Original Study

Expression of CTGF and TNFa in alveolar macrophages of patients with idiopathic pulmonary fibrosis before and after treatment with azathioprine or interferon-γ-1b

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SUMMARY.
BACKGROUND: Idiopathic pulmonary fibrosis (IPF) is a fatal lung disorder the aetiology of which is unknown and for which there is no effective therapy. Connective tissue growth factor (CTGF) and tumour necrosis factor alpha (TNFα) have been reported to participate significantly in the pathogenesis of the disease. The role of alveolar macrophages in the expression of these cytokines remains unclear. **MATERIALS AND METHODS:** Samples of bronchoalveolar lavage fluid (BALF) derived from 20 newly diagnosed patients with IPF before and after 6 months of treatment with either interferon (IFN-γ-1b) and prednisolone (10 patients) or azathioprine (AZA) and prednisolone (10 patients) and from 10 normal subjects (control group) were used for the analysis of CTGF and TNFα protein expression in the alveolar macrophages. The effectiveness of the two drug regimes on the pulmonary function tests (FEV_1, FVC, DLCO) and PaO_2 and $PaCO_2$ of the patients with IPF was investigated. **RESULTS:** Decreased CTGF protein expression was detected in the patients with IPF compared with the control group (p=0.001). TNFα expression in IPF patients did not differ from that of the normal control subjects. Neither of the drug regimes affected the protein expression of these factorsor the pulmonary function parameters. **CONCLUSION:** These findings suggest that the alveolar macrophages are not the main source of CTGF and TNFα in IPF. Treatment with either AZA or IFN-γ-1b did not result in any significant change in the protein expression of these factors. ***Pneumon 2011, 24(2):149-156.***

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most frequent idiopathic

interstitial pneumonia which leads to respiratory failure and death, with a mean survival of 2.5 – 3.5 years[1,2]. Histopathologically, the disease is characterized by fibroblast proliferation, extracellular matrix deposition and progressive lung scarring[3].

For several years it had been considered that IPF is due to an uncontrolled healing response, resulting from persistent inflammation. However, important new concepts derived from recent studies suggest that recurrent injury to the alveolar epithelium and resident macrophages results in overexpression of profibrotic cytokines and growth factors, independently of inflammatory events, leading to fibrosis[4,5]. Several pathways have been implicated in the promotion of fibrosis, involving cytokines, growth factors, chemokines and mediators of apoptosis[6].

Connective tissue growth factor (CTGF) is a growth and chemotactic factor for fibroblasts, which has been found to be overexpressed in a variety of fibrotic disorders, including lung, pancreas, liver and kidney fibrosis. It is a downstream effector of transforming growth factor β (TGFβ), mediating profibrotic activities[7]. It has been found in increased quantities in the lung tissue of patients with IPF and its localization has been confined predominantly to type II alveolar epithelial cells and activated fibroblasts[8].

An important cytokine, known to be upregulated in IPF is tumour necrosis factor α (TNFα), which induces inflammation and limited fibrosis[2,9]. It is produced mainly by monocytes/macrophages and lymphocytes. It is an early response cytokine in the development of a fibrotic and proinflammatory cytokine cascade[10]. It is known to trigger fibroblast proliferation via the action of platelet-derived growth factor, but it also inhibits TGFβ induced fibroblast production of type I and type III procollagens[11]. TNFα can both stimulate and inhibit the evolution of pulmonary fibrosis[12]. Increased expression of TNFα has been observed in animal models of this disease[11].

Concerning the treatment of IPF, after disappointing results from treatment with corticosteroids, the use of immunosuppressive agents, such as azathioprine (AZA) and antifibrotic agents such as interferon-γ-1b (IFN-γ-1b), which also has antiproliferative and immunoregulatory properties, has been recommended[13-16]. The disease, however, is unresponsive to treatment and no regime has been shown to be efficacious[17]. Currently lung transplantation is the only mode of treatment that prolongs survival[18,19].

