

Predominant T_{H1} cytokine pattern in peripheral blood from subjects with chronic obstructive pulmonary disease

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Background: Previous studies have provided evidence for an inflammatory process in the large airways of subjects with chronic obstructive pulmonary disease (COPD), consisting predominantly of activated T cells. No data are available on the T_{H1}/T_{H2} T-cell cytokine pattern in this disease.

Objective: We sought to characterize the T_{H1}/T_{H2} T-cell cytokine pattern in subjects with COPD.

Methods: We examined the IFN- γ and IL-4 expression in peripheral blood CD4+ and CD8+ T cells from 20 patients with COPD and 25 control subjects by using a flow cytometric method of intracellular cytokine detection. We also examined the expression of 2 surface activation markers (CD25 and HLA-DR) on peripheral blood CD4+ and CD8+ T cells.

Results: There was an increased percentage of IFN- γ -producing cells (30.3% [range, 12.9% to 60.4%] vs 19.1% [range, 4% to 31.2%], $P = .003$) and a decreased percentage of IL-4-producing cells (4.55% [range, 0.6% to 11.3%] vs 9.5% [2.1% to 21.3%], $P = .0008$) among peripheral blood CD4+ T cells from the patients with COPD compared with control subjects. There was no significant difference between the 2 groups in the percentage of peripheral blood CD8+ T cells producing IFN- γ or IL-4 or in the percentage of peripheral blood CD4+ and CD8+ T cells expressing CD25 and HLA-DR.

Conclusion: These findings provide evidence for a T_{H1}-like immune response of peripheral blood CD4+ T cells in subjects with COPD. (J Allergy Clin Immunol 1999;103:458-62.)

Key words: Chronic obstructive pulmonary disease, peripheral blood, lymphocytes, cytokines

Chronic obstructive pulmonary disease (COPD) is defined as a disease state characterized by the presence of airflow obstruction caused by chronic bronchitis or emphysema. The airflow obstruction is generally progressive, may be accompanied by airway hyperreactivity, and may be partially reversible.¹ Like asthma, COPD is also associated with an infiltration of the airway mucosa by activated T cells.²

T-helper cells can be classified into different types that produce characteristic clusters of cytokines, some respon-

Abbreviations used

COPD: Chronic obstructive pulmonary disease
PE: Phycoerythrin
PerCP: Peridin chlorophyll protein
PMA: Phorbol myristate acetate

sible for cell-mediated inflammatory reactions, delayed-type hypersensitivity, and tissue injury in infectious and autoimmune diseases (T_{H1}), and others enhancing antibody production by B cells (T_{H2}).³⁻⁵ The cytokine most characteristic of T_{H1} responses is IFN- γ .^{6,7} Conversely, IL-4 production can be used to define T_{H2} response.⁸

Although much is known about the T-cell cytokine pattern in bronchial asthma,⁹⁻¹¹ there are no such studies in COPD. The aim of this study was to characterize the T_{H1}/T_{H2} T-cell cytokine pattern in subjects with COPD, and for this purpose we examined the IFN- γ and IL-4 expression in peripheral blood from 20 subjects with COPD and 25 control subjects by using a flow cytometric method of intracellular cytokine detection (FastImmune Cytokine System; Becton-Dickinson, Mountain View, Calif). This technique is based on the pioneering methodological report of Jung et al,¹² who first showed the feasibility of using intracytoplasmic staining and flow cytometry to assess T-cell cytokine production.

METHODS

Subjects

The study population was composed of 20 patients with COPD (19 men and 1 woman; mean age, 66.8 $\pm$ 2.1) and 25 control subjects (17 men and 8 women; mean age, 63.4 $\pm$ 1.6). Table I shows the characteristics of the study population. Subjects with COPD were characterized by cough and mucoid sputum production on most days of the month for at least 3 months per year during at least the previous 2 years.

The patients met the following criteria: (1) stable airflow limitation with FEV₁ ($\leq$ 80 % of predicted value); (2) no improvement in FEV₁ of over 15% after inhalation of 200 μ g of salbutamol; (3) no history of atopy or evidence of atopy on skin prick testing for 8 common inhalant allergens (*Dermatophagoides pteronyssinus*, *D farinae*, mixed grass pollen, cat allergen, dog dander, birch pollen, feathers, and *Aspergillus fumigatus*); (4) no history of other pulmonary disease; (5) no exacerbation of their disease; (6) no rise in titer in paired complement fixation tests for *Coxiella burnetii*, *Chlamydia psittaci*, *Mycoplasma pneumoniae*, common respiratory viruses (influenza A, B, C; adenovirus; respiratory syncytial virus), or *Legionella* fluorescent antibody assays; or (7) therapy with oral or inhaled glucocorticoids or antibiotics during the month before entering the study. Because an imbalance in the T_{H1}/T_{H2}

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cytokine secretion has been reported in other conditions, such as autoimmune diseases¹³⁻¹⁵ and osteoarthritis,¹⁶ we excluded them by clinical background and laboratory assessment.

