

Cytokine production by bronchoalveolar lavage T lymphocytes in chronic obstructive pulmonary disease

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Background: T lymphocytes (predominantly CD8⁺ cells) have previously been implicated in the pathogenesis of chronic obstructive pulmonary disease (COPD).

Objective: We sought to describe the profile of cytokine production by CD8⁺ and CD4⁺ cells isolated from bronchoalveolar lavage fluid.

Methods: Bronchoalveolar lavage was performed in 11 patients with COPD (median FEV₁, 63.3% of predicted value) and 9 healthy control subjects. CD8⁺ and CD4⁺ T cells were isolated by means of positive selection after macrophage depletion. CD8⁺ and CD4⁺ cells were activated with anti-CD3/CD28 antibodies for 60 hours before restimulation with phorbol 12-myristate 13-acetate–ionomycin and brefeldin. Three-color flow cytometry was used to simultaneously measure levels of intracellular cytokines.

Results: IL-4 was expressed by a higher percentage of stimulated CD8⁺ T cells (T_C2) compared with CD4⁺ T cells (T_H2) in patients with COPD ($P = .01$). In contrast, IFN- γ was expressed in a significantly higher percentage of stimulated CD4⁺ T cells (T_H1) than CD8⁺ T cells (T_C1) in the COPD group ($P = .04$). TNF- α was expressed by almost all T_C1 and T_H1 cells, with virtually no expression by T_C2 and T_H2 cells. In addition, a small number of T cells expressing TNF- α alone without concomitant IFN- γ or IL-4 expression were seen in the majority of subjects. There was a higher percentage of T_C2 cells in subjects with COPD compared with that seen in the control group ($P = .03$). Stimulation with anti-CD3/CD28 antibodies increased the percentage of T_C2 cells and decreased the percentage of T_H2 cells.

Conclusion: Our results suggest that there are increased numbers of T_C2-like cytokine-expressing cells in the lungs of patients with COPD.

Clinical implications: These cells might be a source of T_H2 cytokines, which might, at least in part, explain the lung eosinophilia associated with COPD exacerbations. (J Allergy Clin Immunol 2006;117:1484-92.)

Key words: Cytokines, chronic obstructive pulmonary disease, T cells, bronchoalveolar lavage

Chronic obstructive pulmonary disease (COPD) is characterized by irreversible airflow limitation and chronic inflammation of the airways and lung parenchyma. Neutrophils, alveolar macrophages, and T lymphocytes (predominantly CD8⁺ cells) have all been implicated in the pathogenesis of COPD.¹ For many years, the main focus of interest has been neutrophils and macrophages, but more recent pathologic and bronchoalveolar lavage (BAL) studies have suggested an important role of CD8⁺ T cells in the development of COPD.²⁻⁵

CD4⁺ T lymphocytes are subclassified into T_H1 and T_H2 cells. T_H1 cells produce IFN- γ , IL-2, and TNF in the absence of IL-4, IL-5, IL-9, and IL-13. In contrast, T_H2 cells produce mainly IL-4, IL-5, IL-9, and IL-13 and no IFN- γ or TNF. A predominant T_H1/T_H2 polarization is seen in murine models, but the distinction between T_H1 and T_H2 cells is less distinct in human immune responses.^{6,7}

Cytotoxic CD8⁺ cells have recently been shown to also be efficient cytokine producers. CD8⁺ T cells can also be divided into T_C1 cells, which produce IFN- γ , and T_C2 cells, which produce IL-4 and IL-5.⁸ The major factor that determines the differentiation of precursor T cells is the T-cell cytokine microenvironment. IL-12, IL-18, and IL-23 promote the development of T_H1 and T_C1 cells from naive CD4⁺ and CD8⁺ T cells, respectively, whereas IL-4 is essential to induce differentiation toward T_H2 and T_C2 cells.^{7,9} BAL offers many advantages for the analysis of cells involved in COPD because the cells come from distal smaller airways, which is the site of inflammation and obstruction in COPD.¹⁰ However, analysis of BAL T cells is problematic because of the moderate number (usually 1% to 10%) of T cells present.^{11,12} Here we describe a method to isolate CD8⁺ and CD4⁺ T cells from the BAL fluid of patients with COPD, which can be combined with flow cytometry, ELISA, RT-PCR, or other methods to study the profile of cytokine production by these cells.

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Abbreviations used

APC: Allophycocyanin
BAL: Bronchoalveolar lavage
COPD: Chronic obstructive pulmonary disease
FACS: Fluorescence-activated cell sorting
FITC: Fluorescein isothiocyanate
PE: Phycoerythrin
PMA: Phorbol 12-myristate 13-acetate

The relatively low number of T cells isolated from BAL fluid allowed us to examine only 3 cytokines in this study. IFN- γ and IL-4 were selected as being markers of T_H1 (T_H1) and T_H2 (T_H2) subsets.^{7,13} In addition, it has been proposed that repeated viral infections cause CD8⁺ cells to release TNF- α , leading to damage of the lung in susceptible smokers.¹⁴ However, no studies have investigated this in cells from the site of inflammation in COPD, and there is no information as to whether TNF- α is produced only by T_H1/T_H1 or also by T_H2/T_H2 cells. Our results show that there is an increased population of CD8⁺ cells differentiated into the T_H2 profile.

