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Transcription Factor and Cytokine Profiles in Peripheral Blood T Helper Cells in Patients with Idiopathic Pulmonary Fibrosis

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a severe lung disease with a poor prognosis, characterized by immune cell activation. The role of T helper (Th) cell transcription factors in IPF pathogenesis remains unclear. In this study, we investigated Th cell transcription factors and related cytokines in IPF patients.

Twelve IPF patients and eight healthy controls (HC) were enrolled in this pilot study. Serum levels of fibrosis-associated mediators (Interferon-inducible protein 10 (IP-10), tumor necrosis factor-α (TNF-α), tumor growth factor-β (TGF-β), CXCL-8, interferon-γ (IFN-γ)) were measured by enzyme-linked immunosorbent assay (ELISA). Flow cytometry assessed Th transcription factors T box transcription factor (*T-bet*,), GATA-binding protein 3 (*GATA-3)*, Retinoic acid-related orphan recepto (*ROR-γt)*, forkhead box P3 (*FOXP3*)) and intracellular cytokines (IL-4, IL-17).

SerumTGF-β, CXCL-8, TNF-α, and IFN-γ were significantly elevated, while IP-10 (*þT-bet*, *GATA3*, *ROR-γt*, or *FOXP3* were observed. Positive correlations were found between *T-bet* and *GATA3*, IL-4, *ROR-γt*, and TNF-α expression with age, while *FOXP3* expression negatively correlated with age.

T-cell transcription factors were unchanged in IPF despite changes in inflammatory protein expression. Reduced IP-10 may serve as a potential marker.

Keywords: Cytokines; *FOXP3*; *GATA-3*; IPF; *ROR-γt*, *T-bet*

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INTRODUCTION

The family of interstitial lung diseases (ILDs) is characterized by cellular proliferation, interstitial

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inflammation, fibrosis, or a combination of these, within the alveolar wall, without links to infection or cancer. In most cases, interstitial fibrosis is the predominant phenotype. Idiopathic pulmonary fibrosis (IPF) is a severe, age-related lung disease of unknown cause, with a 50% mortality rate within 3–5 years of diagnosis.[1,2] While previously considered a chronic inflammatory process, current evidence suggests that IPF is driven by abnormally activated alveolar epithelial cells (AECs).[1] The disease is associated with chronic, progressive symptoms such as cough, dyspnea, and impaired quality of life, occurring more frequently in older male individuals.[3] Immune dysregulation in IPF may exacerbate the interplay between pro-fibrotic pathways, contributing to disease progression.[4]

T helper 1 (Th1) and Th2 cells derive from common T-precursor cells through the activity of distinct transcription factors, including *GATA-3* and *T-bet*, which are crucial for Th1/Th2 differentiation.[5] T-cells are widely present in active disease regions of the lungs and contribute to the development of pulmonary fibrosis.[6] An imbalanced Th1/Th2 immune response has been reported as playing a central role in the pathogenesis of IPF.[7] Type 2 (T2) cytokines, such as IL-4 and IL-13, promote pro-fibrotic responses, whereas Th1 cytokines, including IFN-γ and IL-12, may have protective roles in IPF pathogenesis.[8–11] Other T cell subtypes are also likely to play a role in IPF, including Th17 cells, Th9 cells, regulatory T cells (Tregs), T follicular helper cells (Tfhs), cytotoxic T lymphocytes (CTLs), natural killer T cells (NKTs), and γδ-T cells.[12]

T-bet and *GATA-3* regulate the plurality of T-cell cytokines by mediating Th1/Th2 differentiation. *T-bet* specifies Th1 lineage commitment by inducing lineage-restricted genes such as IFN-γ and IL-12 receptor β2 genes. In contrast, *GATA-3* specifies Th2 lineage commitment by inducing IL-4, IL-13, and IL-5 expression.[13] Activation of *T-bet* or inactivation of *GATA-3* has an advantage over the targeting of single T-cell cytokines in therapeutic strategies against airway remodeling.[14,15]

