

Predictive value of response to treatment of T-lymphocyte subpopulations in idiopathic pulmonary fibrosis

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ABSTRACT: T-cell types are important in maintaining immune homeostasis in the lung and their imbalance may be associated with several diseases. We examined the relationship between bronchoalveolar lavage (BAL) T-cell subset profiles and the clinical course of 46 patients with idiopathic pulmonary fibrosis (IPF).

A flow cytometry cell sorter (FACS) was used to analyse the T-cell subsets. Pulmonary function tests (PFT) were performed at baseline and 6–12 months later. Patients were divided into two groups according to their CD4/CD8 ratio: CD4/CD8 >1 (group 1, n=21); and CD4/CD8 <1 (group 2, n=25).

A lower percentage of lymphocytes, a higher percentage of CD8/S6F1 cells (cytotoxic T-lymphocytes) and a higher percentage of neutrophils were found in the BAL in group 2 compared to group 1 ($11\pm 7.5\%$ versus $19\pm 13.2\%$; $p=0.024$ and $29.8\pm 17.6\%$ versus $13.3\pm 6.9\%$; $p=0.068$, respectively for lymphocytes and cytotoxic T-lymphocytes; and $8\pm 11\%$ versus $29\pm 27\%$; $p=0.003$ for neutrophils). Inversely, in the peripheral blood, the distribution of CD8/S6F1 cells was lower in group 1 than in group 2 ($8.3\pm 6.9\%$ versus $33.4\pm 16.5\%$; $p=0.0048$). The patients were followed over a period of 1 yr in order to test whether those findings could determine efficacy of therapy. The baseline transfer factor of the lung for carbon monoxide ($TLCO$) capacity in group 1 and group 2 was $59\pm 22\%$ and $51\pm 21\%$, respectively ($p=0.29$), but only in group 1 was the $TLCO$ capacity improved significantly in response to steroids treatment after 6–12 months.

IPF patients with a higher percentage of lymphocytes, a lower percentage of neutrophils, CD4/CD8 >1 and a low percentage of CD8/S6F1 may have a more benign course of disease. These parameters may identify an early stage of reversible disease responsive to therapy. We conclude that these measurements may be a useful tool in monitoring response to treatment in patients with idiopathic pulmonary fibrosis.

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Idiopathic pulmonary fibrosis (IPF) is a chronic progressive disorder, characterized both by inflammation and fibrosis of the lung parenchyma [1]. The management of patients with IPF is difficult due to their variable clinical evolution and the inability to accurately assess and monitor the severity of the disease [2]. The role of differential cellular analysis and T-lymphocyte subsets in bronchoalveolar lavage (BAL) for diagnostic and prognostic purposes in interstitial lung diseases remains controversial. The mere presence of BAL T-lymphocytosis is nonspecific. In addition to sarcoidosis and hypersensitive pneumonitis, other diseases such as berylliosis, radiation pneumonitis, tuberculosis, opportunistic lung infection and IPF have also been associated with an increase in T-lymphocytes [3]. Moreover, ratios have been reported to be normal [4] or decreased [5, 6] in IPF lymphocyte subsets.

In order to better understand the dynamics of these inconsistencies, we performed a multicentre study to evaluate the BAL profile in patients with IPF. We separated the patients with a normal CD4/CD8 from those with dec-

reased CD4/CD8 ratios and compared these two groups according to the results of clinical, laboratory and pulmonary function tests. In an effort to develop new markers to link the lymphocyte profile to the outcome of disease, we employed CD8/S6F1 monoclonal antibody (specific for cytotoxic effector T-cell) and tested the percentage of positive cells in BAL and peripheral blood. Patients were followed-up over a period of 1 yr in order to test whether findings could determine efficacy of therapy.

