnitude in subjects with atopic asthma compared with normal subjects for peripheral blood CD4<sup>+</sup> and CD8<sup>+</sup> T cells ( $p = 0.02$  and  $0.08$ , respectively), confirming that *in vitro* stimulation with PMA and ionomycin of peripheral blood CD4<sup>+</sup> and CD8<sup>+</sup> T cells induces significant skewing of cytokine repertoire toward type 1 cytokine production and that skewing is greater in cells from atopic subjects with asthma than from normal subjects.

#### Effect of *In Vitro* Stimulation on Differences in Production of Type 1 and Type 2 Cytokines Between Peripheral Blood and Airway CD4<sup>+</sup> and CD8<sup>+</sup> T Cells

In asthmatic subjects, when comparing stimulated cells between the two compartments (Figure 3C), the pattern of type 2 cytokine production was the same as for unstimulated cells (Figure 3A), in that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells type 2 cytokine production was significantly greater in airway (whether stimulated [Figure 3C], or not [Figure 3A]) than in *in vitro* stimulated peripheral blood T cells ( $p < 0.01$ ). In contrast, for IFN- $\gamma$  production in which the response to *in vitro* stimulation in blood was much greater than that observed for type 2 cytokines, the staining frequencies in the two compartments were reversed in comparison to unstimulated cells. IFN- $\gamma$  production in *in vitro* stimulated airway T cells was lower than in *in vitro* stimulated peripheral blood T cells for both CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets ( $p = 0.05$  and  $p = 0.06$ , respectively; Figure 3C).

A similar but less robust reversal in patterns of IFN- $\gamma$  staining in the two compartments in response to *in vitro* stimulation was also observed in normal subjects (Figure 3D). These data confirm that the relevance of observations made in peripheral blood to those in airway T cells can be altered as a result of *in vitro* stimulation.

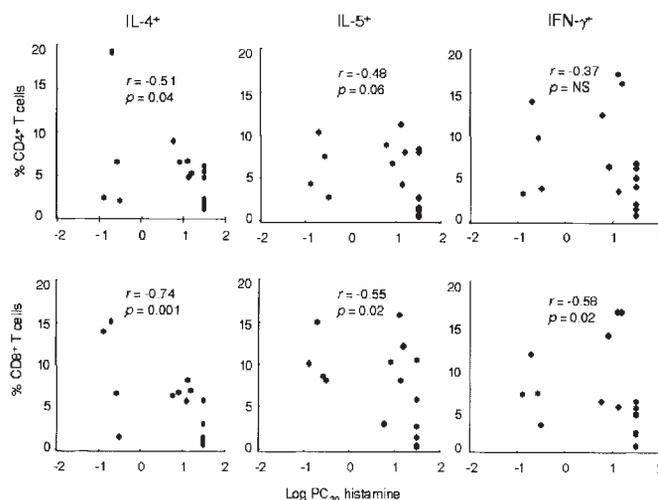
#### Relationships Between Bronchial Hyperresponsiveness, Blood Eosinophils, and Serum IgE and Airway and Blood CD4<sup>+</sup> and CD8<sup>+</sup> T-Cell Cytokine Production

Significant inverse correlations were observed between log PC<sub>20</sub> histamine and the frequencies of airway CD4<sup>+</sup> and CD8<sup>+</sup> T cells spontaneously producing IL-4 ( $r = -0.51$ ,  $p = 0.04$  and  $r = -0.74$ ,  $p = 0.001$ , respectively; Figure 4). A strong trend and a significant inverse correlation were also observed between log PC<sub>20</sub> histamine and the frequencies of airway CD4<sup>+</sup> and CD8<sup>+</sup> T cells spontaneously producing IL-5 ( $r = -0.48$ ,  $p = 0.06$  and  $r = -0.55$ ,  $p = 0.02$ , respectively; Figure 4).

There was a significant inverse correlation between log PC<sub>20</sub> histamine and the frequencies of airway CD8<sup>+</sup> T cells spontaneously producing IFN- $\gamma$  ( $r = -0.58$ ,  $p = 0.02$ ), but not for CD4<sup>+</sup> T cells ( $r = -0.37$ ,  $p = \text{NS}$ , Figure 4).