T-bet expression is strongly correlated with IFN-γ expression and is specifically up-regulated in the Th1 pathway.[16] In comparison, *GATA-3* is induced downstream of STAT6 activation following IL-4 binding to its receptor.[17] *GATA-3* regulates Th2 cytokine expression not only at the transcription level by binding to the IL-5 and IL-13 gene promoters but also remodels the chromatin structure at the T2 locus.[17] A deficiency in *T-bet* expression in $CD4^+$ T cells increases the susceptibility to bleomycin (BLM)-induced lung fibrosis in BALB/c mice, which are usually relatively resistant to BLM.[10] Although Th1-type immune responses are involved in the normal scar healing process, a clinical trial of IFN-γ1b was not efficacious in IPF.[18]

Several groups have evaluated the production of cytokines and inflammatory mediators in the lungs of IPF patients.[19–21] IL-17 has important pro-inflammatory and pro-fibrotic effects.[22] For example, an anti-IL-17 antibody attenuated the ability of 2,4,6-trinitrobenzene sulfonic acid (TNBS) to induce colorectal fibrosis in mice.[23,24] Cytokines such as IL-2, IL-8, IL-10, and tumor growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and tumor necrosis factor-α (TNF-α) have also been shown to be increased in the serum of IPF patients with a concomitant reduction in IFN-γ levels.[19] Furthermore, high levels of IL-6 and IL-8 were reported in early stages of acute exacerbations of IPF (IPF-AEs), and levels were associated with worse disease outcomes.[20]

We hypothesized that the expression of Th2 and Th1 transcription factors in peripheral blood, together with analysis of downstream cytokines, would provide insight into the key cytokines and mechanisms involved in IPF pathophysiology. To this end, this pilot study examined the intracellular expression of *GATA-3* and *T-bet*, IL-4, IL-17, and serum levels of cytokines CXCL-8, IP-10, IFN-γ, TNF-α, and TGF-β in IPF patients.

MATERIALS AND METHODS

Patients

Twelve IPF patients, including 2 outpatients and 10 inpatients, were enrolled in the study at Masih Daneshvari Hospital of Shahid Beheshti Medical University (Tehran-Iran) between 10th October–9th December 2022. Eight healthy control (HC) subjects were also included in the study. All patients had clinical symptoms consistent with the diagnosis of IPF as diagnosed following a clinical and physical examination by a trained pulmonologist.

Data Collection

The clinical records of patients were reviewed by the research team in the Pulmonology Department of Masih Daneshvari Hospital, Shahid Beheshti University, Tehran, Iran. Data were extracted from electronic medical records and included demographic details, medical history, underlying comorbidities, symptoms,

clinical signs, laboratory results, chest computed tomography (CT) findings, and treatment measures.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Three mL of whole blood was collected in tubes containing ethylene-diamine-tetra-acetic acid (EDTA) for plasma, or another 3 mL of whole blood was collected in clotting tubes for serum after obtaining informed consent (IR.SBMU.MSP.REC.1398.568). All participants were informed about the study's objectives and procedures, assured of data confidentiality, and provided written consent. For minors, consent was obtained from legal guardians. Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation with Lymphoprep (density: 1.076 g/mL; Ficoll-Paques, Histosep, Histocompatibility Biotechnology Ltd., Tehran, Iran) as previously described.[25] Whole blood hematology tests including a complete blood count (CBC) and erythrocyte sedimentation rate (ESR) were performed. Serum samples were obtained by centrifugation at 1200*g* for 5 minutes and stored at −80°C until analysed. Biochemical tests, including creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) levels, were performed.

Flow Cytometry Analysis

The antibodies used for surface and intracellular staining are listed in Table 1. For surface staining, cells were suspended in FACS buffer (1× PBS, 0.01% sodium azide, 1% BSA) and incubated with antibodies (CD3-APC, CD4-FITC, CD25-PE) for 30 minutes at 4°C. After surface staining, cells were fixed and permeabilized using a kit (BD Biosciences, San Diego, USA) for 15 minutes at 4°C. Intracellular staining was then performed by incubating the cells with antibodies (T-bet-PE, GATA3-APC, ROR-γt-PE, FOXP3-APC, IL-4-PE, IL-17-Cy5-PE) for 30 minutes at 4°C. After staining, cells were washed with cold PBS, and 10 000 events were acquired for analysis using FlowJo Software (version 10, BD Biosciences).