Materials and methods

Patient population

Forty six patients (mean age 64.8 ± 12.8 yrs; 23 females and 23 males) with IPF were included in this study. All of these patients were identified in part by our current diagnostic and monitoring assessment for interstitial lung diseases. Others were referred from four other centres as part

of a national multicentre programme, supported by the Chief Scientist Office of the Israeli Ministry of Health, for prospectively studying patients with interstitial lung diseases who have undergone transbronchial biopsy (TBB) and BAL. A questionnaire was used to collect demographic and medical information. Data from all patients are reported in table 1

The diagnosis of IPF was based on accepted criteria [7] which included either evidence of diffuse parenchymal infiltrates (peripheral and reticular nodular with lower lobe predominance) on chest radiography or restrictive lung function with a lung biopsy demonstrating varying degrees of interstitial fibrosis and intra-alveolar inflammatory cells. Strict exclusion criteria were established and consisted of a clinically relevant environmental or exposure history or an occupational exposure index, as well as clinical findings of hypersensitivity pneumonitis, left ventricular failure, systemic diseases and granuloma or vasculitis in the biopsy specimen. Patients with evidence of bacterial, mycobacterial and fungal infections were also excluded.

Patients were administered oral prednisone at a dose of 40 mg·day⁻¹ for at least 2 months followed by a tapering off by 5 mg·day⁻¹ every 2 weeks until a maintenance dose of 10–20 mg·day⁻¹ was reached. Clinical exacerbations were treated with oral prednisone at a dose of 40–60 mg·day⁻¹ for at least 2 weeks which was then tapered off. Patients with clinical or functional deterioration to steroids received in addition, cyclophosphamide at a dose of 100–150 mg·day⁻¹ as a maintenance dose.

Characterization of smoking history

Participants were classified as "never smokers" (zero pack-years of cigarette smoking), "former smokers" (>20

Table 1. – Demographic and clinical parameters of all patients

Sex n (%)		
Female	23	(50)
Male	23	(50)
Age yrs mean±SD (range)	64.8±12.8	(32–84)
Duration of symptoms months n (%)		
<3	5	(11)
3–6	13	(28)
6–12	1	(3)
>12	27	(58)
Smoking n (%)		
Current	7	(15)
Former	6	(13)
Never	33	(72)
Dyspnoea at rest n (%)	37	(80)
Clubbing	11	(24)
Cyanosis	3	(6)
Crepitations	31	(67)
Treatment n (%)		
None	5	(11)
Intermittent steroids	16	(33)
Continuous steroids	20	(44)
Cytotoxic drugs	5	(12)
Final diagnosis made by n (%)		
BAL and TBB	43	(93)
Open lung biopsy	3	(7)

BAL: bronchoalveolar lavage; TBB: transbronchial biopsy.

lifetime-packs of cigarettes but cessation at least 3 months prior to evaluation) and "current smokers" (active smoking at the time of evaluation).

Pulmonary function tests

The pulmonary function tests consisted of standard spirometry, lung volumes *via* body plethysmograph and single-breath transfer factor of the lung for carbon monoxide ($T_{L,CO}$) (MasterLab E. Jaeger, Würzburg, Germany). The measurements of lung function were performed using standard protocols, and the American Thoracic Society guidelines [8] were used to determine acceptability. The predicted normal values used were those of forced vital capacity (FVC), forced expiratory volume in one second (FEV₁) for spirometry, total lung capacity (TLC) for lung volumes and $T_{L,CO}$ for diffusing capacity >80%.

Radiographic studies

Chest radiographs were performed in the postanterior projection. All patients underwent computer-assisted tomography (CT) less than 4 weeks prior to BAL. All scans were performed on an Elscint 2002 scanner (Elscint, Haifa, Israel) and independently interpreted by two readers.

BAL

BAL was performed using a flexible fiberoptic video bronchoscope (Pentax, Hamburg, Germany). Subjects were premedicated with pethidine 50 mg and atropine 0.5 mg, and the airways anaesthetized by inhalation of 4% xylocaine. Fifty millilitre boluses of saline (0.9%), previously warmed to 37°C, up to a total volume of 150–200 mL were instilled with the bronchoscope wedged into a subsegmental bronchus of the right or left lower lobe. The cells were recovered by gentle aspiration. The percentage of BAL fluid was 58.2±9.0%. The average total cells recovered was higher among the smokers compared to the nonsmokers (38.2±8.2 *versus* 17.0±7.2×10⁶ cells·mL⁻¹; *p*=0.02)