There were no significant relationships between bronchial hyperresponsiveness and the frequencies of peripheral blood T cells of either subset staining positively for any cytokine, with or without *in vitro* stimulation ( $p = \text{NS}$ ).

Peripheral blood eosinophil counts were significantly correlated with the frequencies of airway stimulated CD4<sup>+</sup> and CD8<sup>+</sup> T cells staining positively for IL-5 ( $r = 0.58$ ,  $p = 0.02$  and  $r = -0.65$ ,  $p = 0.01$ , respectively). There were no significant correlations between serum total IgE levels and frequencies of airway CD4<sup>+</sup> or airway CD8<sup>+</sup> T cells staining positive for IL-4 ( $p = \text{NS}$  in all cases).



**Figure 4.** Relationships between bronchial hyperresponsiveness and spontaneous type 1 and type 2 cytokine production by airway CD4<sup>+</sup> and CD8<sup>+</sup> T cells. There was a strong statistically significant relationship between bronchial hyperresponsiveness and airway CD8<sup>+</sup> T cell cytokine production manifest by inverse correlations between logPC<sub>20</sub> histamine and CD8<sup>+</sup> T-cell staining frequencies for interleukin-4 ( $r = -0.74$ ,  $p = 0.001$ ), interleukin-5 ( $r = -0.55$ ,  $p = 0.02$ ) and IFN- $\gamma$  ( $r = -0.58$ ,  $p = 0.02$ ), respectively. In contrast, the relationship between bronchial hyperresponsiveness and airway CD4<sup>+</sup> T-cell cytokine production was relatively weak, manifested by a significant inverse correlation between logPC<sub>20</sub> histamine and CD4<sup>+</sup> T-cell staining frequency for interleukin-4 ( $r = -0.51$ ,  $p = 0.04$ ), a weak trend toward a correlation for interleukin-5 ( $r = -0.48$ ,  $p = 0.06$ ) and no significant relationship for IFN- $\gamma$  ( $p = \text{NS}$ ).

## DISCUSSION

We have demonstrated that human airway CD4<sup>+</sup> and CD8<sup>+</sup> T cells spontaneously produce increased type 1 and type 2 cytokines in subjects with asthma relative to normal subjects; that type 1 and type 2 cytokine production by both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets have significant relationships with disease severity (more strongly for CD8<sup>+</sup> than for CD4<sup>+</sup> T cells); that both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets are important sources of both type 1 and type 2 cytokines in the lung; and that observations made in peripheral blood CD4<sup>+</sup> and CD8<sup>+</sup> T cells do not reflect observations in airway CD4<sup>+</sup> and CD8<sup>+</sup> T cells. We demonstrate that *in vitro* stimulation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in peripheral blood results in skewing of the cytokine repertoire toward a type 1 response and that this skewing is greater in subjects with asthma than in normal subjects. Finally, we demonstrate that airway CD4<sup>+</sup> and CD8<sup>+</sup> T cells in sputum are fully activated and do not respond to further stimulation and that the patterns of cytokine staining observed are therefore not a result of polarization as a result of *in vitro* stimulation. These data provide direct support for the Th2 hypothesis—that subjects with asthma have increased production of type 2 cytokines by CD4<sup>+</sup> T cells in the diseased organ and that production of Th2 cytokines is related to disease severity. We further demonstrate increased production of type 2 cytokines by CD8<sup>+</sup> T cells in the diseased organ and show that the relationship of CD8<sup>+</sup> T cell type 2 cytokine production to disease severity is stronger than that of CD4<sup>+</sup> T cells. Finally IFN- $\gamma$  production by both T-cell subsets was increased in asthma and production by CD8<sup>+</sup> T cells related to bronchial reactivity, suggesting that the role of IFN- $\gamma$  in asthma may be complex.

