

Original article

Correlation between peripheral blood T-cell profiles and airway inflammation in atopic asthma

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Background: Recent studies suggest that T_H1/T_H2 and T_C1/T_C2 cell imbalances are implicated in asthma pathogenesis.

However, the relationship of T-cell profiles to airway inflammation has not been understood.

Objective: The purpose of this study was to clarify the correlation between peripheral blood T-cell profiles and airway inflammatory parameters.

Methods: Subjects included 21 patients with atopic asthma and 20 healthy control subjects. After stimulation with phorbol 12-myristate 13-acetate and ionomycin, flow cytometry was used to analyze intracellular cytokine staining for IFN- γ and IL-4 in peripheral blood CD4⁺ and CD8⁺ T cells. Airway inflammation was assessed by using exhaled nitric oxide analysis and induced sputum eosinophil counts.

Results: There were a significantly increased proportion of IL-4-producing CD4⁺ T cells (median, 2.7% [interquartile range, 2.4% to 3.3%] vs 1.8% [interquartile range, 1.3% to 2.7%]) and a lower ratio of IFN- γ -producing/IL-4-producing CD4⁺ T cells (7.3% [interquartile range, 5.8% to 9.5%] vs 10.0% [interquartile range, 8.8% to 13.2%]) in asthmatic patients compared with in healthy subjects ($P < .01$) but no increase or decrease in the proportion of IFN- γ -producing CD4⁺ T cells. No significant difference was found in the proportion of IFN- γ - or IL-4-producing CD8⁺ T cells between the 2 groups. The proportion of IL-4-producing CD4⁺ T cells was significantly correlated with exhaled nitric oxide ($r = 0.48$, $P < .05$) and induced sputum eosinophil counts ($r = 0.50$, $P < .05$).

Conclusion: These findings indicate a correlation between peripheral blood T_H2 cytokine production and markers of airway inflammation.

Clinical implications: There is an association between immune function and clinical indicators in asthma. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Airway inflammation, atopic asthma, correlation, exhaled nitric oxide, flow cytometry, induced sputum, IFN- γ , IL-4, peripheral blood, T cell

Abbreviations used

FITC: Fluorescein isothiocyanate
NO: Nitric oxide
PE: Phycoerythrin
PEF: Peak expiratory flow
PerCP: Peridinin chlorophyll protein
PMA: Phorbol 12-myristate 13-acetate
RT: Room temperature

Asthma is characterized by airway inflammation with eosinophils, mast cells, and T lymphocytes. It is known as the T_H2 hypothesis, which suggests that an increased production of T_H2 cell-derived cytokines (IL-4, IL-5, and IL-13), a decreased production of T_H1 cell-derived cytokines (IFN- γ and IL-2), or both are essential for the development of airway inflammation.¹⁻³ A critical role for T_H2 cells in asthma is now widely accepted.

The flow cytometric method to examine intracellular cytokine production at the single-cell level is sensitive and does not need purification of PBMCs, which might modify the cell activation.⁴⁻⁶ A study of the balance of T_H1/T_H2 cells in the peripheral blood from patients with asthma by using the flow cytometric method is controversial. Some reports have shown an increased proportion of IL-4-producing CD4⁺ T cells, a lower ratio of IFN- γ -producing/IL-4-producing CD4⁺ T cells, or both,⁶⁻⁸ whereas others have demonstrated an increased proportion of IFN- γ -producing CD4⁺ T cells.⁸⁻¹⁰ Furthermore, CD8⁺, as well as CD4⁺, T cells have been classified into 2 distinct cell types, T_C1 and T_C2 cells, on the basis of their cytokine profiles.¹¹ Whether IL-4- or IFN- γ -producing CD8⁺ T cells from peripheral blood are predominant in asthma is also controversial.^{9,10,12,13} Recent studies have investigated cytokine production in bronchoalveolar lavage fluid or induced sputum T cells, raising the possibility that pulmonary T-lymphocyte profiles might be different from those of peripheral blood, and a consensus has not been established either.^{10,14,15} Very few studies have assessed the relationship between T-cell profiles and airway hyperresponsiveness or atopic status of asthma.^{6,8,10,13} To our knowledge, no relationship with airway inflammation has been reported.