METHODS

Subjects

The study protocol was approved by the ethics committees of the Silesian Medical Academy and The Royal Brompton and Harefield Hospital. All subjects provided written informed consent before entering the study.

Eleven patients with moderate stable COPD (7 male and 4 female patients) were recruited for the BAL study, and 4 patients were recruited for the blood study. The inclusion criteria were as follows: FEV₁/forced vital capacity ratio of less than 70%, FEV₁ of less than 85% of predicted value, smoking history of at least 10 pack-years, and no use of inhaled or systemic steroids in the last month. Patients with any history of asthma or patients with an exacerbation of the disease in the previous 4 weeks were excluded. In the BAL study 6 patients were treated with short- and long-acting β_2 -agonists, whereas 5 patients received no treatment at all.

The control group consisted of 9 healthy volunteer subjects for the BAL study and 4 smokers for the blood study without any respiratory symptoms. The additional exclusion criteria included abnormal spirometry, history of atopy, and respiratory infection for 4 weeks before the study. None of 9 healthy subjects received treatment. The characteristics of the studied patients are shown in Table I (BAL study) and Table EI (available in the Online Repository at www.jacionline.org).

Bronchoscopy and BAL

BAL fluid was collected by means of sequential instillation and aspiration of up to 4 separate aliquots of 50 mL of warmed 0.9% normal saline, as previously described.¹⁵

Isolation and culture of CD8⁺ and CD4⁺ T cells from BAL fluid

BAL cells were spun and washed twice with HBSS. A differential cell count was performed on cytospin preparations of BAL cells with May-Grunwald-Giemsa stain. Cells were resuspended in culture medium (RPMI-1640, 10% [vol/vol] FCS, 2 mM l-glutamine, 100 IU/mL penicillin, and 100 μ g/mL streptomycin). Macrophages were

TABLE I. Characteristics of BAL study subjects

Group	Patients with COPD	Control subjects
N	11	9
Age (y)	70 (51-84)*	43 (22-79)
Sex (M/F)	7/4	6/3
Cigarette smoking (yes/exsmoker/no)	5/6/0	3/3/3
Pack-years	40 (20-100)†	10 (0-50)
FEV ₁ (% predicted)	63.3 (42.0-82.6)‡	104.0 (89.0-130.6)
FEV ₁ /FVC (%)	62.1 (51.1-67.9)‡	80.2 (78.1-88.8)

Data are expressed as medians and ranges.

M, Male; F, female; FVC, forced vital capacity.

* $P < .05$, † $P < .01$, and ‡ $P < .001$ compared with the control group.

depleted from cells by means of adhesion to plastic plates in an incubator (37°C, 5% CO₂, humidified air), and the process was repeated. Resulting nonadherent cells consisted mainly of lymphocytes, but variable amounts of dead cells, granulocytes, and epithelial cells were also present. Initially, dead cells were eliminated with the Dead Cell Removal Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Negative cells were collected as the live cell fraction. Subsequently, CD8⁺ T cells were isolated by means of positive selection with MACS CD8 MicroBeads (Miltenyi Biotec), according to the manufacturer's instructions. Positive cells were counted and used as CD8⁺ T cells for further experiments. The negative fraction from the first separation was used for the subsequent isolation of CD4⁺ T cells by using MACS CD4⁺ MicroBeads. The purity of isolated cells was assessed by means of flow cytometry. Both the CD8⁺ and CD4⁺ cells were consistently more than 95% pure, as assessed by means of double staining, respectively, with fluorescein isothiocyanate (FITC)-labeled anti-CD8 and phycoerythrin (PE)-labeled anti-CD3 and FITC-labeled anti-CD4 and PE-labeled anti-CD3 (all antibodies from PharMingen, Oxford, United Kingdom). Purified CD8⁺ and CD4⁺ T cells were cultured separately at 1 to 2 $\times 10^5$ /mL and stimulated for 60 hours with anti-CD3 (1 μ g/mL) and anti-CD28 (1 μ g/mL) antibodies in culture medium. In the case of high T-cell yields ($>4 \times 10^5$), part of the CD8⁺ and CD4⁺ T cell-purified fraction was also cultured without stimulation.

Isolation and culture of CD8⁺ and CD4⁺ T cells from blood

See text in the Online Repository at www.jacionline.org.