It is possible that some of the observations made in the subjects with asthma were influenced by the fact that five of the nine subjects with asthma were taking inhaled corticosteroids. In view of the small numbers of patients taking and not taking steroids (five and four, respectively) and the varied dosage of inhaled steroids among those taking, it is not appropriate to carry out statistical analysis between groups. Further studies will be needed to determine whether our observations are valid for asthmatic subjects taking or not taking inhaled steroids.

Studies on T-cell clones derived from two subjects with asthma showed most were of a Th2 profile (23), but no normal individuals were included in this study. When clones from subjects with asthma and normal individuals were systematically compared, no evidence of any Th2 bias was observed (29).

Robinson and Kay were the first to show increased IL-4 and IL-5 mRNA expression in bronchial lavage from subjects with asthma (2). The same investigators then went on to demonstrate that more than 70% of IL-4 and IL-5 mRNA-positive cells in lavage and biopsies were activated CD3<sup>+</sup> T cells (30), though they did not investigate CD4<sup>+</sup> T cells alone. However, when type 2 cytokine protein production was studied, the researchers observed that the predominant cells types staining for IL-4 and IL-5 in bronchial biopsies from subjects with asthma were mast cells and eosinophils, not T cells (4). The authors concluded that immunochemistry was not the best technique for studying T-cell type 2 cytokine production.

For this reason, other investigators have used intracellular cytokine staining: increased IFN- $\gamma$  production by BAL CD3<sup>+</sup> T cells was observed, with no increase in either IL-4 or IL-5 production in subjects with asthma relative to normal subjects (16). In another study, no differences were observed between subjects with asthma and normal subjects in BAL CD4<sup>+</sup> T-cell production of IL-4, IL-5, IL-13, or IFN- $\gamma$  (21). However, this technique has been criticized because the methods of *in vitro* stimulation could preferentially induce type 1 cytokines and therefore not reflect the *in vivo* situation (20).

We have investigated spontaneous T-cell cytokine production without *in vitro* stimulation in subjects with asthma and normal subjects and have demonstrated increased spontaneous production of both IL-4 and IL-5 in airway CD4<sup>+</sup> T cells from asthma subjects relative to normal subjects (Figure 2). These data provide confirmation of increased type 2 cytokine production by CD4<sup>+</sup> T cells in human asthmatic lung.

In addition to CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells have also been shown to produce a range of type 2 cytokines, production that may exceed that of CD4<sup>+</sup> T cells in some instances (17, 18, 31). Recent studies have shown that CD8<sup>+</sup> T cells can be classified into Tc1, which secrete predominantly IL-2 and IFN- $\gamma$ , and Tc2, which secrete IL-4 and IL-5 (17, 32–34). However, the role of type 2 cytokine production by CD8<sup>+</sup> T cells in asthma has not been extensively studied.

We reported the frequencies of peripheral blood CD8<sup>+</sup> T cells producing IL-4, IL-5, IL-10, and IL-13 in response to PMA and ionomycin were comparable to CD4<sup>+</sup> T cells (19). In the present study, we confirm that lung CD8<sup>+</sup> T cells are capable of producing both IL-4 and IL-5 spontaneously and that production of both IL-4 and IL-5 by airway CD8<sup>+</sup> T cells was increased in subjects with asthma (Figure 2). As observed previously in blood (19), we confirmed that production of both IL-4 and IL-5 was similar in CD8<sup>+</sup> T cells and in CD4<sup>+</sup> T cells in both airway and blood, in both stimulated and unstimulated cells in both subjects with asthma and normal subjects. Furthermore, the relationships between cytokine staining frequencies and disease severity were stronger and more statistically significant for CD8<sup>+</sup> T cells than for CD4<sup>+</sup> T cells (Figure 4). These data combined indicate that IL-4 and IL-5 production by airway CD8<sup>+</sup> T cells

is as great as production by CD4<sup>+</sup> T cells and that the relationships with disease severity are stronger for CD8<sup>+</sup> T cells than for CD4<sup>+</sup> T cells.