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Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication December 14, 2005; revised May 10, 2006; accepted for publication May 10, 2006.

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0091-6749/\$32.00

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doi:10.1016/j.jaci.2006.05.005

TABLE I. Characteristics of the study population

	Atopic asthmatic subjects (n = 21)	Healthy control subjects (n = 20)
Age, y	30 (26-42)	34 (26-46)
Sex (male/female), n	9/12	9/11
Onset of asthma (childhood/adulthood), n	7/14	—
Blood eosinophils, per μ L	477 (301-580)	112 (78-212)
Total serum IgE, IU/mL	680 (260-1100)	15 (9-87)
FEV ₁ , % predicted	79 (73-96)	118 (105-131)
PEF variability, %	28 (14-35)	ND
PC ₂₀ FEV ₁ , mg/mL	0.6 (0.4-1.2)	>20.0*
Exhaled NO, ppb	111 (85-179)	14 (13-17)
Sputum eosinophils, %	7 (2-33)	ND

Data are expressed as medians with interquartile ranges.

ND, Not done; PC₂₀FEV₁, provocative concentration of methacholine causing a 20% decrease in FEV₁.

*All subjects had a PC₂₀FEV₁ of greater than 20.0 mg/mL.

The purpose of this study was to clarify the balances of peripheral blood T_H1/T_H2 and T_C1/T_C2 cells in atopic asthma compared with those in healthy subjects and the correlation between T_H and T_C profiles and parameters of airway inflammation, including exhaled nitric oxide (NO) and induced sputum eosinophils.

METHODS

Subjects

Twenty-one patients with atopic asthma and 20 nonatopic healthy control subjects were enrolled in the study (Table I). These consecutive patients with atopic asthma first visited Fujinomiya City General Hospital from December 2003 through November 2004, satisfied the American Thoracic Society criteria,¹⁶ and had asthmatic symptoms within 1 week before the first visit. The severity of asthma was intermittent or mild persistent according to the classification of the Global Initiative for Asthma.¹⁷ Atopy was defined by the positive specific IgE antibodies to at least one inhalant allergen, including house dust mites, cedar pollen, grass pollens, molds, cockroach, cat, or dog (CAP system; Pharmacia, Uppsala, Sweden). According to age of onset, patients were classified as having childhood-onset (<18 years) or adult-onset ($\geq$ 18 years) asthma. No subject was a current smoker (14 subjects who never smoked and 7 exsmokers [mean pack years, 5.1]), had received oral or inhaled corticosteroids or other medications for asthma for at least 6 months, or reported acute viral infections for at least 1 month before the first visit. All subjects provided informed consent.

Study design

At the first visit, the patients underwent blood tests, exhaled NO measurement, spirometry, methacholine inhalation challenge, and sputum induction. Exhaled NO levels were measured by using the online method with an NO analyzer (Sievers NOA 280i; Sievers, Boulder, Colo), according to the American Thoracic Society/European Respiratory Society recommendations.¹⁸ Methacholine inhalation challenges were performed as reported previously,¹⁹ and a provocative concentration of methacholine causing a 20% decrease in FEV₁ was expressed as PC₂₀FEV₁. Sputum induction was performed according to the method described previously,²⁰ with minor modifications. The patients were also instructed to record their peak

expiratory flow (PEF) values and the mean diurnal PEF variation for 1 week since the first visit was calculated.

Preparation of peripheral blood cells

Ten milliliters of heparinized blood was collected from each patient or control subject and diluted 1:20 with a culture medium consisting of RPMI-1640 supplemented with 10% FCS (Gibco, Rockville, Md), 100 U/mL penicillin (Gibco), and 100 μ g/mL streptomycin (Gibco).

Stimulation of cells and intracellular cytokine staining

One milliliter of peripheral blood cells diluted 1:20 in culture medium were stimulated with phorbol 12-myristate 13-acetate (PMA; Sigma, St Louis, Mo) and ionomycin (1 mg/mL, Sigma) in the presence of brefeldin-A (10 mg/mL, Sigma) for 4 hours at 37°C.