Table 1. The Antibodies Used for Extracellular and Intracellular Staining

Surface Abs	mAB	Clone	Manufacture
	CD4-FITC	Tü36	BioLegend, San Diego, USA
	CD3-APC	94	Beckman coulter, Brea, California, USAS
	CD25-PE	Mφp9	BD Biosciences, USA
Intracellular Abs	**mAB**	**Clone**	**Manufacture**
	T-bet-PE	H198	eBioscience, San Diego, USA
	GATA3-APC	RPA-T4	Immunostep, Salamanca, Spain
	ROR-γt-PE	SK1	BioLegend, San Diego, USA
	FOXP3-APC	PC61.5	eBioscience, San Diego, USA
	IL-4-PE	8D4-8	eBioscience, San Diego, USA
	IL-17-PE-Cy5		eBioscience, San Diego, USA

Statistical Analysis

Data analysis was performed using GraphPad Prism software (version 8, GraphPad Software, Inc.). The mean (± SEM) values of cytokine levels were calculated to describe an estimate of the precision of the mean, which is more appropriate for highlighting differences between groups in this context. The unpaired t-test was used for parametric variables. Pearson's correlation coefficient was used to assess correlations through linear regression.

RESULTS

Demographic and laboratory characteristics of participants

The mean age of IPF patients significantly differed from HC (p=0.026). The rates of ESR (mean ± SEM, 20.56±2.45 mm/hr), LDH (431± 31.15 U/L), and CPK (28.5±4.33 U/L) were measured in IPF patients but were not available for the HC group (Table 2).

Table 2. Demographic and Clinical Characteristics of Participants

	Patients (n=12)	Healthy control (n=8)	*p* value
Age (mean ± SEM)	68.83 ± 2.36	55.63 ± 5.79	0.0269
Female, n (mean ± SEM)	4 (76.75 ± 2.68)	5 (64.4 ± 5.19)	0.0926
Male, n (mean ± SEM)	8 (64.88 ± 2.14)	3 (41 ± 7.2)	0.0016
LDH (U/L)	431 ± 121.8 (n=12)	NA	NA
ESR (mm/hr)	20.56 ± 7.37 (n=9)	NA	NA
CPK (U/L)	28.5 ± 12.25 (n=8)	NA	NA
WBC count (cell × 10^3/μL)	6.15 ± 1.88 (n=12)	NA	NA

Values were presented as mean ± SEM. ESR: erythrocyte sedimentation Rate; LDH: lactate dehydrogenase; CPK: creatine phosphokinase; NA: not available; WBC: White blood cells

Expression of T-bet, GATA3, ROR-γt, FOXP3, IL-4 and IL-17 by PBMCs

Representative FACs plots showing the gating strategy for the expression of $CD3^+CD4^+$T-bet^+ (Figure 1A), $CD3^+CD4^+$ROR-γt^+IL-17^+ (Figure 1B), $CD4^+GATA3^+$IL-4^+ (Figure 1C), and $CD4^+CD25^+FOXP3^+$ (Figure 1D) cells in PBMCs of IPF patients are shown in Figure 1. There was no significant difference in the expression of *T-bet* (18.11 ± 2.2% vs. 16.45 ± 2.8%, Figure 2A) or of *GATA3* (11.53 ± 1.83% vs. 9.84 ± 3.35%, Figure 2B) in IPF patients compared to healthy control subjects (Table 3). Expression of intracellular IL-4 in $CD4^+GATA3^+$ T helper cells was 21.9 ± 1.96% in IPF patients and 18.88 ± 1.48% in HC (p=0.26) (Table 3 and Figure 2C). The expression of *ROR-γt* in the $CD3^+CD4^+$ population was similar in IPF patients (13.18 ± 1.2%) compared with HC (10.84 ± 1.13%, Table 3 and Figure 2D). There was a similar level of *FOXP3* expression in IPF patients compared with HC (20.69 ± 2.52% vs 15.44 ± 0.6%, Table 3 and Figure 2E). In contrast, there was a significantly greater expression of intracellular IL-17 in $CD3^+CD4^+$ROR-γt^+ T helper cells in IPF patients (4.63 ± 0.16%) compared with HC (3.54 ± 0.2%, p=0.0011, Table 3 and Figure 2F). The expression of *GATA3* was significantly lower than that of *FOXP3* in IPF patients (p=0.012) (Table 3 and Figure 2G).