Intracellular cytokine staining

CD8⁺ and CD4⁺ cells were washed and restimulated with phorbol 12-myristate 13-acetate (PMA; 10 ng/mL) and ionomycin (400 ng/mL) in the presence of brefeldin A (5 μ g/mL) for 6 hours. Cells were then washed twice in wash buffer (PBS/0.5% BSA/0.1% NaN₃), fixed in cold 4% formaldehyde, washed in PBS, and frozen in PBS/1% BSA/10% dimethyl sulfoxide at -70°C for later use. Subsequently, cells were thawed in a water bath at 37°C, washed in wash buffer, permeabilized in PBS/0.5% BSA/0.1% saponin, washed, and stained with FITC-labeled anti-IFN- γ , PE-labeled anti-IL-4, and allophycocyanin (APC)-labeled anti-TNF- α mouse anti-human antibodies (all from PharMingen) or respective mouse isotype control antibodies (FITC-IgG1, PE-IgG1 and APC-IgG1) for 30 minutes at room temperature in the dark. After being washed twice, the cells were resuspended in 1% formaldehyde in PBS and analyzed within 24 hours by means of flow cytometry (FACScalibur; Becton Dickinson, Franklin Lakes, NJ). CD8⁺ and CD4⁺ T cells stained with isotype control antibodies were used as negative control to set quadrant markers.

TABLE II. Characteristics of BAL cells from the study population

Group	Patients with COPD	Control subjects
Recovery (mL)	50 (30-100)*	110 (85-140)
Total cell numbers ($\times 10^6$)	10.5 (2.4-50.0)	17.0 (8.5-47.4)
Total cell number per volume of BAL recovered ($\times 10^4$ /mL)	19.2 (4.0-104.2)	17.0 (7.7-35.7)
Macrophages (%)	93.2 (66.4-99.1)	92.5 (80.0-97.7)
Neutrophils (%)	0.7 (0-8.7)	0 (0-10.0)
Eosinophils (%)	0 (0-1.4)	0 (0-0.2)
Lymphocytes (%)	6.5 (0.2-23.6)	8.5 (1.5-22.0)

Data are expressed as medians and ranges.

* $P < .001$ for differences between groups, Mann-Whitney U test.**ELISA for IL-4 and TNF- α** See text in the Online Repository at www.jacionline.org.**Quantitative real-time RT-PCR for IL-4**See text in the Online Repository at www.jacionline.org.**Statistical analysis**

Values are expressed as medians with maximal and minimal values. Differences between the studied groups were analyzed by using a Mann-Whitney U test. Differences between CD4⁺ and CD8⁺ cells (paired data) within the studied group or groups were analyzed with the Wilcoxon paired rank test. P values of less than .05 were accepted as significant.

RESULTS**Patient and BAL cell characterization**

The total and differential BAL cell counts obtained from the 2 subject groups are shown in Table II. Recovery of BAL fluid was significantly lower in patients with COPD compared with that seen in control subjects, although the total number of cells recovered was no different between the 2 groups. The number of CD8⁺ (median, 0.8×10^5 ; range, 0.3 – 21.5×10^5) and CD4⁺ (median, 1.28×10^5 ; range, 0.15 – 10×10^5) T cells isolated from all subjects was similar. The number of CD8⁺ ($P = .01$), but not CD4⁺ ($P = .052$), cells was significantly higher in healthy control subjects. Interestingly, the number of CD4⁺CD8⁺ T cells was also significantly higher in the control group ($P = .043$). Treatment with β -agonists had no effect on any parameters measured. Both CD8⁺ T cells and CD4⁺ T cells produced cytokines after stimulation with anti-CD3/CD28 antibodies.

Percentage of anti-CD3/CD28 antibody-stimulated CD4⁺ and CD8⁺ T cells from BAL fluid expressing IFN- γ , IL-4, and TNF- α of all subjects

Representative examples of flow cytometric analysis of intracellular cytokines in CD8⁺ and CD4⁺ T cells of 2 patients with COPD and 2 healthy subjects are shown in Figs E1 and E2 (available in the Online Repository at

www.jacionline.org). IL-4 was expressed by a higher percentage of stimulated BAL CD8⁺ T cells (median, 21.6%; range, 4.4% to 66.9%) compared with CD4⁺ T cells (median, 7.2%; range, 1.9% to 53.8%), and this difference (T_{C2} rather than T_{H2} cells) was significant across all subjects studied ($P = .003$) and also within the COPD group (median of 34.8% and range of 5.7% to 66.9% vs median of 21.1% and range of 2.8% to 53.8%, respectively; $P = .01$) but not within the control group when examined independently ($P = .09$, Fig 1). In contrast, IFN- γ was expressed in a significantly higher percentage of stimulated BAL CD4⁺ T cells (T_{H1}; median, 23.0%; range, 1.3% to 76.8%) than in CD8⁺ T cells (T_{C1}; median, 10.5%; range, 0.2% to 43.9%) across all subjects studied ($P = .02$) and within the COPD group (median of 8.5% and range of 1.3% to 76.8% vs median of 2.8% and range of 0.2% to 43.9%, respectively; $P = .04$) but not within the control group when examined independently ($P = .06$, Fig 1). Similarly, TNF- α was expressed by more CD4⁺ BAL T cells (median, 38.4%; range, 0.5% to 90.0%) than CD8⁺ T cells (median, 21.3%; range, 0.6% to 53.2%; $P = .048$). However, this difference was discernible only when all subjects were considered together and the statistical significance was very close to the limit (Fig 1).