After stimulation with PMA/ionomycin, the cells were incubated with either peridin chlorophyll protein (PerCP)-conjugated anti-CD4 or anti-CD8 antibody (Becton Dickinson, San Jose, Calif) for 15 minutes at room temperature (RT). In the preparation of peripheral blood cells, erythrocytes were lysed by adding FACS Lysing Solution (Becton Dickinson). After centrifugation, the cells were washed with 0.1% BSA/PBS and subsequently incubated in FACS Permeabilizing Solution (Becton Dickinson) for 10 minutes at RT. After washing with 0.1% BSA/PBS, the permeabilized cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-IFN- γ and phycoerythrin (PE)-conjugated anti-IL-4 antibodies for 30 minutes at RT. After staining, they were washed with 0.1% BSA/PBS, fixed in 1% paraformaldehyde, and analyzed. Negative controls consisted of unstimulated cells. We confirmed that the viability of the cells freshly obtained from peripheral blood was greater than 97% by using trypan blue exclusion. After PMA/ionomycin activation, the viability of each cell population was greater than 95%.

Flow cytometric analysis

The samples were analyzed on a FACScan flow cytometer (Becton Dickinson) equipped with a 15-mW argon ion laser and appropriate filters for FITC (530 nm), PE (585 nm), and PerCP (>650 nm) by using the Cell Quest Software (Becton Dickinson). Lymphocytes were first gated on the basis of forward and side light scatter. Subsequently, within this gated area, a second gate was further selected. The following analyses were done on cells in the second gate. For 3-color analysis, we measured the number of positive cells for anti-IFN- γ (FITC), anti-IL-4 (PE), or both antibodies simultaneously in gated cell populations that were stained with either anti-CD4 or anti-CD8 antibody (PerCP). The number of positive cells for each cytokine was expressed as a percentage of CD4⁺ or CD8⁺ T cells. In all experiments parallel incubations were performed with FITC- or PE-conjugated irrelevant antibodies matched for the isotypes of the anticytokine antibodies. The cutoff level for definition of positive cells was thus set so that less than 1% of irrelevant antibody-stained cells were positive.

Statistical analysis

All data are expressed as the median with the interquartile range. Comparisons between groups were made with the Mann-Whitney U test or the Kruskal-Wallis test. Correlations between variables were done by using the Spearman rank correlation coefficient. Stat View Version 5.0 (SAS Institute, Cary, NC) was used for the statistical calculations. A *P* value of less than .05 was considered significant, and all tests were 2 sided. It was determined that a total target sample size of 17 subjects per group would provide 80% power at a 2-sided α level of .05 to detect a difference of 1% or greater in the proportion of IL-4-producing CD4⁺ T cells (the primary end point), assuming an SD of 1%.

