Endogenous blood maximal interferon-γ production may predict response to interferon-γ 1β treatment in patients with idiopathic pulmonary fibrosis

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Abstract. Background: Idiopathic pulmonary fibrosis (IPF) is an untreatable lung disorder with a mean survival of 3 years after diagnosis. Treatment with interferon-gamma (IFN-γ) 1β has been reported to significantly improve lung function and arterial oxygen saturation in a first randomized controlled trial; unexpectedly, these findings have not been confirmed in a subsequent large placebo-controlled randomized study. Another larger placebo-controlled randomized trial has been stopped because data analyzed at interim analysis excluded the possibility that treatment with IFN-γ 1β would cause a significant reduction in the risk of death. Methods: Seven Italian male patients diagnosed with IPF were treated with IFN-γ 1β (200 µg/die subcutaneously three times a week), accordingly to the indications of the Italian Drug Agency. Based on available studies the response to treatment was pre-defined as changes in either lung function (FVC and DLCO) or oxygen arterial saturation. All patients consented to provide a peripheral blood sample for endogenous IFN-γ production measurement with the ELISpot assay before treatment and 6 months thereafter. Results: Four of 7 patients improved or stabilized their lung function after 6 months treatment. Using the ELISpot assay to quantify the maximal production of endogenous IFN-γ on peripheral blood samples, these 4 patients had a significantly higher endogenous IFN-γ production before therapy, as compared to the 3 patients who deteriorated (91.3±49.6 vs 277.8±34.2 spot forming cells, p=0.023). No significant differences were observed after 6 months of treatment. Discussion: These preliminary results suggest that some IPF patients might benefit from treatment with IFN-γ 1β and may help to interpret the results of large randomized trials, suggesting that individual susceptibility could determine clinical response to treatment. (Sarcoidosis Vascul Dis Diffuse Lung Dis 2009; 26: 64-68)

Key words: IPF, therapy, prognosis, ELISpot

Introduction

Idiopathic pulmonary fibrosis (IPF) is a severe and progressive disease limited to the lung with an estimated prevalence ranging from 14.0 to 42.7 per 100,000 persons in the United States (1); it is characterized by insidious onset of symptoms and by a median survival of less than 3 years after diagnosis (2, 3). IPF etiology is unknown, and no therapy has been shown to affect survival to date (4), although some patients may respond to steroids and immuno-
suppressors (3). One randomized trial on 18 IPF patients treated for 12 months with subcutaneous interferon-gamma (IFN-γ) 1β and low dose prednisone generated great enthusiasm; this study showed a statistically significant improvement of lung function and arterial oxygen in all the 9 patients treated with IFN-γ and prednisone (5). Based on these encouraging preliminary findings, patients with a compatible clinical and radiological presentation and with a surgical lung biopsy showing a definite usual interstitial pneumonia (UIP) pattern can be treated in Italy with subcutaneous IFN-γ 1β and the cost of the drug is reimbursed by the Italian National Health Service. The study by Ziesche and co-workers also prompted the largest IPF trial ever performed on 330 patients treated with either IFN-γ or placebo: unexpectedly, this study failed to demonstrate a significant effect on progression-free survival, although a statistical trend toward lower mortality was observed in the treated group, as compared with the placebo arm (6). Finally, a recent meta-analysis of three studies pooling together 198 IPF patients indicated that IFN-γ may improve survival overall (7). A post-hoc sub-group analysis of the Raghu study showed evidence for an effect of IFN-γ in patients with less advanced disease, as measured by a forced vital capacity (FVC) above 55% of predicted values and carbon monoxide transfer (DLCO) above 35%. A differential effect of IFN-γ in IPF patients at different stages of diseases has been therefore postulated, although other factors, such as genetic susceptibility, might account for individual response to treatment. Based on these analyses, a randomized controlled trial in over 800 patients with mild to moderate IPF treated with IFN-γ 1β or placebo (the INSPIRE trial) has been stopped because data analyzed during a pre-defined interim analysis excluded the possibility that treatment with IFN-γ 1β might result in a significant reduction of the risk of death. On the other hand, IFN-γ therapy has been reported to have severe side effects and it has been suggested that it may in fact worsen the disease course in patients with lower FVC values and possibly in those with a familial form of the disease (8, 9).

To explore the reasons of these contrasting results, it might be relevant to note that in the Ziesche study all patients were assessed for the level of expression of different inflammatory cytokines in lung tissue samples before entering the study; in addition to a significant down-regulation of the expression of both transforming growth factor (TGF)-β1 and connective-tissue growth factor (CTGF) after IFN-γ therapy, all patients had virtually no transcription signal for IFN-γ in lung biopsies before treatment (5). A subsequent randomized trial on 32 IPF patients confirmed that IFN-γ therapy may modulate at the protein level the lung expression of several molecules related to fibrosis, angiogenesis, immunomodulation and cellular proliferation (10), but no data were available on the lung cytokine profile before IFN-γ therapy.