Preparation of bronchoalveolar cells

The recovered fluid was collected in specimen traps, filtered through sterile gauze and centrifuged at 400 × *g* for 15 min at 4°C. The pellet was washed three times with cold phosphate-buffered saline (PBS), (Biological Industries, Beit Haemek, Israel) and cells adjusted to a final concentration of 10⁶ cells·mL⁻¹ in RPMI 1640 medium supplemented with 2% foetal calf serum (Biological Industries). A volume of 100 µL of this suspension was loaded into the cuvettes of a cyto centrifuge (Cytospin; Shandon, Southern Products Ltd, Runcorn, UK). The slides were spun at 1,000 revolutions per minute (rpm) for 5 min before the fluid blotters were removed. After the slides were dried in air, a differential count was performed on Giemsa-stained preparations (Merck, Darmstadt, Germany) by counting a minimum of 500 cells.

Evaluation of the phenotype of bronchoalveolar cells

Flow cytometric analysis was performed on a dual-laser flow cytometry cell sorter (FACS) 440 equipped with an Ar⁺ and Kr laser (Becton-Dickinson, San Jose, CA, USA). Data were collected and analysed using the Consort 20 VAX and Disp4 and Disp2D programs (Becton-Dickinson). Cells were collected on a logarithmic scale. The selection of lymphocyte population was based on side scatter and expression of CD45. A total of 1 mL of BAL (10^6 cells·mL⁻¹) was examined.

Lymphocyte subsets were identified by monoclonal antibodies as follows: CD3 = total T-cells; CD4 = T helper cells; CD8 = T suppressor-cytotoxic cells; CD8/S6F1 = cytotoxic T-cells; CD56 = natural killer (NK) cells; CD20 = B cells. Monoclonal antibodies were directly conjugated to either phycoerythrin (RD1) or fluorescein isothiocyanate (FITC). Cells were incubated for 10 min with Epics Coulter Q-Prep (Coulter Immunology, Hialeah, FL, USA) and read either immediately or after 24 h.

Follow-up

Patients underwent regular clinical examinations and pulmonary function 6 months and 1 yr after enrolment in the study.

Statistics

Results are expressed as mean percentage±SD. The comparison between the two groups was done by the two-sample t-test for continuous variables and the Chi-squared test for discrete variables. Log transformation was performed in order to compare the percentage of lymphocytes in the two groups. Analysis of covariance using the first measurement as a covariate was performed to compare the pulmonary function test findings between the groups during the year of follow-up.

Results

Using the lower value of the normal range of the CD4/CD8 ratio as a yardstick, we divided the patients into two groups: group 1 = CD4/CD8 >1 and group 2 = CD4/CD8 <1. The demographic and clinical findings for all patients are shown in table 1. When both groups were compared in correlation to those findings, it emerged that no differences were found between them for sex, smoking habits,

Table 2. – Analysis of the differential cell profile in peripheral blood in group 1 (n=21) and group 2 (n=25)

Cells	Group 1 %	Group 2 %	p-value*
Peripheral WBC ⁺	7.6±1.8	10.6±2.9	0.002
Peripheral Eosin	3.8±2.6	2.6±4.0	0.360
Peripheral Lymph	27.0±6.8	22.0±10.0	0.090
Peripheral Neut	60.0±8.0	63.0±17.0	0.531

Values are presented as mean±SD. Group 1: CD4/CD8 >1; Group 2: CD4/CD8 <1. WBC: white blood cell; Eosin: eosinophils; Lymph: lymphocytes; Neut: neutrophils. +: ×10³ cells·μL⁻¹. *: p<0.05 is considered significant.

dyspnoea, cough, fever, symptoms, duration of symptoms, cyanosis and interstitial pattern (as demonstrated on radiographs and CT). However, group 1 patients showed fewer crepitations and clubbing than those in group 2 (p<0.005). A total of 19 of 21 patients in group 1 were treated as were 22 of 25 in group 2.

Analysis of the results of peripheral blood counts revealed that patients in group 1 had a lower white blood cell (WBC) count ($7.6±1.8 × 10^3$ cells·μL⁻¹) than patients in group 2 ($10.6±3.9 × 10^3$ cells·μL⁻¹), p=0.002. There were no differences among the other types of cells (table 2).