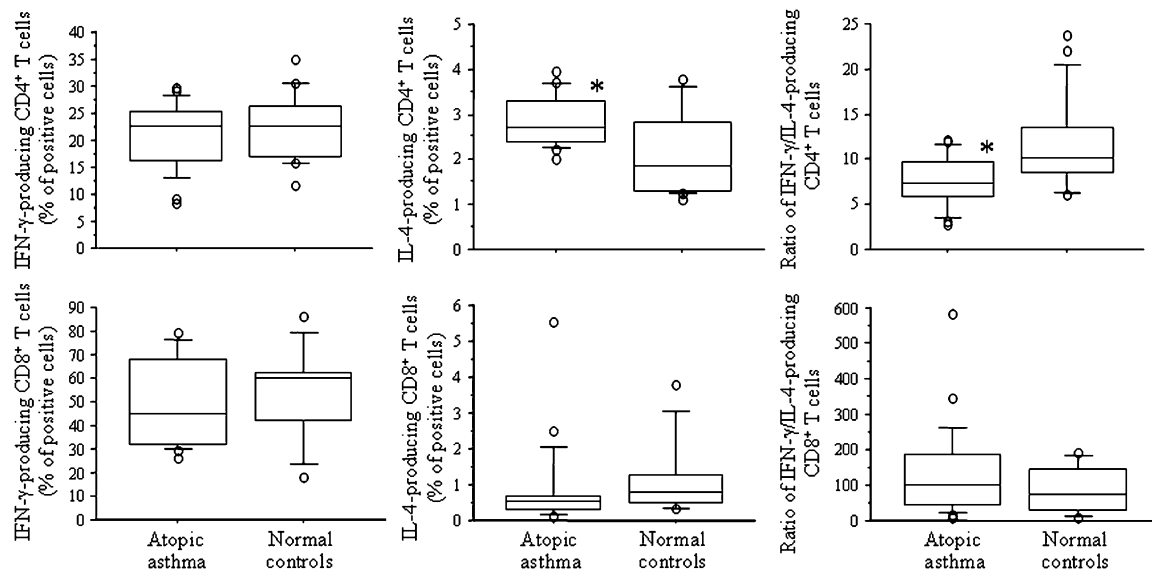


FIG 1. The percentage of IFN- γ - and IL-4-producing CD4⁺ and CD8⁺ T cells and the ratio of IFN- γ -producing/IL-4-producing CD4⁺ and CD8⁺ T cells obtained from peripheral blood after stimulation with PMA/ionomycin. Horizontal lines, boxes, whiskers, and circles represent median values, the interquartile range, 10th or 90th percentile, and data apart from 10th or 90th percentile, respectively. * $P < .05$.

RESULTS

Peripheral blood T-cell profiles: Comparison between atopic asthmatic and healthy subjects

Under unstimulated conditions, we could find no cytokine-expressing cells in the CD4⁺ or CD8⁺ T-cell population of peripheral blood cells in patients or healthy control subjects.

In response to PMA/ionomycin, no significant difference was found in the proportion of IFN- γ -producing CD4⁺ T cells between patients with atopic asthma and healthy control subjects (Fig 1, upper left panel). However, the percentage of IL-4-producing CD4⁺ T cells was significantly higher in the patients (Fig 1, upper middle panel). In addition, the ratio of IFN- γ -producing/IL-4-producing CD4⁺ T cells was significantly lower in the patients than in the control subjects (Fig 1, upper right panel).

There was no difference in the proportion of IFN- γ - or IL-4-producing CD8⁺ T cells or in the ratio of IFN- γ -producing/IL-4-producing CD8⁺ T cells between the 2 groups (Fig 1, lower panels).

Peripheral blood T-cell profiles: Comparison between childhood-onset and adult-onset asthmatic subjects

There was no difference in the proportion of IFN- γ - or IL-4-producing CD4⁺ T cells or in the ratio of IFN- γ -producing/IL-4-producing CD4⁺ T cells between the 2 groups.

However, the percentage of IFN- γ -producing CD8⁺ T cells was significantly lower in patients with childhood-onset asthma than in patients with adult-onset asthma and healthy control subjects (childhood-onset asthma:

median, 31.1% [interquartile range, 30.2% to 35.8%]; adult-onset asthma: median, 58.4% [interquartile range, 45.3% to 71.4%]; and healthy control subjects: median, 59.8% [interquartile range, 41.6% to 62.6%]; $P = .0116$). No significant difference was found in the proportion of IL-4-producing CD8⁺ T cells or in the ratio of IFN- γ -producing/IL-4-producing CD8⁺ T cells between the 2 groups.

Correlation between T-cell profiles and clinical parameters in asthmatic patients

As shown in Table II, the proportion of IL-4-producing CD4⁺ T cells was positively correlated with exhaled NO levels (Fig 2, left panel) and sputum eosinophil counts (Fig 2, right panel) but not with peripheral blood eosinophil counts, total serum IgE levels, FEV₁, PEF variability, or PC₂₀FEV₁. No correlation was found between the ratio of IFN- γ -producing/IL-4-producing CD4⁺ T cells and exhaled NO levels, sputum eosinophil counts, or other parameters.

The proportion of IFN- γ -producing CD4⁺ T cells and the ratio of IFN- γ -producing/IL-4-producing CD8⁺ T cells were positively correlated with peripheral blood eosinophil counts. No correlation was found between other T-cell profiles and parameters.