Percentage of anti-CD3/CD28 antibody-stimulated CD4⁺ and CD8⁺ T cells from BAL fluid expressing IL-4, IL-4/TNF- α , TNF- α , IFN- γ /TNF- α , or IFN- γ of all patients

As expected, stimulated BAL fluid CD8⁺ and CD4⁺ T cells did not coexpress IL-4 and IFN- γ . In all subjects studied, both CD8⁺ and CD4⁺ T cells were clearly divided into 3 populations: those expressing IFN- γ alone (T_{C1} [T_{H1}]); those expressing IL-4 alone (T_{C2} [T_{H2}]); and those not polarized (expressing neither IFN- γ nor IL-4; Fig 2). Furthermore, there was virtually no expression of TNF- α by T_{C2} and T_{H2} cells. Moreover, almost all T_{C1} and T_{H1} cells (IFN- γ positive) also expressed TNF- α . Additionally, a majority of studied subjects also had some T cells that expressed TNF- α alone (without IFN- γ , Fig 2).

IL-4 alone and TNF- α alone were expressed by a significantly higher percentage of CD4⁺ T cells compared with IL-4/TNF- α -expressing cells across all subjects studied and within the COPD subgroup (Fig 2, A). In addition, CD8⁺ T cells expressing IL-4 alone and TNF- α alone were present to a significantly greater extent compared with IL-4/TNF- α -expressing cells across all subjects studied and within the COPD subgroup (Fig 2, A).

Similarly, CD4⁺ T cells expressing only TNF- α and TNF- α /IFN- γ -expressing CD4⁺ T cells were present to a significantly higher degree compared with CD4⁺ T cells expressing IFN- γ only across all subjects studied and within the COPD subgroup (Fig 2, B). In addition, there was a higher percentage of CD8⁺ T cells expressing TNF- α alone and TNF- α /IFN- γ compared with IFN- γ alone in all subjects and within the COPD subgroup (Fig 2, B).

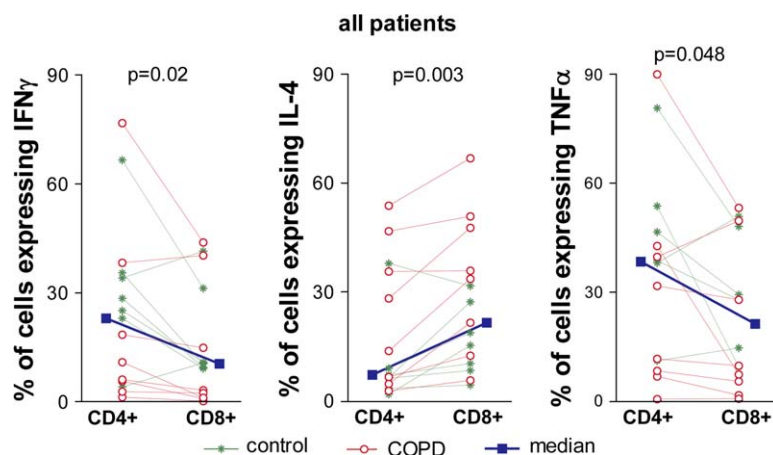


FIG 1. Percentage of anti-CD3/CD28 antibody-stimulated and PMA/ionomycin/brefeldin A-restimulated CD4⁺ and CD8⁺ T cells from BAL fluid expressing IFN- γ , IL-4, and TNF- α in all subjects. Median values are shown as a square connected with a line.