Macrophages are an important source of cytokines and growth factors which are implicated in IPF. Alveolar macrophages (AM) present two polarized phenotypes, the M_1 and M_2 AM which can be activated by T-helper type 1 (Th1) and T-helper type 2 (Th2) cytokines, respectively[20,21]. Th2 cytokines have been related to the pathogenesis of IPF[6,22,23]. While M_1 AM promote IL-2 mediated Th1 response and overproduce inflammatory cytokines such as IL-6 and TNFα, M2 AM support a fibroproliferative microenvironment, secreting growth factors, such as TGFβ, and Th2 cytokines, such as IL-10[24-26]. Thus, macrophages appear to have a crucial role in the pathogenesis of IPF.

The aim of this study was to determine the role of AM obtained from the bronchoalveolar lavage fluid (BALF) of patients with IPF in the expression of CTGF and TNFα, compared with that of normal subjects. It was also aimed to evaluate the comparative effectiveness of AZA and IFN-γ-1b on these markers in the AM from the BALF of patients with IPF on their lung function tests and blood gases.

MATERIALS AND METHODS

Study population

Twenty patients with newly diagnosed, untreated, symptomatic IPF were included in the study. The diagnosis of IPF was based on surgical lung biopsy (i.e., the histological pattern of Usual Interstitial Pneumonia) in 9 of the patients and on the ATS/ERS diagnostic criteria for IPF (presence of all major and at least three of the four minor criteria)[1] in 11: Major criteria: Non-identification of a secondary cause of interstitial lung disease (drug toxicity, environmental exposure, collagen vascular disease), lung function tests showing a restrictive pattern and blood gas abnormalities (increased alveolar-arterial gradient for PaO_2 at rest or after exercise), typical HRCT findings, BAL or transbronchial biopsy not supportive of an alternative diagnosis. Minor criteria: Age above 50 years, progressive dyspnoea on exercise, disease duration of more than 3 months, bilateral fine crackles on lung auscultation.

Ten normal subjects, 3 female and 7 male, with a median age of 61 years, who underwent bronchoscopy and BAL because of cough, a history of haemoptysis or hoarseness, were included in the study as control subjects. Three of the control subjects were smokers, 3 ex-smokers and 4 were never smokers. None of them suffered from chronic obstructive pulmonary disease (COPD), malignancy, or interstitial lung disease.

Study design

The study was approved by the Bioethics Committee of the Sismanoglio Hospital and all participants provided written informed consent. The 20 patients with IPF were

randomised into two groups for treatment: the first group was treated with a combination of IFN-γ-1b 200 μg subcutaneously 3 times per week and 10mg of oral prednisolone daily, while the second group was treated with 150mg of oral AZA and 10 mg of oral prednisolone daily.

Bronchoalveolar lavage

Bronchoscopy and BAL were performed according to recommended guidelines[27,28] in all IPF patients at the beginning of treatment and after a 6-month course of treatment. The patients in the control group underwent bronchoscopy and BAL once.

BAL was performed in the middle lobe or the lingula, depending on the high resolution computed tomography (HRCT) findings. Aliquots of 60 ml of sterile normal saline were instilled through the bronchoscope and the fluid was retrieved by mechanical suction. The standard introductory volume was 180 ml.

Cells were separated from the BALF by low-speed centrifugation at 300g for 5 minutes and were washed three times with cold minimal essential medium (MEM) containing 25Mm Hepes buffer. BALF cell counts were made as reported previously. The total cell count was determined using an improved Neubauer counting chamber and expressed as the total number of cells per ml of aspirated fluid. Slide preparations for differential percentage count of the cells were made with a Shandon cytocentrifuge (Cytospin II, Shandon Ltd, Runcorn, Cheshire, U.K.) using 100-μl aliquots of the lavage cell suspensions, adjusted to 1.25x10 6 cells per ml in MEM. The differential count was determined on a stained preparation stained by May-Grunwald Giemsa staining and Papanicolaou staining, counting more than 1,000 cells.

