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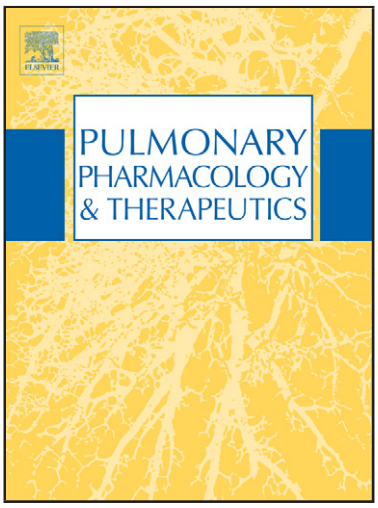


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PII: S1094-5539(08)00069-2
DOI: doi:10.1016/j.pupt.2008.06.005
Reference: YPUPT 848

To appear in: *Pulmonary Pharmacology & Therapeutics*

Received date: 14 July 2007
Revised date: 24 June 2008
Accepted date: 30 June 2008

Cite this article as: Katerina M. Antoniou, Nikolaos Tzanakis, Eleni G. Tzortzaki, Katerina Malagari, Anastassios V. Koutsopoulos, Michael Alexandrakis, Athol U. Wells and Nikolaos M. Siafakas, Different angiogenic CXC chemokine levels in bronchoalveolar lavage fluid after interferon gamma-1b therapy in idiopathic pulmonary fibrosis patients, *Pulmonary Pharmacology & Therapeutics* (2008), doi:10.1016/j.pupt.2008.06.005

# Different angiogenic CXC chemokine levels in bronchoalveolar lavage fluid after Interferon gamma-1b therapy in idiopathic pulmonary fibrosis patients

[1] Katerina M. Antoniou MD, PhD, [1]Nikolaos Tzanakis MD, PhD, [1]Eleni G. Tzortzaki, MD, PhD, FCCP, [2]Katerina Malagari, MD, PhD, [3]Anastassios V Koutsopoulos MD, [4]Michael Alexandrakis, [5]Athol U Wells, MD, FRACP and [1]Nikolaos M. Siafakas MD, PhD, FRCP, FCCP

[1] Department of Thoracic Medicine, University of Crete, Heraklion, Greece
[2] Department of Radiology, University of Athens, Greece
[3] Department of Pathology, University of Crete, Heraklion, Greece
[4] Department of Haematology, University of Crete, Heraklion, Greece
[5] Interstitial Lung Disease Unit, Royal Brompton Hospital, London, England

**Corresponding author:**
Nikolaos M. Siafakas, MD, PhD, FRCP
Professor of Thoracic Medicine
Dept of Thoracic Medicine,
University General Hospital, Medical School, University of Crete,
Heraklion 71110 Crete, Greece
Tel: +30-2810 392 433, Fax: +30 2810 542 650,
Email: siafak@med.uoc.g

## Abstract

**Background and aim**: Pulmonary fibrosis is a devastating disease with few treatment options. Angiogenesis that leads to aberrant vascular remodeling is regulated by an opposing balance of angiogenic and angiostatic factors. The present study aims to evaluate the role of three angiogenic (IL-8, ENA-78 and GRO-a) and three angiostatic (MIG, IP-10, ITAC) chemokines in bronchoalveolar lavage fluid (BALF), before and after treatment with interferon gamma-1b (IFN gamma-1b).

**Patients and methods:** We studied prospectively 20 patients (16 males, 4 females) of median age 68 yr (range, 40-75) with histologically confirmed IPF/UIP. Patients were assigned to receive IFN gamma-1b 200 μg sc thrice a week. Angiogenic and angiostatic mediators' levels were measured by ELISA kits.

**Results:** The levels of the angiogenic chemokines significantly decreased after 12mo of IFN-γ-1b treatment (median values in pg/ml, IL-8/CXCL8: 640 vs 81, $p < 0.05$, ENA-78/CXCL5: 191 vs 51, $p< 0.005$ and GRO-α: 1827 vs 710, $p<0.005$). No significant differences were detected in the levels of the angiostatic chemokines after therapy (median values in pg/ml, IP-10/CXCL10: 56 vs 56.5, p=0.6, ITAC/CXCL11: 43 vs 47, p=0.11). However, a significant decrease in the MIG/CXCL9: 66 vs 31, p=0.006, has been detected.