The possible role of CD8<sup>+</sup> T cells in the pathogenesis of asthma has received relatively little attention. Numerically, CD8<sup>+</sup> T cells are present in the lung in similar numbers to CD4<sup>+</sup> T cells (35), and CD8<sup>+</sup> T cells have recently been implicated in asthma deaths (36). Our data and previous data showing that resting peripheral blood CD8<sup>+</sup> T cells in patients with asthma contained significantly more IL-4 than those of healthy nontopic subjects (26 versus 16 pg/10<sup>6</sup> cells) (18) suggest that the role of type 2 cytokine production by CD8<sup>+</sup> T cells in asthma merits further investigation.

The relationships we have observed between frequencies of type 2 cytokine production by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells and disease severity (Figure 4) are novel and important. Bronchial hyperresponsiveness is a cardinal feature of asthma, and the relationships reported here indicate that production of type 2 cytokines by both CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets, but particularly that of CD8<sup>+</sup> T cells, is likely to play a causal role in disease pathogenesis.

The relationships we have observed between airway CD4<sup>+</sup> and CD8<sup>+</sup> T cell IL-5 production and peripheral blood eosinophilia are consistent with its biological function (37, 38). They also suggest that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are important sources of IL-5 in the asthmatic lung and that both subsets are likely to be important regulators of eosinophil recruitment from the bone marrow. That the relationship for CD4<sup>+</sup> T cells was only a trend toward significance ( $r = -0.48$ ,  $p = 0.06$ ), whereas that for CD8<sup>+</sup> T cells was statistically significant ( $\rho = -0.55$ ,  $p = 0.02$ ), suggests that CD8<sup>+</sup> T cells may play a more important role in eosinophil recruitment than has previously been recognized.

We did not observe any relationship between lung CD4<sup>+</sup> or CD8<sup>+</sup> T cell production of IL-4 and serum total IgE levels. This lack of correlation may be because IL-13 may be more important in regulating serum IgE than is IL-4 (39)—unfortunately, we were unable to assess IL-13 production in this study. A second possibility is that lung IL-4 production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells may be closely related to production of allergen-specific IgE (40), but not to serum total IgE (41).

In the present study, we elected to investigate sputum T-cell cytokine production. From the limited data available, it appears the cellular distribution of sputum correlates well with cell numbers in BAL (42, 43). Although lymphocyte numbers in induced sputum are low, we have been able to detect spontaneous production of both IL-4 and IL-5 by CD4<sup>+</sup> and CD8<sup>+</sup> T cells. These data indicate that noninvasive sampling of induced sputum permits the study of airway T-cell cytokine production without the need for bronchoscopy.

Reported observations in BAL and our data in sputum indicate possible differences in T-cell phenotypes between the two lung compartments. T cells in BAL produce very little type 2 cytokine without *in vitro* stimulation (16, 21). In contrast, T cells in sputum produced maximal levels of type 2 cytokines spontaneously and did not further increase production with stimulation. These observations suggest that airway T cells in sputum are already fully activated and may be different in their functional activity from BAL T cells. Further studies are needed to clarify the functional differences between BAL and sputum-derived cells from the same subjects.

*In vitro* stimulation can polarize T-cell cytokine production (20) and result in skewing of cytokine production patterns (18). Previous studies (19, 44) and the present study have observed an increased frequency of peripheral blood T cells staining positively for IFN- $\gamma$ , but not for IL-4 and IL-5, after stimulation

with PMA and ionomycin in subjects with asthma compared with healthy control subjects. The same increased T-cell type 1 but not type 2 cytokine production has been reported for BAL T cells stimulated *in vitro* in the same manner (16). A possible explanation for these findings, which all appear to contradict the Th2 hypothesis, is that the method of *in vitro* stimulation used preferentially enhances type 1 cytokine production relative to type 2 (20).

In the present study, we show that stimulation of peripheral blood T cells preferentially enhances IFN- $\gamma$  over IL-4 and IL-5 production by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Furthermore, this skewed induction in favor of IFN- $\gamma$  production is significantly greater in the subjects with asthma. In the present study, our ability to investigate airway T-cell cytokine production in unstimulated sputum CD4<sup>+</sup> or CD8<sup>+</sup> T cells demonstrated staining positivity for IL-4 or IL-5 was indeed increased in subjects with asthma compared with control subjects. We believe that the previous reports demonstrating increased T-cell IFN- $\gamma$  production in blood and BAL in subjects with asthma (16, 19, 44) may result from the greater induction of IFN- $\gamma$  production in asthmatic subjects relative to normal subjects as a consequence of the *in vitro* stimuli used (33).