Pulmonary Function Tests (PFTs)

PFTs (FEV_1, FVC, DLCO) and measurement of PaO_2 and $PaCO_2$ were performed at the beginning of treatment and after the 6-month course of treatment.

Antibodies and Immunohistochemistry (IHC)

Antibodies were selected and applied, including anti-CTGF goat polyclonal (L-20, Santa Cruz Biotechnology, Inc, USA) at dilution 1:100, and anti-TNFa mouse monoclonal (clone 28401.111, Santa Cruz Biotechnology, Inc, USA) at a dilution of 1:100, respectively. The immunohistochemistry (IHC) protocol for this antigen was carried out on slides of the corresponding BAL samples. The slides were firstly dipped in acetone, following which EN Vision (DAKO, Denmark) assay was applied using an automated staining system (I 6000 – Biogenex, USA) and according to corresponding manufacturer's instructions. This specific assay is based on a soluble, dextran-polymer system, preventing endogenous biotin reaction and increasing the quality of the stained slides. Briefly, the slides, after peroxidase blocking, were incubated with primary antibody for 30 to 40 min - depending on the antibody - at room temperature and then incubated with Horseradish peroxidase labelled polymer-HRP LP for 30 min. The antigen-antibody reaction was visualized using 3-3 diaminobenzidine tetrahydrocloride (DAB) as a chromogen substrate. The slides were slightly counterstained with haematoxylin for 30 seconds, dehydrated and mounted. For negative control slides, the primary antibodies were omitted. Cytoplasmic-extracellular localization and membranous-cytoplasmic staining patterns were considered to be acceptable for CTGF and TNFα, respectively.

Computerized Image Analysis (CIA)

In order to evaluate the immunohistochemistry results not only qualitatively, but in a unbiassed accurate way, computerized image analysis (CIA) was performed using a semi-automated system with the hardware features: Intel Pentium IV, Sony Camera (resolution of 800x600), Microscope Olympus BX-50 and Windows XP/Image software, Pro Plus version 3.0-Media Cybernetics 1997. Measurements of protein expression of the markers described above were performed in 5 optical fields per case at a magnification of 400 (40x10). In a rectangular active window on the computer screen, covering a stable area of approximately 16848μm^2, each pixel contained a 24-bit value, called RGB "TRIPLE". This RGB-triple is made up of 3 separate 8-bit samples. Each sample represents the level of brightness of its respective colour channel: red, blue or green. In a 0-255 range, values decreasing to 0 correlate with overexpression, while values increasing to 255 correspond to progressive loss of expression. A macro based on Basic Pro Plus algorithms was implemented, according to which all stained macrophages (i.e., round to oval objects demonstrating light to dark brown colour due to DAB chromogen and with a diameter of more than 15μm for the exclusion of lymphocytes and neutrophils) per case in the corresponding optical fields were measured and the final number was filed in Excel sheets for statistical analysis. Areas of significant cellularity including isolated macrophages or small clusters were considered to be eligible for measurement. Semi-automated segmentation was performed by splitting the

small clusters (Figures 1, 2, 3, 4).

Statistical analysis

All CIA values were expressed as mean ± standard deviation (±SD). Comparison between groups at baseline was performed with the Student's t-test or chi-squared test. Paired differences of CTGF and TNFα were assessed with Student's paired t-statistic. The influence of treatment on the alteration of the values of PFTs and blood gases over time was examined by mixed model analysis using the unstructured covariance structure. A p-value of <0.05 was considered significant.

RESULTS

There was no difference in age, sex and smoking history between the two groups of patients, and the patients of both groups had similar FEV_1, FVC, DLCO and $PaCO_2$ values at entry, but the group receiving AZA had a statistically significant lower mean PaO_2 value than the group receiving interferon. None of the patients had indications of respiratory failure. The demographic profile of the study patients is shown in table 1.