**Conclusion:** These findings support the notion that IFN gamma may be one of the important mediators regulating angiogenetic balance in IPF. However, IFN gamma-1b decreases MIG levels, finding that in association with no alteration in IP-10 and I-TAC levels, could explain in part the non beneficial effect of this drug in IPF.



## Key words

angiogenesis, angiostasis, CXC chemokines, pathogenesis, treatment, IFN gamma-1b

**Abbreviation List**

BALF: Bronchoalveolar Lavage Fluid

CXCL: CXC ligand

CXCR: CXC receptor

ELISA: Enzyme-linked immunosorbent assay

$FEV_1$: Forced Expiratory Volume in 1 sec

FVC: Forced Vital Capacity

TLco: Diffusing capacity for carbon monoxide

$P\alpha O_2$: partial pressure of oxygen

HRCT: High Resolution Computed Tomography

IL-8 : interleukin -8

ENA-78: Epithelial Neutrophil Activating protein-78

GRO-a: Growth-related gene alpha

ITAC: Interferon-γ -inducible T cell alpha chemoattractant

IFN- γ: Interferon-gamma

IP-10: Interferon- γ inducible protein-10

IPF: Idiopathic Pulmonary Fibrosis

MIG: Monokine induced by Interferon- γ

Th1: T helper 1

## Introduction

Idiopathic Pulmonary Fibrosis (IPF) is a chronic and often fatal pulmonary disorder with a 2- to 3- year median survival of 50% [1]. To date no management approach has proven to be efficacious for the treatment of this disease [2, 3]. IPF has been defined as an ''epithelial –fibroblastic disorder'', characterized by abnormal wound healing with excessive fibrosis and minimal inflammation [4]. These emerging data have focused attention on antifibrotic drugs [2, 5]. Interferon gamma-1b (IFN gamma-1b) has been proposed as a promising candidate for IPF. The reason is that IFN gamma-1b has the ability to modulate the Th1/Th2 imbalance and to suppress fibroblast activation [6]. A recent meta-analysis of 390 patients from three long-term randomized controlled trials [7-9] has reported that IFN gamma-1b therapy decreases mortality in patients with IPF [10].

The mechanism, however, for the potential survival advantage in the IFN gamma-1b treated patients remains to be determined. Based on the report by Ziesche and coworkers [7], we initially hypothesized that IFN gamma-1b would down-regulate mRNA for transforming growth factor beta (TGF-b) or connective tissue growth factor (CTGF) [11]. As we did not reproduce Ziesche's data, in agreement with Strieter's report [12] we reported that IFN gamma-1b acts through other pathways [5]. Our laboratory has shown decreased levels of the angiogenic cytokine, interleukin-18 (IL-18) [13] after IFN gamma-1b therapy in both bronchoalveolar lavage fluid (BALF) and induced sputum samples from IPF patients [14,15]. Neovascularization in IPF was originally identified in 1963 by Turner-Warwick, who demonstrated that within areas of pulmonary fibrosis there was extensive neovascularization with anastomoses between the systemic and pulmonary microvasculature [16]. Angiogenesis in fibroproliferative disorders is regulated by an opposing balance between angiogenic and angiostatic factors [17]. In the last decade, several studies suggested a potential role of neovascularization in the etiopathogenesis of IPF [18-24]. However, the contribution of aberrant vascular remodeling in IPF is still controversial [25, 26]. Molecules that originally promote angiogenesis include members of the CXC chemokine family that contain a three amino-acid motif (ELR) such as IL-8/CXCL8, epithelial neutrophil activating protein ENA-78/CXCL5 and growth-related genes (GROs, a, β, γ/CXCL1, 2, 3). By contrast, other members of this unique chemokine family that do not contain the angiogenic ELR motif behave as potent inhibitors of angiogenesis. These are interferon (IFN)-γ

inducible monokines such as (MIG)/CXCL9, IFN-γ-inducible protein (IP)-10/CXCL10, and IFN-γ-inducible T-cell a chemoattractant (ITAC)/CXCL11 [17].