The patients' smoking history was carefully checked, and a minimum of 15 pack-years smoked was required.

The control group was composed of 25 healthy subjects: 14 smoking subjects similar with regard to age, sex, and smoking history to COPD patients but without cough or sputum production and 11 nonsmoking subjects. They all had normal spirometric values without clinical or laboratory signs of sensitization to common allergens. Each subject gave signed, informed consent to participate in the study.

Study design

Before entry, patients and control subjects were screened with a clinical history, physical examination, chest radiograph, sputum analysis, blood analysis, and electrocardiogram. All subjects underwent spirometric function tests (Spiroflow; Morgan, Kent, UK), and peripheral venous blood samples were taken for lymphocyte analyses and complete blood cell counts. An aliquot of serum was frozen at -80°C for further analysis.

Antibodies and reagents

Anti-IFN- γ conjugated to phycoerythrin (PE); anti-IL-4 PE; anti-CD3 (T cells) peridin chlorophyll protein (PerCP); anti-CD4 (T helper-inducer) FITC and PE; anti-CD8 (T suppressor-cytotoxic) FITC and PE; anti-CD25 (p55 IL-2 receptor) PE; anti-HLA-DR (Class II MHC) PE; anti-CD23 (B cells expressing the low affinity Fc IgE receptor) PE; isotype-matched, directly conjugated (FITC, PE, and PerCP) control antibodies; FACS Lysing Solution; and FACS Permeabilizing Solution were obtained from Becton Dickinson. Phorbol myristate acetate (PMA), ionomycin, brefeldin A, paraformaldehyde, L-glutamine, RPMI 1640, and AB serum were obtained from Sigma Chemical Co (St Louis, Mo).

Processing of cells and staining procedure

For surface marker analysis of peripheral blood lymphocytes, 100 μ L of heparinized whole blood was incubated with 10 μ L of one of the FITC-labeled antibodies along with 10 μ L of one of the PE-labeled antibodies at room temperature for 20 minutes. Erythrocytes were lysed by adding 2 mL of lysing solution for 10 minutes. Cells were washed twice with PBS containing 2% FCS and 0.1% sodium azide.

For intracellular cytokine detection, 500 μ L of heparinized whole blood was added to RPMI 1640 with 2 mmol/L L-glutamine. Cells were stimulated with PMA (25 ng/mL) and ionomycin (1 μ g/mL) in the presence of brefeldin-A (10 μ g/mL) and kept in tubes (Falcon, Becton Dickinson) for 4 hours at 37°C in a humidified atmosphere at 5% CO₂ in air. As previously described,¹⁷ brefeldin-A was used to block intracellular transport mechanisms, thereby leading to an accumulation of cytokines in the cells.

Ten microliters of anti-CD3 PerCP and 10 μ L of anti-CD8 FITC were added to 100 μ L of stimulated whole blood and incubated for 15 minutes at room temperature. Erythrocytes were lysed by adding 2 mL lysing solution for 10 minutes. Cells were washed in PBS containing 0.5% BSA and 0.1% sodium azide and subsequently resuspended in 500 μ L FACS Permeabilizing Solution. After 10 minutes, the cells were washed with PBS and incubated with saturating concentrations of anti-IFN- γ or anti-IL-4 PE for 30 minutes at room temperature. After a final wash in PBS, cells were resuspended in 500 μ L paraformaldehyde 1% and kept at 4°C for no

TABLE I. Characteristics of the study population

Subject no	Sex (M/F)	Age (y)	Smoking history (packs/y)	FEV ₁ (% predicted)	Treatment
1	M	54	19	44	None
2	M	74	49	36	B
3	M	69	66	39	T
4	M	69	32.5	40	B,T
5	M	72	20	40	None
6	M	68	126	27	B
7	M	68	15	58	None
8	F	71	20.2	42	B
9	M	68	48	19	None
10	M	69	15	53	B,T
11	M	70	30	71	None
12	M	66	90.5	29	T
13	M	67	44.7	38	T
14	M	50	60	23	B
15	M	64	65.5	43	None
16	M	77	41	40	B,T
17	M	74	100	41	B
18	M	71	51.5	37	None
19	M	76	16	55	B
20	M	40	60	80	None
Mean	—	66.8	48.5	42.4*	—
SEM	—	2.1	7	3.4	—
Control subjects					
Smokers					
(10 M/4 F)					
Mean	—	63	45.2	96	—
SEM	—	1.4	6.3	0.4	—
Nonsmokers					
(7 M/4 F)					
Mean	—	63.9	—	98.6	—
SEM	—	2	—	0.3	—

T, Sustained-release theophylline; B, β -agonist.