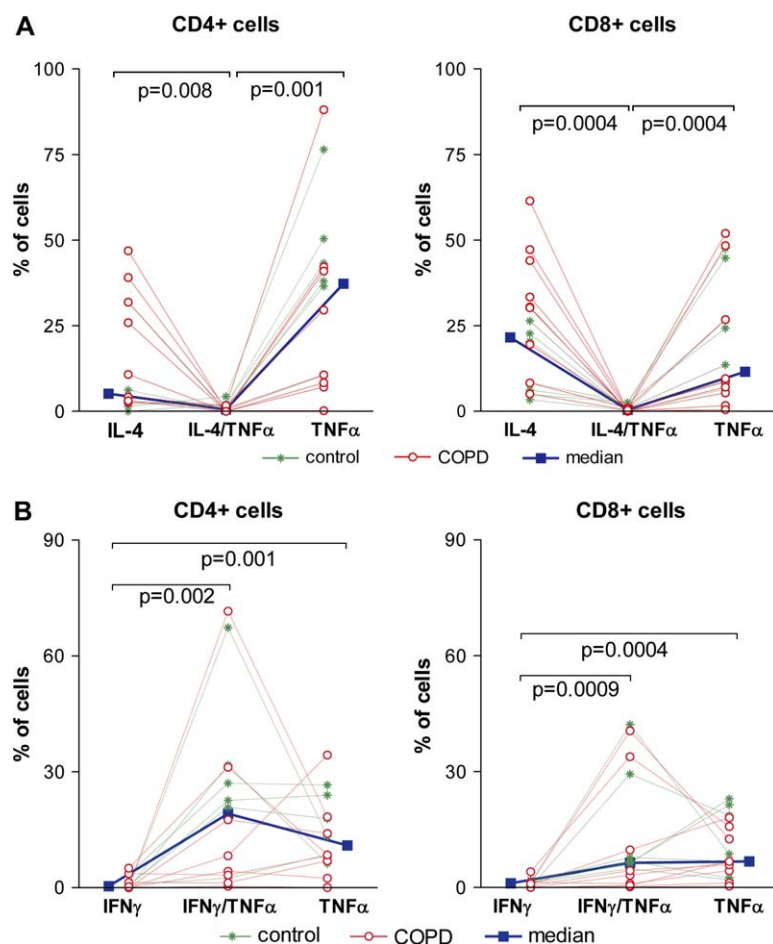


FIG 2. A. Percentage of anti-CD3/CD28 antibody-stimulated and PMA/ionomycin/brefeldin A-restimulated CD4⁺ and CD8⁺ T cells from BAL fluid expressing IL-4 alone, TNF- α alone, or both IL-4 and TNF- α in all patients. **B.** Percentage of anti-CD3/CD28 antibody-stimulated and PMA/ionomycin/brefeldin A-restimulated CD4⁺ and CD8⁺ T cells from BAL fluid of all patients expressing IFN- γ alone, TNF- α alone, or both IFN- γ and TNF- α .

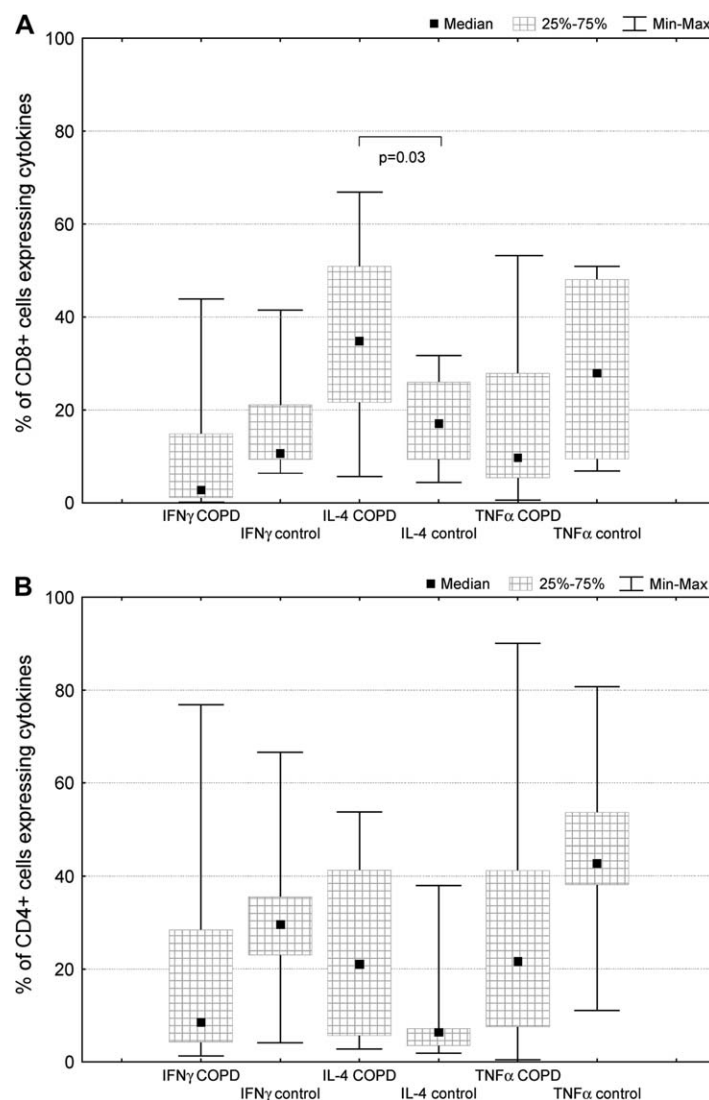


FIG 3. Percentage of anti-CD3/CD28 antibody-stimulated and PMA/ionomycin/brefeldin A-restimulated CD8⁺ T cells (**A**) and CD4⁺ T cells (**B**) from BAL fluid expressing IFN- γ , IL-4, and TNF- α in patients with COPD and control subjects. Results are expressed as the median and range. The *hatched area* represents the 25% to 75% interquartile range.