BAL cells recovered from patients in group 1 showed a lower percentage of neutrophils (p=0.003) and eosinophils (p=0.057) than those from patients in group 2. Log transformation was performed for lymphocyte values as they were not normally distributed. An overlapping pattern was noted in the two groups: 19±13.2% lymphocytes in group 1 and 11±7.5% lymphocytes in group 2 (p=0.024). The profile of T-cell subtypes showed that 25 patients in group 1 (55%) had a CD4/CD8 ratio of 2.11±1.2 and that 21 patients in group 2 (45%) had a CD4/CD8 ratio of 0.45±0.2 (p=0.01). No significant differences were found

Table 3. – Analysis of the differential cell profile in bronchoalveolar lavage in group 1 (n=21) and group 2 (n=25)

Cells	Group 1 (n=21) %	Group 2 (n=25) %	p-value*
Alveolar Macro	62±21	47±27	0.059
Alveolar Lymph	19±13.2	11±7.5	0.024
Alveolar Neut	8±1	29±27	0.003
Alveolar Eos	3±2	6±7	0.057
Alveolar Lymph CD4/CD8	2.1±1.2	0.45±0.2	0.010
Alveolar Meta cells	0.68±0.9	0.66±0.6	0.923
Alveolar Lymph CD56	2.9±3.4	3.3±4.4	0.737
Alveolar Lymph CD20	2.2±2.7	3.1±6.1	0.592

Values are presented as mean±SD. Group 1: CD4/CD8 >1; Group 2: CD4/CD8 <1. Macro: macrophages; Neut: neutrophils; Eos: eosinophils; Lymph: lymphocytes; Meta: metachromatic. *: p<0.05 is considered significant.

Table 4. – CD8/S6F1 profile in peripheral blood and in bronchoalveolar lavage (BAL) cells in group 1 (n=13) and group 2 (n=13)

	Peripheral blood %	BAL %	p-value*
Group 1	33.4±16.5	13.5±6.9	0.005
Group 2	8.3±6.9	29.8±17.6	0.001

Values are presented as mean±SD. Group 1: CD4/CD8 >1; Group 2: CD4/CD8 <1. *: p<0.05 is considered significant.

Table 5. – Pulmonary function test findings in group 1 (n=21) and group 2 (n=25) at enrolment

	Group 1	Group 2	p-value
FVC % pred	77.6±19.2	71.8±18.7	0.328
FEV ₁ % pred	83.7±21.4	73.7±22.4	0.147
TLC % pred	73.7±17.3	77.7±19.0	0.480
TL _{CO} % pred	59.5±22.5	51.6±21.7	0.290

Values are presented as mean±SD. Group 1: CD4/CD8 >1; Group 2: CD4/CD8 <1. FVC: forced vital capacity; % pred: percentage of predicted value; FEV₁: forced expiratory volume in one second; TLC: total lung capacity; TL_{CO}: transfer factor of the lung for carbon monoxide.

for macrophages, metachromatic cells, NK cells (CD56) or B-cell lymphocytes (CD20) between the two groups (table 3).

Since the low ratio of CD4/CD8 which characterized group 2 was due to an increase in the cytotoxic/suppressor subset ($48 \pm 19\%$ versus $29 \pm 9\%$ for group 1 $p < 0.0001$), we considered the possibility of these cells being cytotoxic and evaluated the CD8/S6F1 cluster of differentiation. We succeeded in showing that the cytotoxic subset was lower in group 1 ($13.5 \pm 6.9\%$) compared to group 2 ($29.8 \pm 17.6\%$), $p = 0.007$. The distribution of the same subset in the peripheral blood was a mirror image, verifying a high percentage in group 1 and low percentage in group 2 ($33.4 \pm 16.5\%$ and $8.3 \pm 6.9\%$, respectively, $p = 0.005$) (table 4).