The aim of this prospective study is to further investigate the angiogenetic balance in patients with IPF and to characterize the effects of IFN gamma-1b on angiogenic and angiostatic chemokines in BALF.

## Materials and Methods

### 1. Subjects

From January 2000 to January 2003 we recruited 20 newly diagnosed patients with IPF (14 males and 6 females), 16 ex-smokers, and 4 nonsmokers, median (range) age 68 (40-75) years. The diagnosis of IPF was based on accepted clinical and imaging criteria [1] and was confirmed histologically in all the included patients. Lung biopsies were taken using video-assisted thoracoscopic surgery (VATS). The histologic diagnosis was usual interstitial pneumonia (UIP) in all patients [1].

The Ethics committee of our hospital approved the protocol and all patients gave their consent.

Protocol started with a two months run-in period. During the run-in period, all eligible patients received 50 mg of oral prednisolone per day for 4 weeks orally, with subsequent tapering of the dose to 10 mg per day over a 1 month period, regardless of any previous treatment. If the glucocorticoid treatment was ineffective, the patients were assigned to receive 200 μg of IFN gamma-1b (Imukin; Boehringer Ingelheim Pharma KG, Biberach, Germany) subcutaneously three times per week for 12 months.

### 2. Spirometry

Spirometry was performed using MasterLab system, Jaeger 2.12, Germany, according to standard protocols [27].

### 3. High resolution computed tomography (HRCT) evaluation

Scoring of disease extent and progression: Two readers (AUW and KM), blinded to the clinical, functional data and type of treatment, examined the HRCT images.

HRCT slices at five predetermined levels were evaluated (the great vessels, the aortic arch, the carina, the right inferior pulmonary vein, and two centimetres above the right hemidiaphragm), were evaluated.

HRCT extent of disease score: At each level, the overall extent of disease was visually estimated to the nearest 5% including a reticular pattern or ground-glass opacification with or without traction bronchiectasis. To obtain the mean fibrosis score percentages from all slices examined were summed and divided by the number of slices (five). This mean value was considered the extent of fibrosis, irrespective of the predominant pattern. This visual method of disease extent quantification has been extensively used for HRCT scoring in interstitial lung disease with good functional correlations by Wells and co-workers [28, 29].

HRCT disease progression score: Repeat HRCT studies at twelve months after initiation of treatment were compared to baseline. HRCT changes were measured in a scale of -2 to +2 representing likelihood of improvement or deterioration (HRCT progress score). A score of -2 indicated definite improvement, a score of -1 indicated that subtle improvement is most likely, a score of 1 was given when subtle or little deterioration was most likely, and a score of 2 when definite deterioration was seen. Stable disease was recorded as 0. This scoring system forms a 5 point scale for disease changes at each HRCT slice (range 5-25 for each patient). To obtain the average HRCT disease progression score the rating values from all slices examined were summed and averaged.

Definite improvement was agreed to be recognised when unquestionable resolution of ground glass or interstitial abnormalities was seen. Definite deterioration was recognised when unquestionable new areas of ground glass opacities or reticulation emerged or when a microcystic reticular pattern changed into macrocystic disease (coarsening of reticulation to honeycomb) or when unquestionable traction bronchiectasis had developed in an area of previous ground glass opacification. In addition, consensus agreement upon level of "certainty" was agreed to be also based on the relative predominance of the above signs of improvement or deterioration within each individual slice.

Finally, a qualitative comment of the type of change was noted including (a) ground glass development or resolution, (b) reticulation development or resolution, (c) coarsening of the reticulation (microcystic reticular pattern replaced by macrocystic

disease). The above qualitative parameters were roughly quantitatively characterized overall.

**4. Bronchoalveolar lavage**

Fiberoptic bronchoscopy with bronchoalveolar lavage (BAL) was performed as part of routine clinical management, according to recommended guidelines [30]. The BAL was conducted before the run-in period and after twelve months of treatment with IFN gamma-1b.