Percentage of anti-CD3/CD28 antibody-stimulated CD8⁺ and CD4⁺ T cells from BAL fluid expressing IFN- γ , IL-4, and TNF- α in patients with COPD and control subjects

The percentage of stimulated BAL CD8⁺ T cells expressing IL-4 (median, 34.8%; range, 5.7% to 66.9%) was significantly higher in patients with COPD compared with that seen in healthy subjects (median, 17.1%; range, 4.4% to 31.7%; Fig 3, A; $P = .03$). In contrast, there was no significant difference between the 2 subject groups in the percentage of CD8⁺ cells expressing either IFN- γ (median of 2.8% and range of 0.2% to 43.9% vs median of 10.6% and range of 6.4% to 41.5%, $P = .09$) or TNF- α (median of 9.7% and range of 0.6% to 53.2% vs

median of 27.9% and range of 6.9% to 50.9%; Fig 3, A). In addition, there were large variations in the percentage of CD4⁺ cells expressing IL-4, IFN- γ , and TNF- α , and as a result, there were no differences between the 2 groups of subjects studied in the percentage of CD4⁺ BAL T cells expressing IFN- γ , IL-4, or TNF- α (Fig 3, B).

The effect of anti-CD3/anti-CD28 antibody stimulation on the expression of IFN- γ , IL-4, and TNF- α by BAL CD8⁺ and CD4⁺ T cells

In a majority of patients, we did not obtain sufficient CD8⁺ and CD4⁺ T cells to culture both with stimulation and without stimulation. However, results from 3 subjects

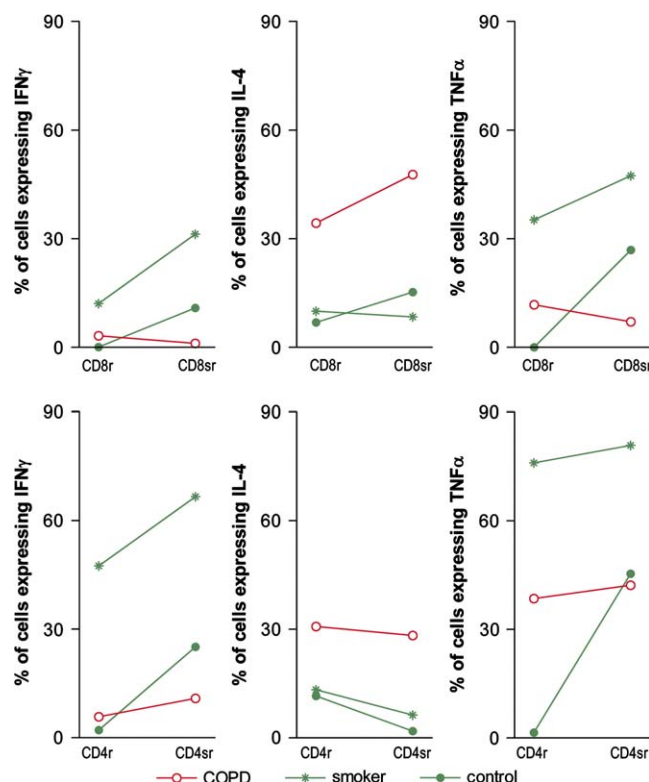


FIG 4. The effect of anti-CD3/anti-CD28 antibody stimulation on the expression of IFN- γ , IL-4, and TNF- α by CD8 $^{+}$ (upper panels) and CD4 $^{+}$ T cells (lower panels). Results are presented as changes for individual patients. r, Restimulated cells; sr, stimulated-restimulated cells.

whose BAL fluid produced high enough yields of CD8 $^{+}$ and CD4 $^{+}$ T cells (Fig E1 [available in the Online Repository at www.jacionline.org] and Fig 4) indicates that stimulation of both CD8 $^{+}$ and CD4 $^{+}$ cells results in enhanced numbers of cells expressing IFN- γ and TNF- α . In contrast, the numbers of IL-4-expressing cells increases after stimulation of CD8 $^{+}$ cells and decreases in CD4 $^{+}$ cells. Because of a lack of cell numbers, we were unable to detect IL-4 mRNA or cytokine expression in culture supernatants from these cells either before or after restimulation with PMA/ionomycin.

Expression of cytokines in CD8 $^{+}$ and CD4 $^{+}$ T cells isolated from blood

Thus the results in blood cells shows that at rest, by using fluorescence-activated cell sorting (FACS) analysis, there are few CD4 $^{+}$ cells expressing IL-4 (T_H2-like, 1.2% median value) compared with BAL cells (7.2% median value) and that the numbers of IL-4-expressing cells does not alter greatly after restimulation (Fig E3, available in the Online Repository at www.jacionline.org). This is paralleled by the failure to detect IL-4 in the supernatant of the cultured cells either before or after stimulation (Fig E4, available in the Online Repository at www.jacionline.org), the lower level of IL-4 mRNA transcripts found in these cells (Fig E5, available in the Online Repository at www.jacionline.org), and the failure to

induce IL-4 mRNA expression after restimulation. The data from the 3 methods used to examine IL-4 production show a good correlation. In contrast, the numbers of CD8 $^{+}$ cells expressing IL-4 (T_C2-like cells) were higher at baseline (3.5% median value) but still less than that seen in BAL cells (21.6% median value) and showed an induction with PMA/ionomycin restimulation (Fig E3). This mimics the data seen in BAL cells (Fig 1). In addition, the results seen with FACS analysis in blood was supported by the data examining IL-4 release (Fig E4) and IL-4 mRNA expression (Fig E5). Furthermore, the differing ability of blood CD4 $^{+}$ and CD8 $^{+}$ cells to induce IL-4 production (Figs E3-E5) was similar to that seen with BAL cells (Fig 4).