Spirometric data disclosed impairment in either FEV₁ and/or FVC in 30 patients (65%), while 10 patients (22%) had a normal ventilatory pattern. Five study patients (10.8%) had a mild decrease of TLCO ($70\text{--}80$ mmol·min⁻¹ kPa range) while in 31 (67%) there was a severe impairment of TLCO ($25\text{--}70$ mmol·min⁻¹ kPa range). Two of the three patients (4.3%) whose TLCO was within normal ranges had reduced lung volumes (TLC of 69% and 73%). There was a severe restrictive pattern (range of 50–70 % pred) in lung volumes in 19 patients (41.3%), and only a moderate reduction (range 70–80 % pred) in 12 (26%). Four patients (4.8%) did not undergo pulmonary function tests before BAL.

These results, which include pulmonary function tests of the 46 patients on the date of enrolment, are shown in table 5. No significant differences were found in any of the parameters. A total of 42 patients (91%) finished the 1 yr follow-up. Four patients died: one in group 1 (unrelated myocardial infraction) and three in group 2 (one during

Table 6. – Pulmonary function test findings in group 1 (n=21) and group 2 (n=25) after 12 months

	Group 1	Group 2	p-value	95% CI
FVC % pred	80.2 ± 17.2	73.4 ± 11.4	0.241	-4.17–17.78
FEV ₁ % pred	85.1 ± 15.7	75.6 ± 15.1	0.191	-2.09–21.09
TLC % pred	79.1 ± 14.2	73.4 ± 30.9	0.950	-12.39–23.79
TLCO % pred	77.4 ± 24.0	54.0 ± 20.0	0.009	6.78–40.02

Values are presented as mean \pm SD. Group 1: CD4/CD8 >1; Group 2: CD4/CD8 <1. 95% CI: 95% confidence interval. For further definition see legend to table 5.

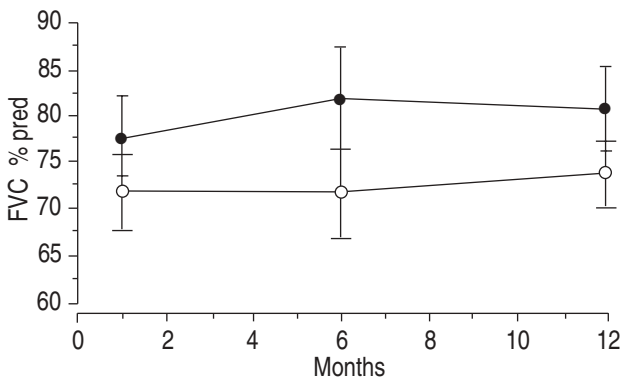


Fig. 1. – Monitoring of forced vital capacity (FVC) measurements in group 1 (CD4/CD8 >1 (●—)) and group 2 (CD4/CD8 <1 (○—)) during the 1 yr follow-up. No differences were found between the two groups.

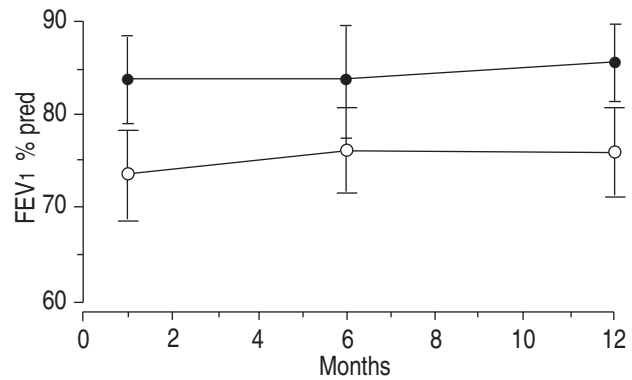


Fig. 2. – Monitoring of forced expiratory volume in one second (FEV₁) measurements in group 1 (CD4/CD8 >1 (●—)) and group 2 (CD4/CD8 <1 (○—)) during the 1 yr follow-up. No differences were found between the two groups.