*Significantly different from control subjects.

more than 24 hours in the dark until analyzed. Negative controls consisted of unstimulated cells.

Flow cytometric analysis

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) was used.

For surface marker analysis of peripheral blood lymphocytes, 10,000 cells were computed in list mode and analyzed by using appropriate software (Lysis, Becton Dickinson). Simultaneous T-cell subset analysis (CD4+ and CD8+ T cells) and activation study (expression of activation markers CD25 and HLA-DR) were performed on peripheral blood lymphocytes.

For intracellular cytokine detection, 10,000 cells were computed and analyzed with the same software. An electronic gate was set on the lymphocytes on the forward and side-scatter plot, and CD3+ (PerCP+) cells falling within the gated area were then identified and selected in a second gate. As previously described,¹⁷ cytokine production was analyzed by detection of FITC and PE staining, and the number of cells staining for each cytokine was expressed as a percentage of CD4+ and CD8+ T cells. CD4+ T cells were identified

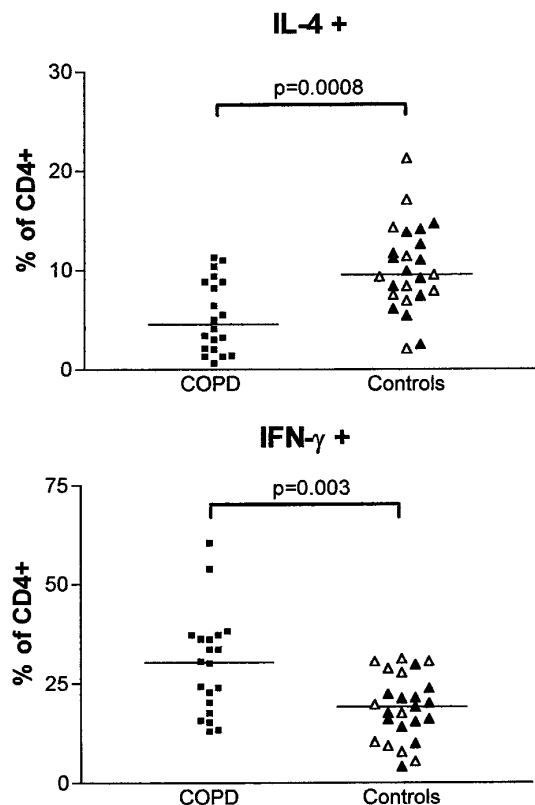


FIG 1. Percentages of peripheral blood CD4+ T cells with positive intracellular staining for IL-4 (4.55% [range, 0.6% to 11.3%] vs 9.5% [range, 2.1% to 21.3%], $P = .0008$) (top) and IFN- γ (30.3% [range, 12.9% to 60.4%] vs 19.1% [range, 4% to 31.2%], $P = .003$) (bottom) from patients with COPD and control subjects. Horizontal bars represent group median values. Filled symbols indicate smoking subjects.

as CD3+ CD8- cells because of the downregulation of CD4 after phorbol ester stimulation.¹⁸ Specific binding of mAb was controlled by subtraction of isotype-matched mouse immunoglobulins.

Statistical analysis

Results were expressed as medians and ranges, and differences between groups were analyzed with the Mann-Whitney U test.

Correlation coefficients were calculated by using Spearman's rank method. Probability (P) values less than .05 were accepted as significant.

RESULTS

The characteristics of the study population are reported in Table I. Subjects with COPD had significantly lower FEV₁ values when compared with control subjects ($P = .001$) (data not shown). In patients with COPD, we observed a significant negative correlation between smoking history and the percentages of predicted values obtained for FEV₁ ($r = -0.6$; $P = .004$) (data not shown).

Flow cytometric analysis of peripheral blood lymphocytes showed no significant differences in either the percentage or absolute numbers of CD3+, CD19+, and

CD23+ cells between COPD and control subjects (Table II). There were also no differences in either the percentage or absolute numbers of CD3+ cells expressing CD4 or CD8 or the expression of CD25 and HLA-DR in either CD4+ or CD8+ T cells in the 2 groups of subjects examined (Table II).