In contrast, the numbers of T_H1 cells detected in blood (3.5% median value) was much lower than that seen in BAL cells (21.6% median value), whereas the numbers of T_C1 cells detected in blood (18.2% median value) were greater than those seen in BAL cells (10.5% median value). Restimulation of the cells produced a small increase in the numbers of IFN- γ -expressing cells in both BAL fluid (Fig 4) and blood (Fig E3), although the results were variable between subjects.

The numbers of TNF- α -expressing CD4 $^{+}$ and CD8 $^{+}$ cells from blood were similar, although there was a spread of values, and restimulation did not enhance TNF- α -expressing cell numbers (Fig E3) or release into the

cytoplasm (Fig E4). This is in contrast to results seen in BAL cells (Figs 1 and 4), which shows reduced numbers of TNF- α -expressing CD8⁺ cells at baseline and an ability of restimulation to enhance TNF- α expression in both CD4⁺ and CD8⁺ cells.

DISCUSSION

In this study we demonstrate the profile of cytokine production by CD8⁺ and CD4⁺ T cells isolated from the BAL fluid of patients with COPD for the first time. IL-4 was expressed by a higher percentage of stimulated CD8⁺ T cells compared with CD4⁺ T cells in patients with COPD. In contrast, IFN- γ was expressed in a significantly higher percentage of stimulated CD4⁺ T cells (T_H1) than CD8⁺ T cells (T_C1) in the COPD group. TNF- α was expressed by almost all IFN- γ ⁺ cells (T_C1 and T_H1 cells), with virtually no expression by T_C2 and T_H2 cells. In addition, a small number of T cells expressing TNF- α alone were seen in most subjects. Furthermore, there was a higher percentage of T_C2 cells in subjects with COPD compared with in the control group. Finally, stimulation with anti-CD3/CD28 antibodies increased the percentage of T_C2 cells and at the same time decreased the percentage of T_H2 cells. Our results suggest that in the lungs of patients with COPD, there is an increased population of CD8⁺ cells differentiated into the T_C2 profile.

After polarization of peripheral blood cells, effector T cells usually show a stable phenotype of cytokine production and very rarely, if ever, revert to the naive state or switch to the opposite pattern.⁷ We stimulated CD8⁺ and CD4⁺ T cells with anti-CD3/CD28 antibodies and restimulated the cells with PMA/ionomycin because this combination provided the best activation of CD8⁺ and CD4⁺ T cells isolated from blood without altering the pattern of synthesized cytokines (unpublished observations). The profile of cytokine production from peripheral blood T cells and lung sections in patients with COPD has been reported previously.^{16,17} Majori and colleagues¹⁶ showed that there were no differences between the percentage of T_C1 or T_C2 cells between the COPD and control groups. However, a significantly increased percentage of IFN- γ ⁺ and a reduced percentage of IL-4⁺ CD4⁺ T cells were demonstrated in the COPD group. In an elegant, recently published study by Grumelli and associates,¹⁷ lymphocytes isolated from lung sections obtained from patients with emphysema undergoing lung resection surgery for lung cancer were studied. In contrast to our results, they showed a bias toward the T_H1/T_C1 phenotype, with almost no presence of T_H2/T_C2 cells. The disparity between these results and those presented here might reflect the severity of disease or the heterogeneous nature of COPD (emphysema vs nonemphysema). In our study, although emphysematous patients were not formally excluded, the very poor recovery of BAL fluid¹⁸ would exclude these patients from our study.

The differences seen between our results in BAL fluid and those reported in peripheral blood might reflect the lung-specific nature of mild-to-moderate COPD or possibly reflect differences in disease severity because Majori and colleagues¹⁶ examined a group of patients with a much lower mean FEV₁. We therefore examined T-cell subsets in peripheral blood to determine the lung-specific nature of our results. Although there were clear differences in T-cell profiles seen between blood and BAL fluid in respect to there being more T_H2 and T_C2 cells in BAL fluid after stimulation-restimulation than in blood, the general pattern was similar, with more T_C2 than T_H2 cells being present. The percentage of T_C1 cells appears to be similar in the lung and in blood; however, there were more T_H1 cells in BAL fluid than in blood. In addition, we were able to show that changes in IL-4 release were similar whether measured on the basis of ELISA, FACS, or mRNA.