**4.1 BAL fluid processing**

The recovered BAL fluid (BALF) was filtered through sterile gauze (Thompson, Ontario, Canada) and centrifuged at 400 g for 15 minutes at $4^0$C. Total cell counts were determined using an improved Neubauer counting chamber, and expressed as the total number of cells per mL of aspirated fluid. The pellet was washed three times with cold PBS-Dulbecco's and the cells were adjusted to a final concentration of $10^6$cells/mL with RPMI1640 plus 2%FCS. The slide preparation was performed as previously reported [30].

**4.2 Assay of chemokine levels using specific enzyme-linked immunosorbent assay**

Measurements of GRO-a/CXCL1, ENA-78/CXCL5, IL-8/CXCL8, MIG/CXCL9, IP10/CXCL10 and ITAC/CXCL11 were performed using specific enzyme linked immunosorbent assays (ELISA). The ELISA capture and detection antibodies for assaying angiogenic and angiostatic chemokines were selected paired reagents optimized for ELISA performance from R&D systems (Quantikine R&D system, Minneapolis MN USA). The detection limits for the assays were as follows: 10 pg/ml for GRO-a/CXCL1, 15 pg/ml for ENA-78/CXCL5, 7.5 pg/ml for IL-8/CXCL8, 3.9 pg/ml for MIG/CXCL9, 1.7 pg/ml for IP10/CXCL10 and 13.9 pg/ml for ITAC/CXCL11. Angiogenic and angiostatic chemokines were quantitated according to the protocols provided by the manufacturer. Although BAL procedures generally have dilution effect on the recovery of chemokines, a good correlation

was observed between the original values and standardized values by albumin concentrations in BALF (data not shown). Thus, the original values of chemokine levels were reported in this study, than the corrected values for albumin concentrations.

**Statistical analysis**

Results are expressed as mean ± SD, in normally and as median (min-max) in non-normally distributed variables. Pair student's t-test for normally or Wilcoxon signed ranks test for non-normally distributed was used to compare variables before and after treatment. A p-value of <0.05 was considered as statistically significant. Statistical analysis was carried out using SPSS 13.0 Chicago IL, USA.

**Results**

Patients' characteristics are shown in Table 1.

The levels of all three angiogenic chemokines (IL-8, ENA-78 and GRO-a) were found significantly decreased after 12 months of therapy with IFN gamma-1b treatment (median values in pg/ml, IL-8/CXCL8: 640 vs 81, $p < 0.05$, ENA-78/CXCL5: 191 vs 51, $p< 0.005$ and GRO-α: 1827 vs 710, $p<0.005$) (Table 2, Figure 1).

No significant differences were detected in the levels of the angiostatic chemokines IP-10/CXCL10 and ITAC/CXCL11 after IFN therapy (median values in pg/ml, IP-10/CXCL10: 56 vs 56.5, p=0.6, ITAC/CXCL11: 43 vs 47, p=0.11). (Table 2, Figure 1). However, a significant decrease in the angiostatic chemokine MIG/CXCL9: 66 vs 31, p=0.006, has been detected.

No significant differences were detected in the lung function tests (FVC % pred, TLC % pred and $TL_{CO}$ % pred) and partial pressure of oxygen ($P\alpha O_2$ , mm Hg) before and after treatment (Table 1).

The mean extent of the disease on HRCT was calculated as 34.2 ± 8.7 % at baseline. Change on HRCT was defined as a score < -0.5 or > + 0.5 indicating a minimal change status of marginal change in three sections. Seven patients improved, seven stabilized while six of them deteriorated after HRCT comparison at baseline and one year after IFN gamma-1b treatment.

No clinically significant differences between before and after IFN gamma-1b were seen in changes in total BAL cell count or changes in percentage of alveolar macrophages, lymphocytes, or neutrophils between baseline and 12 months (Table 3). No significant correlations were detected between chemokines and HRCT score or lung physiology, either before or after treatment.