The IHC staining showed that CTGF expression in the BALF macrophages of patients with IPF was significantly decreased compared with that of the healthy control subjects (Table 2). No difference in CTGF and TNFα expression was detected between the two treatment groups before and after treatment (table 3). The findings are also depicted in bar graphs (figures 5, 6), where the higher the staining intensity values (values between 0-255), the lower the protein expression of CTGF and TNFα.

The PFT parameters (% predicted values) showed no change after treatment in either treatment group (Table 4).

a. CTGF -control b. CTGF –IPF before treatment

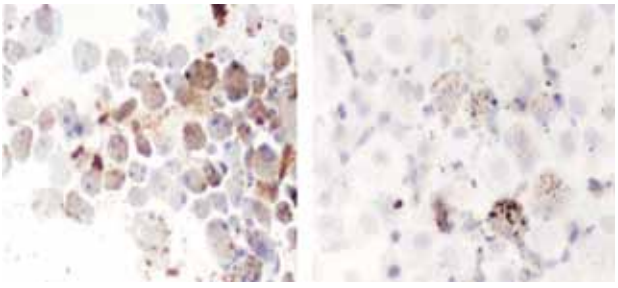

FIGURE 1. Computerized Image Analysis (CIA) of bronchoalveolar lavage fluid (BALF) macrophages of a control subject (a) and a patient with IPF, before treatment (b), stained for connective tissue growth factor (CTGF).

a. CTGF – IPF before treatment b. CTGF – IPF after IFN treatment

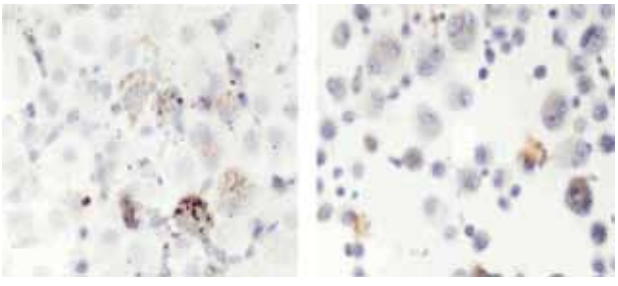

FIGURE 2. Computerized Image Analysis (CIA.) of bronchoalveolar lavage fluid (BALF) macrophages of a patient with IPF before treatment (a) and a patient, after interferon (IFN) treatment (b) stained for connective tissue growth factor (CTGF).

a. TNFα – control b. TNFα – IPF before AZA treatment

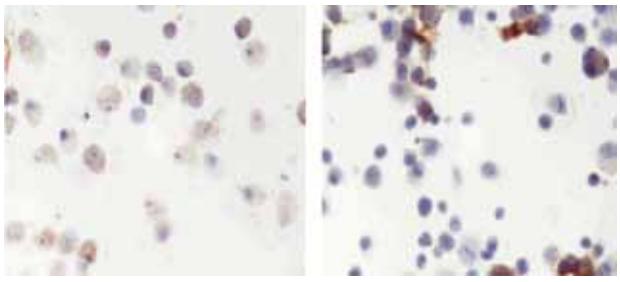

FIGURE 3. Computerized Image Analysis (CIA) of bronchoalveolar lavage fluid (BALF) macrophages of a control subject (a), and a patient with IPF, before azathioprine (AZA) treatment (b), stained for tumour necrosis factor-α (TNFα).

a. TNFα – IPF before treatment b. TNFα – IPF after IFN-γ-1b treatment

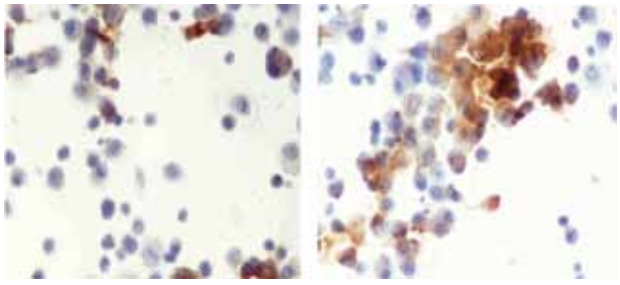

FIGURE 4. Computerized Image Analysis (CIA) of bronchoalveolar lavage fluid (BALF) macrophages of a patient with IPF before (a), and a patient after interferon (IFN-γ-1b) treatment (b), stained for tumour necrosis factor-α (TNFα).