We applied the sensitive enzyme-linked immunospot (ELISpot) assay (11) for single cell IFN-γ secretion to detect the frequencies of IFN-γ-producing T cells freshly isolated from peripheral blood samples in a group of patients with biopsy-proven IPF treated in an open-label fashion with IFN-γ 1β. The ELISpot assay detects secreted cytokine molecules in the immediate vicinity of the cell from which they are derived, while still at a high concentration; each spot in the read-out represents a ‘footprint’ of the original cytokine-producing cell, or spot forming cell (SFC). We hypothesized that in IPF intrinsic capacity to produce IFN-γ may influence the clinical response to exogenous IFN-γ 1β therapy.

**Methods**

Seven Italian male patients (2 were brothers) diagnosed with IPF based on current diagnostic standards (12) and all with evidence of an UIP pattern on surgical lung biopsy were treated with IFN-γ 1β (Imukin, Boehringer Ingelheim, Germany) 200 µg/die subcutaneously three times a week, accordingly to the indications of the Italian Drug Agency. All patients had had already a course of at least 6 months of prednisone and azathioprine without improvement of lung function and were on low dose prednisone (maximum 12.5 mg per day) at the time of IFN-γ treatment start. Based on available studies (5, 6) the response to treatment was pre-defined as changes in either lung function (FVC and DLCO) or oxygen arterial saturation. All patients consented to provide a peripheral blood sample for endogenous IFN-γ production measurement with the ELISpot assay before IFN-γ treatment and 6 months there-
Peripheral blood mononuclear cells (PBMC) were separated from 15 mL of blood by standard means and were suspended in RPMI with 10% foetal calf serum, 1% ampicillin and 0.05% gentamicin (R10, Sigma Aldrich, St. Louis MO) at the final concentration of 2.5x10^6 cells/ml. Non-specific binding was blocked by incubation with R10 at 37°C, 5% CO₂ for at least 30 minutes. After rinsing the blocking solution, 250,000 PBMC were added to each well and incubated overnight at 37°C, 5% CO₂ with medium alone and in presence of 10 µg/ml of phytohemagglutinin (PHA, Sigma Aldrich) or streptokinase-streptodornase (SKSD, Wyeth Farma SA, Madrid, Spain). The next day, the plate was washed with phosphate-buffered saline (PBS) and biotin-conjugated anti-IFN-γ (1-D1K, Mabtech AB, Nacka, Sweden) was added to each well for 2 hours at room temperature. After washing, plate was incubated for 10 minutes at room temperature with chromogen (BCIP/NBT Plus, Mabtech). When spots became visible, a final rising was performed using tap water and SFCs were counted with an automated ELISpot reader (AID Systems, Strassberg, Germany). Results are reported as mean values ± standard deviation; the two-sample t test was used to assess differences between groups and significance was set at p values <0.05.

**Results**

All patients complained about one or more of the following symptoms during IFN-γ treatment: low grade fever, headache, chills, myalgia, fatigue, nausea, arthralgia and injection site tenderness. The effects of 6 months of IFN-γ treatment (i.e. the period with data available for all patients, since 2 of them died soon after this time-point) are reported in the Table 1. Three of the 7 patients deteriorated, based on the reduction of the FVC (-16.7±2.5%), DLCO (-23.0±1.0%) and arterial oxygen (PaO₂, -23.0±7.9%) values; also respiratory symptoms were reported to be worsened during treatment in these 3 patients, with an increase in dry cough and dyspnoea on exertion. However, the 4 other patients showed an improvement or little/no change of lung function and respiratory symptoms after 6 months of IFN-γ treatment (FVC +5.3±5.3%; DLCO +9.3±8.5%; PaO₂ +5.0±1.0%). The results of the ELISpot assay showed a very low IFN-γ production in basal condition, i.e. without any stimulation (Table 1). Regarding maximal systemic endogenous production of IFN-γ using the PHA as a non-specific T cell stimulator before initiation of therapy, we observed significantly lower levels in patients who deteriorated (91.3±49.6 SFC vs 277.8±34.2 SFC in patients stabilized or improved, p=0.023). No significant difference were observed comparing endogenous IFN-γ production in response to PHA at baseline (i.e. before starting treatment) and after 6 months of IFN-γ therapy, both in the “responder” (277.8±34.2 SFC vs 182.0±98.1, p=0.11) and “non-responder” (91.3±49.6 SFC vs 94.3±86.0, p=0.344) groups. Similarly, the results of endogenous IFN-γ production as measured by ELISpot in response to the bacterial protein SKSD showed a lower level among patients with poor outcome, although the difference with patients improved or stabilized did not reach statistical significance (1.7±1.2 SFC vs 66.3±38.8 SFC, p=0.218). Comparing IFN-γ production as measured by ELISpot in response to the bacterial protein SKSD before starting treatment and after 6 months of IFN-γ therapy both in the “responder” (66.3±38.8 vs 44.0±47.3 SFC p=0.74) and “non responder” (1.7±1.2 vs 1.0±1.0

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*At the time of IPF diagnosis
‡Brothers

Table 1. ELISpot responses in IPF patients before IFN-γ treatment and after 6 months of therapy
SFC $p=0.912$) groups no statistical significant differences were observed.