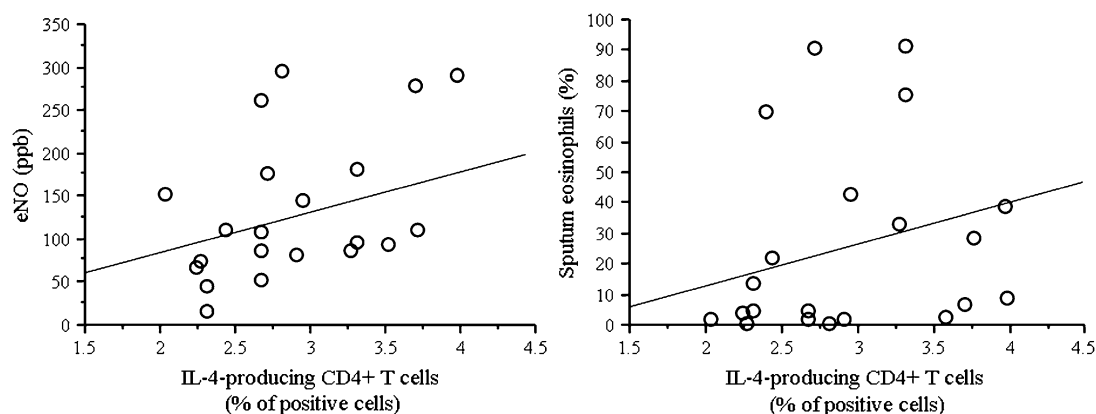
DISCUSSION

In this study we found a significantly higher percentage of IL-4-producing CD4⁺ T cells together with a significantly lower ratio of IFN- γ -producing/IL-4-producing CD4⁺ T cells in peripheral blood after PMA/ionomycin stimulation in patients with atopic asthma

TABLE II. Correlations between T-cell profiles and clinical parameters

	Exhaled NO	Sputum eosinophils	Blood eosinophils	Total serum IgE	FEV ₁	PEF variability	PC ₂₀ FEV ₁
IFN- γ -producing CD4 ⁺ T cells	0.24	0.01	0.52, <i>P</i> = .0246	0.16	-0.16	0.18	-0.13
IL-4-producing CD4 ⁺ T cells	0.48, <i>P</i> = .0337	0.50, <i>P</i> = .0448	0.16	0.22	-0.32	-0.18	-0.07
IFN- γ -producing/IL-4-producing CD4 ⁺ T cells	-0.07	-0.24	0.29	0.03	-0.03	0.22	-0.01
IFN- γ -producing CD8 ⁺ T cells	-0.13	-0.20	0.44	0.15	-0.04	-0.07	0.20
IL-4-producing CD8 ⁺ T cells	-0.16	-0.04	-0.44	-0.30	0.05	-0.32	0.18
IFN- γ -producing/IL-4-producing CD8 ⁺ T cells	0.09	0.02	0.52, <i>P</i> = .0237	0.30	-0.14	0.22	-0.16

Values are the correlation coefficient and statistical significance. If nothing but the number is shown, it is nonsignificant.

**FIG 2.** Correlations between the proportion of IL-4-producing CD4⁺ T cells and airway inflammatory indices. eNO, exhaled nitric oxide.

compared with healthy control subjects. However, there was no difference in the percentage of IFN- γ -producing CD4⁺ T cells between the 2 groups. Our study provides direct support for the T_H2 hypothesis that an increased production of T_H2 cell-derived cytokines, a decreased production of T_H1 cell-derived cytokines, or both are essential for development of airway inflammation.

Other investigators have also reported a lower ratio of IFN- γ -producing/IL-4-producing CD4⁺ T cells in whole blood culture at the single-cell level in patients with atopic asthma compared with those in healthy control subjects. Wong et al⁷ found a reduced proportion of IFN- γ -producing CD4⁺ T cells but no increase in IL-4-producing CD4⁺ T cells, whereas Kuo et al²¹ did not find any significant difference in the proportion of IFN- γ - or IL-4-producing CD4⁺ T cells between atopic asthmatic children and healthy control subjects. Taken together, it is important to assess the relative balance between IFN- γ - and IL-4-producing CD4⁺ T cells. In contrast, Cho et al^{9,10} reported an increased proportion of IFN- γ -producing CD4⁺ T cells in subjects with asthma compared with that in healthy subjects. Furthermore, Nagano et al⁸ found increased proportions of both IFN- γ - and IL-4-producing CD4⁺ T cells. Basically, there is no difference in methodology between theirs and ours. The diversity of the