TABLE 1. Demographics, smoking history and lung function parameters of the patients with interstitial pulmonary fibrosis at entry into the study (n=20)

	IFN-γ-1b Group	AZA Group	p-value**
Median age (yr)	67.9 ± 5.8 (n=10)	71.6 ± 6.3 (n=10)	
Sex (male/female)	3/7	6/4	
Smoker			
Current	1	1	
Past	3	5	0.638
Never	6	4	
FVC (%pred)	74.0 ± 19.0 (n=10)	66.3 ± 20.8 (n=10)	0.399
FEV1 (%pred)	81.3 ± 19.9 (n=10)	71.7 ± 18.8 (n=10)	0.282
DLCO (%pred)	47.7 ±36.8 (n=8)	56.9 ± 17.8 (n=8)	0.538
paO2 (mmHg)*	79.9 ± 8.7 (n=10)	65.7 ± 11.6 (n=10)	0.006
paCO2 (mmHg)*	39.7 ± 3.9 (n=10)	38.1 ± 4.2 (n=10)	0.399

AZA = azathioprine treatment, IFN-γ-1b = interferon treatment
Values are expressed as mean ± SD, and age as median (range)
*Breathing Room Air, **p<0.05: statistically significant

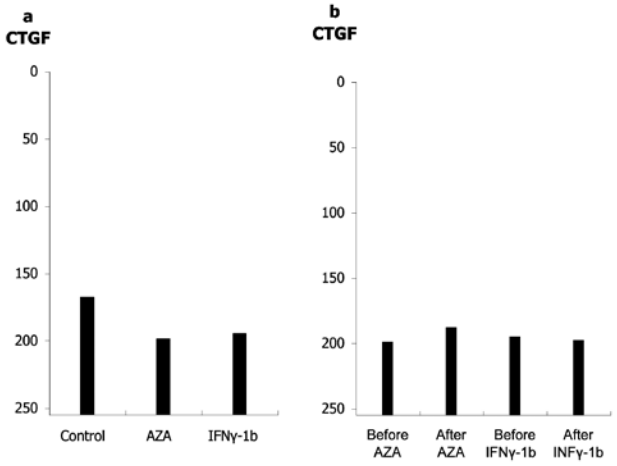

FIGURE 5. Quantification of staining intensities for CTGF (mean values; values ranged between 0-255).
a. Significant decreased CTGF expression of CTGF in IPF patients comparing to normal subjects (p=0,001)
b. No significant difference of CTGF was found in patients before and after therapy
CTGF = connective tissue growth factor
IPF = interstitial pulmonary fibrosis
AZA = azathioprine
IFN-γ-1b = interferon

TABLE 2. CTGF and TNFα expression (CIA mean values) in patients with interstitial pulmonary fibrosis before treatment and control subjects

	Control group N =10	IFN-γ-1b group N = 10	AZA group N = 10	p
CTGF	168.50 ± 19.8	195.48 ± 24.2	199.29 ± 14.6	0.019
TNFα	160.24 ± 43.3	158.43 ± 24.5	157.43 ± 27.3	0.907

CIA = computerized Image Analysis, AZA = azathioprine treatment, IFN-γ-1b = interferon treatment, CTGF = connective tissue growth factor, TNFα = tumour necrosis factor-α
p<0.05: statistically significant

To summarize, low CTGF expression was observed in the BALF macrophages of patients with IPF compared with the control subjects, with no change after IFN-γ-1b or AZA treatment, respectively, in the two groups of patients. No difference of TNFα expression was found between the patients and the control group, or between the two groups of patients before and after treatment. The FVC%, FEV_1%, DLCO%, PaO_2 and $PaCO_2$ in the two patient groups at entry differed to a statistically significant degree only regarding PaO_2 and none of the patients showed respiratory failure. No differences between PFT parameters were found before and after IFN-γ-1b or AZA administration.