Serum Cytokine Levels

The levels of TNF-α (p=0.0312, Figure 3A), CXCL-8 (p=0.0005, Figure 3B), IFN-γ (p=0.0313, Figure 3C) and TGF-β (p=0.001, Figure 3D) in the serum were significantly higher in IPF patients compared to HC (Table 4). Conversely, the serum levels of IP-10 were significantly lower in IPF patients than in HC subjects (*p*).

Correlations between Transcription Factor Expression and Cytokines

We investigated the correlation between serum levels of TNF-α, TGF-β, CXCL-8, IP-10, and IFN-γ with the transcription factors involved in T helper cell differentiation (Table 5). The expression of *T-bet* positively correlated with *GATA-3* (p=0.006, r=0.738) in IPF patients (Figure 4A and Table 5). Additionally, the expression of *ROR-γt* positively correlated with the level of intracellular expression of IL-4 (p=0.045, r=0.59, Figure 4B and Table 5). The expression of *FOXP3* was negatively correlated with age (p=0.0017, r=-0.8) (Figure 4C and Table 5). Lastly, the serum levels of TNF-α positively correlated with age (p=0.0073, r=0.75, Figure 4D and Table 5).

Figure 1. Gating strategy for identification of T helper cells transcription factors; Representative flow cytometric dot plots showing expression of (A) T-bet on CD3+CD4+ T-cells in a sample from a patient with an IPF. (B) Representative flow cytometric dot plots showing expression of ROR-γt on CD3+CD4+ T-cells in a sample from a patient with an IPF. (C) Representative flow cytometric dot plots showing expression of GATA3 and IL-4 on CD4+ T-cells in a sample from a patient with an IPF. (D) Representative flow cytometric dot plots showing expression of FOXP3 on CD4+CD25+ T-cells in a sample from a patient with an IPF.