No cytokine levels were detectable without stimulation, and appropriate isotype-matched control antibodies showed no positive staining. There was an increased percentage of IFN- γ -producing cells (30.3% [range, 12.9% to 60.4%] vs 19.1% [range, 4% to 31.2%], $P = .003$) and a decreased percentage of IL-4-producing cells (4.55% [range, 0.6% to 11.3%] vs 9.5% [range, 2.1% to 21.3%], $P = .0008$) among peripheral blood CD4+ T cells from the subjects with COPD compared with control subjects (Fig 1). In contrast, there was no significant difference between the 2 groups in the percentage of peripheral blood CD8+ T cells producing IFN- γ or IL-4 (Table II).

When control subjects were divided according to smoking habits, no significant differences between smoking and nonsmoking subjects were found for any of the parameters examined. No correlation was found between functional and flow cytometric data in patients with COPD.

DISCUSSION

In this study we showed a T_{H1}-like immune response of peripheral blood CD4+ T cells in patients with COPD whose capacity for IFN- γ expression was significantly increased, whereas that for IL-4 was significantly reduced compared with control subjects.

We can speculate that an increased percentage of T_{H1} cells in peripheral blood from patients with COPD may reflect either a "spill-over" of T_{H1} cells from the bronchial mucosa or an influence of the bronchial microenvironment on circulating T cells. Although analysis of peripheral blood and BAL lymphocytes from atopic asthmatic subjects after allergen bronchial challenge has demonstrated dynamic exchanges of T lymphocytes between these 2 compartments,¹⁹ there are no such studies in subjects with COPD. Thus there is a need to perform a parallel T-cell cytokine pattern analysis in bronchial mucosa and peripheral blood from patients with COPD to support these hypotheses.

On the basis of the description of 2 separate profiles of cytokine production from the 2 subsets of T_{H1} and T_{H2} cells,³⁻⁵ the evidence of increased levels of IL-4 and IL-5 in samples of asthmatic patients have suggested that there might be a T_{H2} cytokine pattern in these patients.⁹ To the best of our knowledge, no studies assessing the T_{H1}/T_{H2} T-cell cytokine pattern in subjects with COPD have been reported so far.

The physiologic significance of T-cell derived IFN- γ in the pathogenesis of COPD is unclear. IFN- γ is known principally for its ability to activate macrophages.²⁰ Macrophages have been shown to be increased in the bronchial mucosa of subjects with COPD,² and theoretically there should be an interaction of this cell type with the T cells. T cells may regulate macrophage function in COPD by means of production of IFN- γ , resulting in

TABLE II. Percentages of lymphocyte subpopulations in peripheral blood from patients with COPD and control subjects

	CD3	CD19	CD23	CD4	CD8	CD4/ CD25	CD4/ HLA-DR	CD8/ CD25	CD8/ HLA-DR	CD8/ IL-4	CD8/ IFN- γ
Patients with COPD											
Median	69.8	9.2	5.3	42.7	35.9	2.5	2.4	0.04	2.3	0.5	13
Range	59-84	2-36.1	1.6-23.8	19.5-55.7	22-61	1.2-3.6	0.9-5.8	0.01-0.2	0.5-14.8	0-1.4	3.5-35.2
Control subjects											
Median	73.3	11	7.1	41.8	36	2.9	1.5	0.03	2.1	0.5	6.4
Range	59-82.6	5.1-14.5	3-12.4	26.8-56.8	20-56.1	1.4-4.1	1-3.9	0-0.4	0.4-9.1	0.1-2.7	0.1-22.3
P value	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

increased secretion of IL-12 and production of macrophage inhibitory factor.²⁰ This would increase macrophage residency and also encourage maintenance of a T_{H1}-like immune response. Activated macrophages may also produce IL-8,²¹ which is a potent chemotactic factor for neutrophils and neutrophil recruitment to the lungs, neutrophil activation, and release of proteolytic enzymes within the tissues. These factors are thought to be central to perpetuation of lung inflammation and progression of chronic obstructive pulmonary disease.²² In turn, IFN- γ may also induce endothelial²³ and epithelial²⁴ cell adhesion molecule expression, supporting the recruitment and activation of inflammatory cells. Expression of the inducible form of nitric oxide (NO) synthase is increased by IFN- γ ,²⁵ and NO can interact with superoxide leading to peroxynitrite generation and nitrotyrosine formation,²⁶ which may result in epithelial damage. Finally, IFN- γ has been shown to impair epithelial regeneration.²⁷

The decrease of IL-4 in our patients with COPD could be regarded as consistent with a relative predominance of IFN- γ because IFN- γ suppresses T_{H2}-cell activity.²⁸

An imbalance in the number of T_{H2} cells secreting IL-4 against T_{H1} cells secreting IFN- γ has been implicated in autoimmune diseases,^{13,14} suggesting that preferential activation of T_{H1} responses is central in the pathogenesis of these disorders. In view of our data, it is tempting to speculate that COPD may be an autoimmune disease.