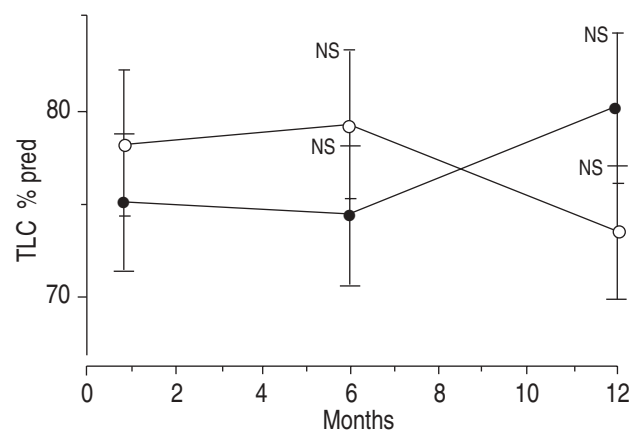


Fig. 3. – Monitoring of total lung capacity (TLC) measurements in group 1 (CD4/CD8 >1 (●—)) and group 2 (CD4/CD8 <1 (○—)) during the 1 yr follow-up. ns: nonsignificant, compared to baseline.

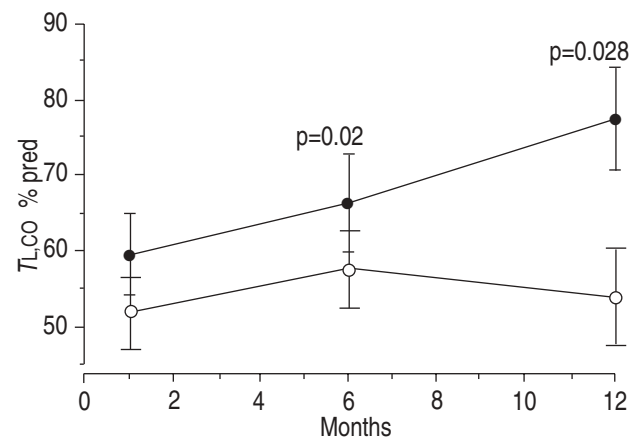


Fig. 4. – Monitoring of transfer factor of the lung fro carbon monoxide (TLCO) measurements in group 1 (CD4/CD8 >1 (●—)) and group 2 (CD4/CD8 <1 (○—)) during the 1 yr follow-up. In group 1 there was an improvement in the TLCO capacity after 6 (p=0.02) and 12 (p=0.028) months in response to steroid treatment.

lung transplantation and two from the progress of disease). Pulmonary function tests were performed 6 and 12 months after enrolment in the remaining patients. No significant changes were seen in FVC and FEV₁ in the two groups during that year. Baseline TLCO values and values 6 months after enrolment did not differ between the two groups: $59 \pm 22\%$ versus $51 \pm 21\%$ ($p = 0.29$) and $66 \pm 23\%$

versus $57\pm 18\%$ ($p=0.30$), respectively. After 12 months from enrolment in the study, the TL,CO values differed significantly: $77\pm 24\%$ for group 1 versus $54\pm 20\%$ for group 2 ($p=0.009$, using the baseline as a covariate) (table 6). Only in patients in group 1 did the percentage of TL,CO improve significantly after 12 months (from $59\pm 22\%$ to $77\pm 24\%$, $p=0.002$) in response to steroid treatment (figs. 1–4).

Discussion

We designed this study with the intent to determine whether differential cell counts and T-cell subsets from BAL may have a predictive value for the response to treatment in patients with IPF. We were able to demonstrate that a group of patients with a $CD4/CD8 >1$ had a better outcome after appropriate therapy. Moreover, we could show that, in these patients, alveolar lymphocytes were higher in percentage and were highly positive for CD8/S6F1 monoclonal antibody, a specific marker for suppressor/cytotoxic T-cells.

Previous studies provided controversial information on the cellular analysis in BAL fluid in patients with IPF. REYNOLDS *et al.* [9] and WEINBERGER *et al.* [10] demonstrated that patients with IPF have increased numbers of neutrophils. Conversely, HASLAM *et al.* [11] reported a slight but significant increase in lymphocytes in patients' BAL fluid. In this report, we included patients with moderate lymphocytosis or marked neutrophilia (acute and chronic phase of disease) who had evidence of abnormalities on pulmonary function tests and whose TBB revealed mild to severe fibrosis. In contrast with previous studies [6], and due to the availability of more sophisticated technology (FACS analysis), we also tested patients with a low lymphocytic content in their BAL (chronic phase).