**Discussion**

Despite the recent increase in the number of randomized controlled trials in IPF, this deadly disease is still untreatable. Until now, subcutaneous IFN-$\gamma$ 1$\beta$ is the therapeutic option that has been used in the largest group of patients; nonetheless, its efficacy remains controversial, although a differential effect in specific subgroups of patients has been postulated. We report here that in a group of clinically homogeneous, biopsy-proven IPF patients treated with IFN-$\gamma$ 1$\beta$ for a period of 6 months, about half of them did show some benefit based on functional parameters; this does not necessarily imply that a significant fraction of IPF patients benefit from IFN-$\gamma$ treatment. However, these findings from an uncontrolled small study suggest that some IPF patients may have at least a short-term benefit from therapy with IFN-$\gamma$ 1$\beta$. These results lend further support to the effect of a “susceptibility factor” in the response to IFN-$\gamma$ treatment in IPF, suggesting that the results of large randomized trials may have been influenced by the coexistence of “responder” and “non-responder” patients. In the small group of patients reported here, those who either stabilized or improved after 6 months of treatment had significantly more blood IFN-$\gamma$-producing T cells after maximal stimulation, as measured by mean of the sensitive ELISpot assay, immediately before starting therapy, but not after 6 months of treatment. Since IPF is a disease of unknown origin, we decided to perform both a non antigen-specific (PHA) and an antigen-specific (SKSD) PBMC stimulation. However, maximal PHA stimulation is likely to represent more accurately the effective cellular capacity (largely innate) of producing IFN-$\gamma$ in vitro and the discrepancy between PHA and SKSD data might be well explained by the different nature of these two antigens.

Although with the limitations of the small sample size, these findings provide a further step to interpret the discordant results obtained in different IPF clinical trials with IFN-$\gamma$. Since the measurement of systemic IFN-$\gamma$ production has not been included in any of these studies, it’s impossible to assess retrospectively the role of this single factor on the response to treatment. Interestingly however, patients included in the original study on IFN-$\gamma$ treatment did not show any expression of IFN-$\gamma$ in lung samples at enrolment (5) and all improved after 12 months treatment. However, any comparison between the latter and the present study is elusive, since the lack of IFN-$\gamma$ expression in the Ziesche study has been observed at the lung tissue level; in contrast, we observed a positive clinical response to IFN-$\gamma$ 1$\beta$ treatment by measuring IFN-$\gamma$ production (both in basal condition and after stimulation with PHA and SKSD) in peripheral blood. On the other hand, the finding of a low endogenous IFN-$\gamma$ production in IPF patients which do not benefit from administration of exogenous IFN-$\gamma$ 1$\beta$ is somewhat counterintuitive. However, this finding might be explained in the context of the so-called “paradoxical” function of IFN-$\gamma$ (13): in addition to its role in establishing a Th1-type immune reaction, IFN-$\gamma$ released by regulatory T cells can indirectly prevent further T cell activation by modulating the function of antigen-presenting cells. Since circulating IFN-$\gamma$ levels are not easily detectable in vivo, most conclusions on the presence or the absence of this cytokine in disease states rely on data obtained from the stimulation of T cells in vitro (14). However, some data support the hypothesis that patients presenting with a high degree of systemic immune activation in vivo show a severely diminished functional response of their peripheral blood mononuclear cells after in vitro stimulation; as an example, HIV infected patients produce low levels of interleukin-2 and IFN-$\gamma$ after stimulation with soluble antigens or mitogens (15, 16). Therefore, the inability of cells to produce IFN-$\gamma$ following stimulation in vitro may be seen as a consequence of the overwhelming production of the cytokine in vivo. In this context, as in other disorders, “footprints” of IFN-$\gamma$ activity, like neopterin production (17) and tryptophan degradation (18) might have a role as surrogate markers for detecting IFN-$\gamma$ production in vivo in IPF patients and to monitor response to therapy. Interestingly, it has been reported that about one third of IPF patients have elevated serum neopterin levels (19).

The main limitation of our study resides in the small population, and for this reason a definite evidence for intrinsic IFN-$\gamma$ production as a prognostic factor for response to treatment in IPF is still lack-
ing. However, as for any rare disease, large randomized studies (unless adequately supported and coordinated by industry) are problematic (20). Our results reflect common clinical practice in the treatment of a rare disease like IPF, showing that some patients may benefit from treatment with IFN-γ 1β and that an already available immunologic test might help to identify these individuals.

References