Because respiratory infection may well be another important factor that drives the T cells toward a T_{H1} phenotype, we took great care to select patients free of respiratory infections in the month before the study. However, without more sophisticated techniques, such as PCR, a latent viral²⁹ or *Chlamydia pneumoniae* infection³⁰ cannot be excluded. Thus we cannot completely rule out the possibility that the T_{H1} cytokine pattern reflects an underlying respiratory infection instead of being a specific marker of COPD. Further studies, including studies of individuals with exacerbations of COPD, are necessary to evaluate the results of this study.

In a previous study, Mueller et al³¹ failed to find a significant difference in the IFN- γ and IL-4 expression in bronchial mucosa between patients with COPD and control subjects. The discrepancy with our data may be explained by a number of factors. First, these authors examined the localization of cytokine protein without identifying the cytokine-producing cells. Second, differences in patient

selection may contribute to the conflicting results. Finally, in some diseases the T_H pattern of nonspecific stimulation of peripheral blood lymphocytes may not reflect the T_H pattern in the target tissue as it occurs in rheumatoid arthritis.¹⁵

Although other authors showed an upregulation of T-cell activation² in bronchial mucosa from patients with COPD, we are unable to show any significant difference in the expression of CD25 and HLA-DR in either peripheral blood CD4+ or CD8+ T cells in our patients with COPD compared with control subjects. These discrepancies may be due to the fact that the authors examined T-cell activation on CD3+ cells in toto, whereas we evaluated the expression of T-cell activation markers CD25 and HLA-DR on CD4+ and CD8+ T cell subsets separately. Moreover, differences of severity and/or chronicity of the disease can make it difficult to draw a direct comparison among the various studies.

Several studies reported a relative prevalence of CD8+ over CD4+ T cells in bronchial biopsy specimens from smokers with chronic bronchitis and chronic airflow limitation.³²⁻³⁴ Increased proportion of CD8+ T cells in bronchial mucosa may be the consequence of a migration process of a homogeneous T-cell subset from the blood to this site of involvement and/or, alternatively, of the proliferation in the lung of a selected cell subpopulation in response to a persistent stimulus that may act independently of clinical exacerbation. When a redistribution of cells with limited phenotype from the blood to the lung is considered, a decrease in T cells with this phenotype would be predicted in the blood of the same subject. Our data did not demonstrate any depletion of CD8+ T cells in the blood of patients with COPD, arguing against a T-cell redistribution from the blood to the lung. However, a parallel T-cell subset analysis in bronchial mucosa and peripheral blood from subjects with COPD should be performed to clarify this point.

All patients with COPD were smokers, and it has been demonstrated that tobacco smoking induces an increased nonspecific IgE response.³⁵ IgE synthesis is controlled by IL-4 and IFN- γ .³⁶ However, in a previous study Le Cam et al³⁷ demonstrated that nicotine, a major pharmacologically active component of tobacco smoke, does not modulate IL-4 and IFN- γ release from PBMCs activated with PMA. Moreover, we have not found differences in the T-cell cytokine pattern in peripheral blood between smoking and nonsmoking control subjects.

Because a variety of cells and mediators can contribute

to the inflammatory processes in COPD, the lack of correlation between flow cytometric and physiologic data was not unexpected. Moreover, airway obstruction in COPD has a multifactorial pathogenesis and is linked not only to inflammatory events, such as tissue edema and cell infiltration, but also to irreversible end-stage lesions, such as connective tissue deposition and distortion of the membranous bronchioles by loss of elastic recoil.³⁸

In conclusion, this study provides evidence for a T_{H1}-like immune response of peripheral blood CD4⁺ T cells in subjects with COPD. Further studies designed to examine the expression of cytokines other than IL-4 and IFN- γ (ie, IL-2 and IL-5) are required to confirm a definite bias toward a T_{H1} phenotype in this disease.

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