We divided the patients into two groups, one with a $CD4/CD8$ ratio >1 and the other with a $CD4/CD8$ ratio <1 , 1 being the lower limit for normal ranges of $CD4/CD8$ ratio [12]. In an experimental model of bleomycin-induced pulmonary fibrosis, it was found that the helper-to-suppressor ratio of T-cell lymphocytes shifted with time, having a value >1 at the beginning of the disease and a value <1 at the latest phases of disease [13]. Although we did not find any difference in the duration of symptoms between our groups, these experimental findings may support the fact that our two groups were at the early and later stages of the same immunological process. This difference may be reflected in the number of neutrophils in the lower respiratory tract. In fact we found a lower percentage of neutrophils in group 1 which has been previously shown to be a sign of improved prognosis [14]. Similar correlation between a low $CD4/CD8$ ratio and a high percentage of neutrophils in BAL was observed in human immunodeficiency virus (HIV) patients with lung involvement [15, 16].

Caution must be exercised in interpreting lymphocyte phenotyping data used to characterize several stages of diseases, since both CD4 and CD8 T-lymphocytes can be composed of different subpopulations. CD8 T-cells were shown to be differentiated into cytotoxic and suppressor precursors [17]. CD8 subpopulations were shown to play a role in the onset of experimental models in rat hypersensitivity pneumonitis [18] and murine interstitial nephritis [19]. In the present study, we used the CD8/S6F1

monoclonal antibody to assess positive cytotoxic effector subsets which were shown to participate in many other immunological disorders [20]. We analysed the percentage of positive cells in the BAL and peripheral blood. By correlating both groups with clinical and functional parameters, it emerged that the first group of patients (helper-to-suppressor ratio >1 , fewer cytotoxic cells in lung and a higher percentage in peripheral blood) showed a better response to therapy. A mirror-image picture was observed in the distribution of these T-cell subsets: they had migrated from the peripheral blood and accumulated in the lung at an active site of disease in the group with the poorer prognosis (helper-to-suppressor ratio <1). The opposite was observed in group 1.

The analysis of the clinical parameters showed that crepitations and clubbing are more often found in patients whose BAL display less lymphocytosis than in the other group and has a $CD4/CD8$ ratio <1 . Smoking history was not found to be significantly different between the two groups. Similar results were found in a case control study of causes of cryptogenic fibrosing alveolitis where smoking history was not related to the disease state [21]. Conversely, other studies showed that cigarette smoking strongly alters the phenotype of T-cell subsets in BAL [22]. The discrepancy between the data may be due to the fact that there was a similar distribution of nonsmokers, smokers and exsmokers in our two study groups.

Functional assessment showed, as reported in other studies [23, 24], that the single breath TL,CO is a very sensitive parameter for following-up deterioration and response to therapy [23]. During the past few years, we have attempted to facilitate the assessment of patients with IPF. In addition to thin-section CT [25], ^{67}Ga lung scanning [26] and exercise testing [23] have been used to predict the progression of disease. We believe that BAL findings provide a more comprehensive answer to the early inflammatory cellular effects taking place in the lung and is required to determine the need for intensive therapy to reverse the disease. Moreover, all the above-mentioned approaches together with BAL analysis provide and predict a predominant fibrotic histology [27]. In IPF, it is known that inflammation precedes and leads to fibrosis [28], as was confirmed by instillation of bleomycin in the lung which induced an influx of inflammatory cells in the lung as the first event [29]. Moreover, a recent publication by BRAUN *et al.* [30] demonstrated that in the bleomycin-induced fibrosis in a mouse model, the first event is accumulation of CD4+ cells but, with progress of fibrosis, the CD8+ lymphocytes increased from 4–68% [30].

In summary, the findings of the present study suggest that the assessment of cells recovered by bronchoalveolar lavage and the analysis of T-cell subsets may detect cellular events of inflammation of pulmonary parenchyma in a group of patients with a more benign form of disease responsive to early treatment. We assume that this is a realistic approach for identifying those patients who need intensive therapy in order to reverse disease and improve prognosis.

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