**Discussion**

To the best of our knowledge this is the first study to investigate the local expression of the six cardinal CXC chemokines (GRO-a, ENA-78, IL-8, MIG, IP-10 and ITAC) in patients with IPF after IFN gamma-1b treatment. In addition, it is the second study in humans to characterize the effects of IFN gamma-1b on lung biomarkers after one year of treatment. Our major finding is the downregulation of the three angiogenic chemokines after 12 months treatment with IFN gamma-1b. On the contrary, we did not detect changes in the levels of angiostatic biomarkers IP-10/CXCL10 and ITAC/CXCL11 after IFN gamma-1b therapy in the BAL fluid. However, a significant decrease in the MIG/CXCL9 levels has been detected.

Studies directed on understanding the pathogenesis of IPF have primarily focused on mechanisms related to fibroplasia and deposition of extracellular matrix [31]. However, multiple disorders associated with fibroproliferative changes are also associated with aberrant vascular remodelling [32]. The contribution of aberrant neovascularization to the pathogenesis of fibrosis in IPF has only recently been appreciated [22-24]. An imbalance in the levels of angiogenic chemokines, as compared to angiostatic chemokines, that favors net angiogenesis has been demonstrated in both animal models and tissue specimens from patients with IPF [18-21]. Furthermore, a recent report suggests increased levels of VEGF, endothelin-1 and IL-8 in serum of patients with Idiopathic Interstitial Pneumonias [33]. Additionally, differential profiles of CXC chemokines in patients with IPF and non-specific interstitial pneumonia have recently been described [34]. In line with these findings, our group has recently demonstrated a distinct local and systemic angiogenic profile in patients with IPF compared to sarcoidosis patients [35]. These findings implicate angiogenesis in relation to Th2 and angiostasis in relation to Th1 immune response, suggesting that neovascularization is actively involved in the development of the fibrogenic process and is associated with detrimental prognosis and clinical course [36]. Therefore it is tempting to speculate that aberrant

angiogenesis is responsible for the switch of the immune response from Th1 to Th2 in these patients.

However, the current study has some limitations and our results should be considered with caution. First, the small number of IPF patients. Secondly, quantifying protein in BALF is notoriously difficult and the lavage procedure may lead to influx of proteins from blood that falsely elevates their concentrations in the fluid. On the other hand, BALF does not include the sampling error of transbronchial lung biopsies [11, 12]. The above technical issues maybe could explain the absence of correlation between conventional measurements of disease activity and angiogenic and/or angiostatic modulators. Moreover, as we did not detect any significant change in lung physiology, in agreement with previous reports [8, 9, 12], our results on the angiogenetic profile can only be speculative and require further research. However, since there is no good animal model for pulmonary fibrosis, descriptive data obtained from well characterized human subjects may serve to generate mechanistic hypotheses.

The downregulation of the angiogenic profile in IPF patients by IFN gamma-1b treatment and the unchanged levels of IP-10/CXCL10 and ITAC/CXCL11 angiostatic mediators after therapy, are partially in agreement with Strieter's report regarding the effects of IFN gamma-1b on biomarker expression in IPF patients [12]. The aforementioned report detected a significant increase in ITAC/CLCL11 levels in both lung tissue and BALF after a six month period therapy with IFN gamma-1b in 17 IPF patients [12], suggesting that this is the cardinal agent through which IFN gamma-1b exerts its beneficial effect on survival. However, this finding should be considered with caution for the following two reasons. Firstly, the INSPIRE trial designed to evaluate the safety and efficacy of IFN gamma-1b in IPF patients with mild to moderate impairment in lung function, clearly showed no benefit on survival[37]. Secondly, the anti-angiogenic effect of CXCL11/ITAC could be damaging, which is consistent with Kao and colleagues[38] suggested that CXCL11/ITAC was involved in heart transplant rejection. Nevertheless, they did not demonstrate the same result in the other two CXCR3 ligands, MIG and IP-10 [12], and this is in agreement with our data. We also found a down regulation of MIG levels after treatment. The finding that IFN-γ therapy did not demonstrate induction the three CXCR3 ligands, CXCL11, CXCL9 or CXCL10 suggests that the potential defect in IPF may not be directly related to IFN-γ, but is rather related to the failure

to fully mount an appropriate CXCR3/CXCR3 ligand response to injury. Additionally, a decrease in ENA-78 levels was reported [12] in BALF of IPF patients treated with IFN gamma-1b, in agreement with our findings.