population examined, whether an atopic or asthmatic phenotype existed, and statistical power could all be causes of the discrepancy. In fact, our patients had asthmatic symptoms, and none had received oral or inhaled corticosteroids or other medications for at least 6 months, whereas the patients studied by Cho et al²¹ were given bronchodilators or inhaled corticosteroid at 200 to 1000 μ g/d. It was reported that the proportion of IL-4-producing CD4⁺ T cells increases during acute exacerbation²¹ and decreases after treatment with inhaled corticosteroid at 400 to 800 μ g/d.⁸

Concerning the balance between IFN- γ - and IL-4-producing CD8⁺ T cells, we could not find any difference between the patients with atopic asthma and healthy control subjects. However, stratification of the asthmatic patients on the basis of onset of asthma revealed a decrease in the proportion of IFN- γ -producing CD8⁺ T cells in patients with childhood-onset asthma compared with patients with adult-onset asthma and healthy control subjects. Grob et al¹² also demonstrated a reduced expression of IFN- γ in CD8⁺ T cells in atopic asthmatic patients, although onset of disease was not described. In contrast, Cho et al^{9,10} reported an increased proportion of IFN- γ -producing CD8⁺ T cells in subjects with asthma compared with that seen in healthy subjects and that the production

of both type 1 and type 2 cytokines by peripheral blood CD8⁺ T cells was comparable with that by CD4⁺ T cells. Some investigators have also shown an increased proportion of IFN- γ -producing CD8⁺ T cells.^{13,15} Thus the meaning of T_H1 and T_H2 cells is still controversial. It is suggested that the CD8⁺ T-cell response might be more important in nonatopic asthma or occupational asthma associated with small-molecular-weight antigens, such as toluene diisocyanate, than in atopic asthma, especially asthma that developed during childhood.¹¹ Further studies will clarify the role of CD8⁺ T cells in different asthma phenotypes.

We also found a positive correlation between the proportion of IL-4-producing CD4⁺ T cells and parameters of airway inflammation, including exhaled NO levels and sputum eosinophil counts. It has been suggested that NO contributes to asthma pathogenesis by means of selective downregulation of T_H1 responses.²² That is, NO produced by epithelial cells and macrophages in asthma has an inhibitory effect on T_H1 cells, allowing the activation of T_H2 cells, which are normally suppressed by IFN- γ . This results in increased secretion of IL-4 and IL-5, which leads to IgE formation and eosinophil recruitment into the airway. Furthermore, it has been suggested that NO modulates the T_H1/T_H2 balance by favoring T_H2 responses, implying that increased release of NO promotes disease severity of asthma not only through tissue destruction but also through enhancement of T_H2 activity.^{23,24} Our findings are consistent with such a hypothesis.

The positive correlation between the proportion of IFN- γ -producing CD4⁺ T cells and peripheral blood eosinophil counts suggests that the T_H2 hypothesis might not apply to the peripheral blood eosinophil counts. Blood eosinophil counts are not a direct marker of airway inflammation and do not always correlate with changes in asthmatic status. We also found a positive correlation between the ratio of IFN- γ -producing/IL-4-producing CD8⁺ T cells and blood eosinophil counts in this study. The meaning of this finding is also unclear. Magnan et al¹³ showed a correlation between the percentage of IFN- γ -producing CD8⁺ T cells and blood eosinophilia in asthma, suggesting a regulatory role for IFN- γ -producing CD8⁺ T cells.

One limitation of this study is that the conclusions only apply to adults with recent-onset asthma and with intermittent or mild persistent asthma. Another limitation might be in the lack of information from a third group, atopic nonasthmatic subjects, because the pattern of T-cell cytokine production differed between atopy and asthma in a previous study.¹³

In conclusion, there is a correlation between peripheral blood T_H2 cytokine production and markers of airway inflammation, including exhaled NO and induced sputum eosinophils, in subjects with mild asthma.

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