Although our study is the first to report the percentage of T_C1/T_H1 and T_C2/T_H2 cells in the BAL fluid of patients with COPD, there are 2 previous reports examining T-cell cytokine profiles in BAL fluid from healthy subjects.^{19,20} In contrast to these earlier reports, we show reduced numbers of T_C1 cells and higher numbers of T_C2 cells in healthy subjects. This probably reflects the fact that we stimulated purified CD4⁺ and CD8⁺ T cells, whereas the other studies stimulated total BAL cells. Therefore an effect of other BAL cell types on the activation of T cells cannot be excluded.

In addition to their traditional cytotoxic role, CD8⁺ T cells can also influence CD4⁺ T cells and other components of immune response through secretion of cytokines and chemokines or by means of direct cell-to-cell contact. CD8⁺ T cells can change the T_H1/T_H2 balance *in vivo*, activate macrophages, or play a role in the recruitment of eosinophils into the lung during viral infection.²¹⁻²³ $\alpha\beta$ T_C2 cells are proinflammatory and participate in allergic airway reactions.²⁴

Although several previous studies have reported a predominance of T_H1 cytokines in patients with COPD, there are also a number of studies showing that T cells polarized to produce T_H2 cytokines are present in the airways of patients with COPD, especially in subjects who fulfill the criteria for diagnosis of chronic bronchitis.²⁵⁻²⁷ BAL studies in some subjects with chronic bronchitis have demonstrated variable numbers of eosinophils and often increased levels of eosinophil cationic protein.^{28,29} Additionally, tissue eosinophilia in bronchial biopsy specimens during exacerbation of nonasthmatic patients with chronic bronchitis has been reported.^{30,31} Finally, increased expression of IL-4 and IL-5 has been shown around the hypertrophied bronchial mucus-secreting glands of smokers with chronic bronchitis.³²

Our results suggest that there is an increased population of antigen-primed CD8⁺ T cells polarized toward a T_C2 phenotype in the lungs of patients with COPD. The interactions between T_H1/T_C1 and T_H2/T_C2 responses are complex. For example, IL-4 release from CD8⁺ T cells is important in the early phase of the T_H1 response,³³

T_{C2} T cells inhibited IgE and promoted T_{H1} cell development and generation of T_{H2} CD4⁺ T cells,³⁴ and IFN-γ is necessary for optimal T_{H2} differentiation.³⁵ Our *in vitro* data show that CD3/CD28 stimulation increases both T_{C2} and T_{H2} cells, possibly through stimulation of effector memory CD8⁺ T cells, and a similar effect might occur *in vivo* in the lung. Two distinct populations of CD8⁺ T cells are present in the lung during viral infections,³⁶ namely T_{C1} cells and memory CD8⁺ T cells primed to produce IL-4, which exhibit the T_{C2} phenotype on restimulation.³⁷ These cells might trigger the eosinophilic airway inflammation seen during viral infections of the respiratory tract.^{36,38}

Our study suggests that in the lungs of patients with COPD, there is an increased population of CD8⁺ cells differentiated into a T_{C2} profile. These cells might be a source of large amounts of IL-4 and perhaps IL-5 during infectious exacerbation of COPD, and we speculate that this could partly explain the lung eosinophilia seen in COPD exacerbations. We further speculate that T_{C2} cells and eosinophils together could help promote tissue damage and the development of emphysema during repeated infectious exacerbations of COPD.

Although our results show that BAL T cells are efficient producers of TNF-α, the fact that no differences in the number of cells expressing TNF-α were observed between patients with COPD and control subjects suggests that the enhanced TNF-α expression seen in patients with COPD is produced by other cells, such as macrophages or epithelial cells. Our study does suggest, however, that TNF-α is associated with T_{C1} cells because we showed that stimulated lung CD8⁺ cells, which express IL-4, do not coexpress TNF-α. Similar results were obtained for CD4⁺ cells, which further supports a close link between TNF-α and T_{C1} (T_{H1}) responses. Moreover, cells expressing IFN-γ without TNF-α were extremely rare.

The COPD group was older than the control subjects. However, the age of studied subjects did not correlate with the percentage of any cytokine produced by CD4⁺ or CD8⁺ cells. Moreover, increased age was associated with increased T-cell production of TNF-α, but not IL-4, in patients with COPD.³⁹ Therefore it seems very unlikely that the increased population of T_{C2} cells in patients with COPD found in our study is due to age. In addition, 3 of 9 healthy subjects enrolled in the study had never smoked. One of these subjects had the highest and the 2 others had close to average percentage of T_{H2}/T_{C2} cells, and therefore the effect of smoking status *per se* was not further studied.

In summary, we have shown an increased percentage of T_{C2} cells in patients with COPD after *in vitro* stimulation. In addition, TNF-α was expressed by almost all T_{C1} and T_{H1} cells, whereas virtually no TNF-α was expressed by T_{C2} and T_{H2} cells, and a majority of subjects also had some T cells that expressed TNF-α alone (without concomitant expression of IFN-γ or IL-4). Our results might suggest that in the lungs of patients with COPD, there is an increased population of CD8⁺ cells differentiated into the T_{C2} profile.

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