We also observed that frequencies of unstimulated airway CD4<sup>+</sup> and CD8<sup>+</sup> T cells spontaneously producing IFN- $\gamma$  were increased in subjects with asthma compared with control subjects. These data suggest that T cells producing IL-4 and IL-5 and T cells producing IFN- $\gamma$  are increased in the airways of subjects with asthma. In addition, we observed a significant relationship between CD8<sup>+</sup> T-cell IFN- $\gamma$  production and bronchial responsiveness and a weak trend toward such a relationship for CD4<sup>+</sup> T-cell IFN- $\gamma$  production (Figure 4), suggesting that IFN- $\gamma$  production was related to disease severity.

These data are in accordance with previous work demonstrating that IFN- $\gamma$  is elevated in acute and chronic asthma (12–14) and correlated with disease activity in subjects with asthma (15). However, none of these clinical studies have specifically investigated lung T cells as the source of IFN- $\gamma$  production. IFN- $\gamma$  has a number of proinflammatory actions relevant to the pathogenesis of asthma (45–47), and Hansen and colleagues showed that Th1 cells did not attenuate Th2-cell-induced airway hyper-responsiveness in mice, but rather caused severe airway inflammation (48). Collectively, these data suggest IFN- $\gamma$  may have dual roles in relation to the pathogenesis of asthma—as a mediator of both acute and chronic inflammation and as a counterregulator of type 2 T-cell responses.

These data emphasize that studying the role of IFN- $\gamma$  in asthma is complex. Studies such as ours, which compare inflamed asthmatic airways with normal control airways, can detect increased IFN- $\gamma$  in asthma as a result of an inflammatory condition being compared with the absence of any inflammatory condition. In contrast, studies comparing normal subjects and subjects with asthma in which both undergo a similar inflammatory stimulus such as a viral infection, can reveal the relative deficiency in IFN- $\gamma$  production in asthma (11).

In conclusion, we have investigated frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> human airway and peripheral blood T cells staining positively for type 2 (IL-4, IL-5) and type 1 cytokines (IFN- $\gamma$ ) in subjects with asthma and normal subjects. We demonstrate that frequencies of airway CD8<sup>+</sup> T cells producing type 2 cytokines are as great as those of airway CD4<sup>+</sup> T cells, that both are increased in asthma, and that both are related to disease severity. The relationship with disease severity was stronger for CD8<sup>+</sup> T cells than for CD4<sup>+</sup> T cells. We also demonstrate skewing of the peripheral blood cytokine repertoire toward type 1 as a result of *in vitro* stimulation, and that this skewing is greater in subjects with asthma. These data provide an explanation for previous

reports that failed to demonstrate increased type 2 cytokine producing T cells in asthma in studies using these techniques. These data provide direct support for the Th2 hypothesis, demonstrating increased Th2 cytokine production by CD4<sup>+</sup> T cells in the target organ in subjects with asthma. They also indicate that the CD8<sup>+</sup> T cell may be an important source of type 2 cytokines in asthma. Further studies investigating the role of CD8<sup>+</sup> T cells are required to further elucidate their importance in type 2 and type 1 cytokine production in the pathogenesis of human allergic disease.

**Conflict of Interest Statement:** S.-H.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; L.A.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; S.T.H. has been reimbursed by a number of pharmaceutical companies to present at sponsored symposia and has participated as a speaker in scientific meetings or courses organized and financed by various pharmaceutical and diagnostic companies (MSD, AstraZeneca, Novartis) and a remuneration for serving as a member of the Board of Scientific Advisors for Ammiral in 2001, 2002, and 2003 as well as the Scientific Advisory Board of Cell Tech and is one of the founders of SynAirgen; S.L.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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