TABLE 3. CTGF and TNFα expression (CIA mean values) in patients with interstitial pulmonary fibrosis before and after treatment with IFN-γ-1b or AZA.

	IFN-γ-1b group			AZA group		
	Before treatment	After treatment	P	Before treatment	After treatment	p
CTGF	195.48 ± 24.2	198.32 ± 37.5	0.844	199.29 ± 14.6	187.58 ± 20.10	0.111
TNFα	158.43 ± 24.5	159.33 ± 28.9	0.947	157.43 ± 27.3	160.35 ± 37.20	0.873

CIA = computerized Image Analysis, AZA = azathioprine treatment, IFN-γ-1b = interferon treatment, CTGF = connective tissue growth factor, TNFα = tumour necrosis factor-α
p<0.05: statistically significant

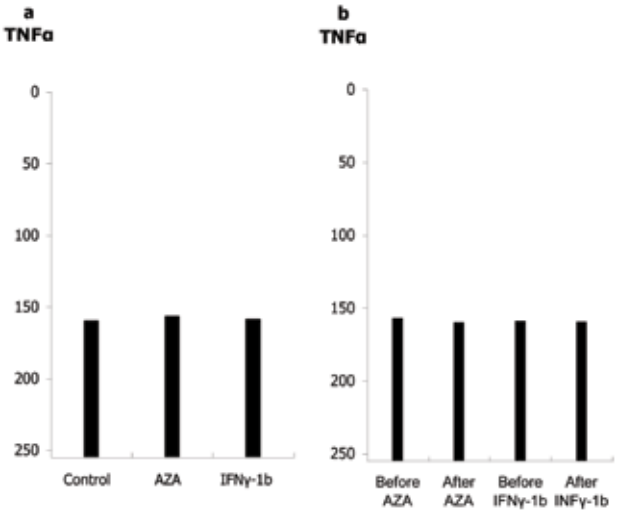

FIGURE 6. Quantification of staining intensities for TNFα (mean values; values ranged between 0-255).
a. No significant difference of TNFα was found between patients and control group.
b. There was no significant difference of TNFα expression between IPF patients before and after AZA or IFN-γ-1b treatment.
TNFα = tumour necrosis factor-α
IPF = interstitial pulmonary fibrosis
AZA = azathioprine
IFN-γ-1b = interferon

DISCUSSION

In the present study we found that the BALF macrophages of patients with IPF express CTGF at a lower level than control subjects, while no difference in TNFα expression was observed. Treatment with either IFN-γ-1b or AZA did not affect the expression of these factors.

The finding that the two groups of patients, allocated randomly, did not differ in terms of CTGF and TNFα expression before treatment suggests that the two groups were comparable. In addition they showed similar performance on PFT, differing only in PaO_2, and none of the patients showed respiratory failure.

CTGF is considered to be a crucial mediator of fibrogenesis and has been found increased in IPF[6,9]. In a study of Pan et al, CTGF mRNA was overexpressed in fibrotic lung tissues, where it was localized in proliferating type II alveolar epithelial cells and activated fibroblasts[8]. Our findings concerning CTGF suggest that BAL macrophages are not the main cellular source of this factor. A possible explanation for the down-regulation of CTGF protein expression in the macrophages of patients with IPF compared with normal subjects, is that the factor had already been consumed, once the fibrosis progressed. The IHC findings reflect only the disease stages at which these markers were evaluated, and therefore cannot predict what lies upstream or downstream of the pathogenetic cascade. The possibility cannot be excluded that the expression of these markers might be different during other stages of the disease process. This is also a caveat in most of the studies of IPF pathogenesis using human samples (tissue or serum or BAL) since we are limited to investigating only a "snapshot" of the pathogenetic cascade and are therefore unable to gain a holistic view based on human data.