Novel data support the notion that IPF could be an immunologically driven disorder [39]. A failure to mount an appropriate Th1 response shifts the polarization of the immune response from type 1 toward type 2 humoral –mediated immunity [39]. IFN gamma is the most pivotal type 1 cytokine and it has the known ability to promote both Th1 response and concomitant attenuation to fibrosis [40]. It has been recently shown that CXCR3 ligands (MIG, IP-10 and ITAC) are critical in recruiting Th1 cells and further amplifying the expression of endogenous IFN gamma [39]. Taken all the above together, our data implicate angiogenesis in the fibrotic (Th2) pathway in IPF and provide some evidence in the underlying mechanism of action of IFN gamma-1b in this disorder.

In conclusion, our data obtained from well characterized human subjects support the hypothesis that IFN gamma does not have benefit in the treatment of IPF, as does not have any induction of angiostatic CXCR3 ligands BALF levels. Further research is needed in order to verify these data.

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Table 1 Demographic and spirometric characteristics of patients with idiopathic pulmonary fibrosis (IPF) Values are expressed as mean ± SD, and age as median (range).

| **Characteristics** | **IPF patients (before treatment)** | **IPF patients (after treatment)** |
|---|---|---|
| Number | 20 | 20 |
| Sex: Male/Female | 14/6 | - |
| Age, median (yr) | 68(40-75) | - |
| Smokers/non smokers | 16/4 | - |
| FVC, (% pred) | 77.3± 13.0 | 83.8±10.1$^{+}$ |
| TLC,( % pred) | 67.4±14.2 | 67.6 ± 13.5$^{+}$ |
| $TL_{CO,}$ (% pred) | 60.3±17.8 | 58.6 ± 17.9$^{+}$ |
| $P\alpha O_{2,}$ (mmHg) | 80.3±10.0 | 74.8± 8.2$^{+}$ |

Abbreviations: FVC: Forced Vital Capacity, TLC: Total Lung Capacity, $TL_{CO}$: Diffusing Capacity for Carbon Monoxide, $P\alpha O_2$: Arterial Partial Pressure of Oxygen

$^{+}$ No statistical difference before/after treatment

Table 2 Median values (pg/ml) and range in parenthesis of angiogenic (GRO-a/CXCL1, ENA-78/CXCL5, IL-8/CXCL8) and angiostatic (CXCL9/MIG, CXCL10/IP10 and CXCL11/ITAC) chemokines in BALF in IPF patients.

| | **IPF patients before-treatment** | **IPF patients after -treatment** | **p value IPF before vs after treatment** |
|---|---|---|---|
| GRO-a/CXCL1 | 1827(28-1827) | 710(376-1500) | 0.003 |
| ENA-78/CXCL5 | 191(46-644) | 51(34-112) | 0. 0001 |
| IL-8/CXCL8 | 640 (86-20000) | 81(24-661) | <0.05 |
| MIG/CXCL9 | 66(29-1141) | 31(28-46) | 0.006 |
| IP10/CXCL10 | 56(31-235) | 56.5(31-290) | NS |
| ITAC/CXCL11 | 43(43-224) | 47(47-89) | NS |

Table 3 Mean ± SD of total and differential cell counts in BALF in 20 IPF patients (and change from baseline at 12 months) .

| CELL TYPE | IPF patients- Before treatment | IPF patients- After treatment | **p value**<br><br>**IPF before vs after treatment** |
|---|---|---|---|
| TCCx$10^5$ /ml | 28.6 ± 3.2 | 26.6 ± 2.3 | NS |
| Macrophages% | 82.2 ± 4.3 | 83.1 ± 3.8 | NS |
| Neutrophils % | 6.23 ± 2.2 | 5.2 ± 1.8 | NS |
| Lymphocytes% | 7.7 ± 3.0 | 6.5 ± 2.5 | NS |
| Eosinophils % | 3.7 ± 2.4 | 2.8 ± 2.4 | NS |

TCC = Total cell counts

## Legends for figures

### Figure 1

Bronchoalveolar lavage fluid angiogenic and angiostaic chemokines' levels (pg/ml) in IPF patients, before and after treatment with IFN gamma-1b.