Tzortzaki et al, showed that mRNA expression of CTGF in lung tissues of patients with IPF remained almost stable after a 24-month course of treatment with IFN-γ-1b[29], a finding consistent with our results regarding the lack of effectiveness of IFN-γ-1b treatment on CTGF expression.

TNFα is known as a T-helper cell type I cytokine with pleiotropic effects on the inflammatory response[6,10]. The study of Martinez et al reported that the level of TNFα mRNA in macrophages from the BALF of patients with

TABLE 4. Lung function parameters in patients with interstitial pulmonary fibrosis before and after treatment with IFN-γ-1b or AZA

	IFN-γ-1b group N=10			AZA group N=10		
	Before treatment	**After treatment**	**p**	**Before treatment**	**After treatment**	**p**
FVC (%pred)	74.0 ± 19.0	65.9 ± 6.6	0.058	66.3 ± 20.8	68.3 ± 6.6	0.554
FEV_1 (%pred)	81.3 ± 19.9	72.6 ± 5.5	0.100	71.7 ± 18.8	73.8 ± 5.5	0.620
DLCO (%pred)	47.7 ±36.8	31.3 ± 4.2	0.165	56.9 ± 17.8	51.4 ± 4.4	0.293
paO_2 (mmHg)	79.9 ± 8.7	74.9 ± 5.5	0.531	65.7 ± 11.6	74.2 ± 4.9	0.224
$paCO_2$ (mmHg)	39.7 ± 3.9	38.8 ± 1.6	0.580	38.1 ± 4.2	40.1 ± 1.5	0.257

AZA = azathioprine treatment, IFN-γ-1b = interferon treatment
$p<0.05$: statistically significant

IPF was not significantly different from that of normal subjects[30], while Homolka et al found increased release of TNFα by BALF cells, compared to controls, which persisted after corticosteroid treatment[31]. Nash et al showed that type II epithelial cells from the lung tissue of patients with IPF overexpressed TNFα, while the macrophages stained only weakly.

According to the M1/M2 alveolar macrophages system, alternatively activated M2 alveolar macrophages do not produce large amounts of TNFα, which is a T-helper cell type I cytokine but they secrete Th2 cytokines such as IL-10, which predominate in fibrosis[20,21,25,26].

The treatment regimes used in this study did not alter the CTGF and TNFα protein expression, or the FVC%, FEV_1%, DLCO%, PaO_2 and $PaCO_2$ values. These findings are consistent with the results of the recent INSPIRE study reported by King et al, in which 15% of the patients in the IFN-γ-1b group had died after a 64-week treatment, compared with 13% of those in the placebo group, and thus IFN-γ-1b was demonstrated to be inefficacious[18,33]. There are insufficient data to show a significantly positive effect of AZA in the treatment of IPF[34] and to date lung transplantation is the only mode of treatment which prolongs survival[18]. A recent study showed that pirfenidone represents a promising treatment for IPF[35,36], and at the time the present study was initiated, IFN-γ-1b also appeared to be promising[34,37], while the combination of an immunosuppressive or cytotoxic agent, such as AZA with prednisolone was recommended by ATS/ERS.

In conclusion, the present study showed that the BALF macrophages of patients with IPF are characterized by lower CTGF protein expression compared with normal subjects, and similar TNFα expression.Treatment with either AZA or IFN-γ-1b did not change either the expression of thesemarkers or PFT parameters.

Further studies are needed to clarify the precise role of the alveolar macrophages in the complex pathogenesis of this dismal disease.

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