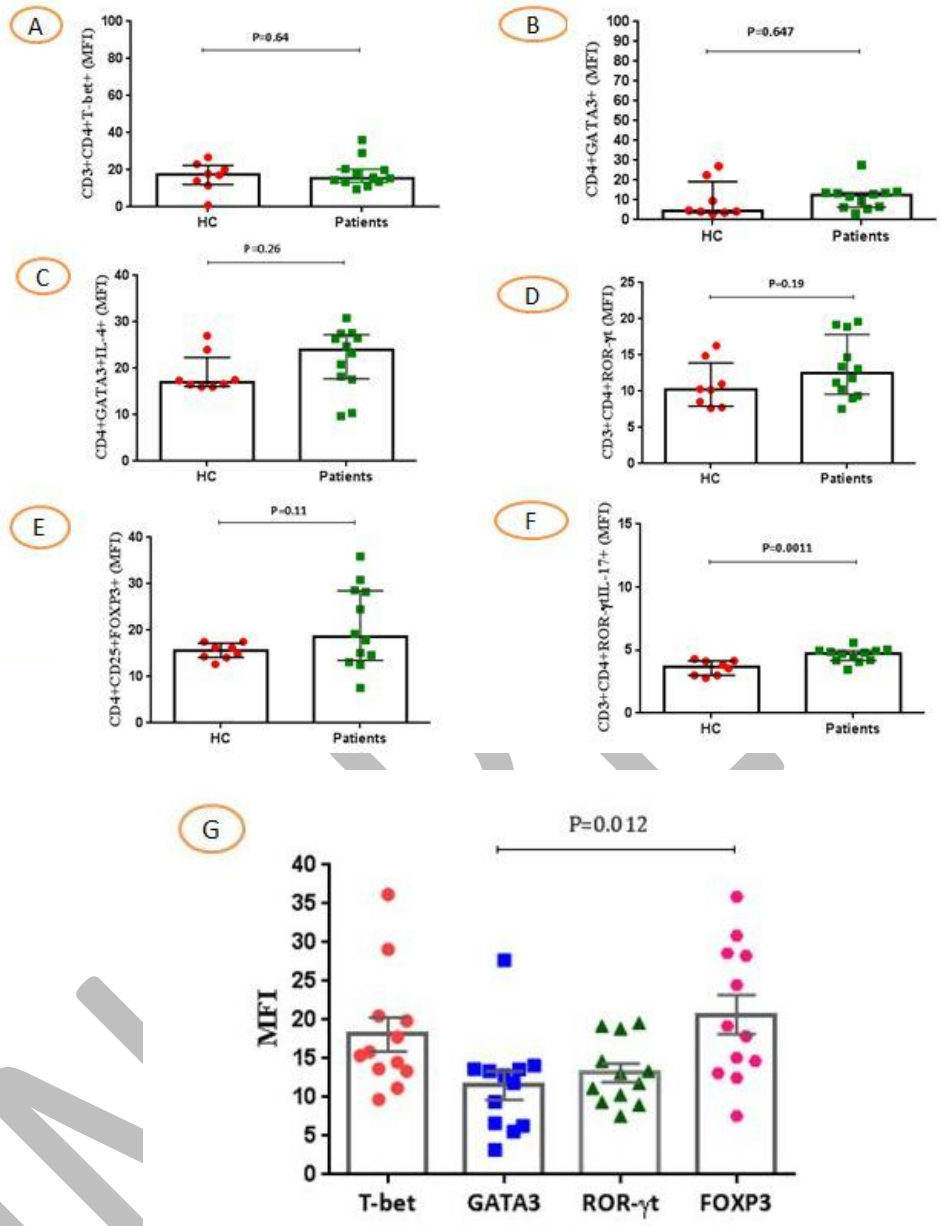

Figure 2. Graphical analysis of T helper cells transcription factors expression. A. The expression of *T-bet* on $CD3^+CD4^+$ T-cells in IPF patients (n=12) and healthy subjects (n=8). B. The expression of *GATA3* on $CD4^+$ T-cells in IPF patients (n=12) and healthy subjects (n=8). C. The expression of IL-4 on $CD4^+GATA3^+$ T-cells in IPF patients (n=12) and healthy subjects (n=8). D. The expression of *ROR-γt* on $CD3^+CD4^+$ T-cells in IPF patients (n=12) and healthy subjects (n=8). E. The expression of *FOXP3* on $CD4^+CD25^+$ T-cells in IPF patients (n=12) and healthy subjects (n=8). F. The expression of IL-17 on $CD4^+$ROR-γt^+ T-cells in IPF patients (n=12) and healthy subjects (n=8). G. The comparison of transcription factors expression in IPF patients. All values are presented as the mean ± SEM and comparisons made between IPF and HC group were performed using the Student's t-test. * *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.*

Table 3. The Expression of Transcription Factors and Cytokines in IPF Patients and Controls

	Patients (n=12)	Healthy control (n=8)	*p*
T-bet (MFI)	18.11 ± 2.2	16.45 ± 2.79	0.6446
Gata3 (MFI)	11.53 ± 1.83	9.84 ± 3.36	0.6375
ROR-γt (MFI)	13.18 ± 1.2	10.84 ± 1.13	0.1948
FOXP3 (MFI)	20.69 ± 2.52	15.44 ± 0.6	0.1142
IL-4 (MFI)	21.99 ± 1.96	18.88 ± 1.49	0.2643
IL-17 (MFI)	4.613 ± 0.16	3.59 ± 0.2	0.0011
		p	
T-bet vs. *Gata3**		0.1085	
T-bet vs. *ROR-γt**		0.3159	
T-bet vs. *FOXP3**		0.7991	
Gata3 vs. *ROR-γt**		0.937	
Gata3 vs. *FOXP3**		0.012	
ROR-γt vs. *FOXP3**		0.0523	

Comparisons of the mean fluorescent intensity (MFI) values between the IPF patient and healthy control groups was performed using a parametric Student's t-test. Values were reported as mean ± SEM. Comparisons of MFI for differential expression of transcription factors across groups was performed using a one-way ANOVA test and post hoc Tukey's multiple comparison test. Values were reported as mean ± SEM.

Table 4. The Levels of Cytokines in IPF Patients and Healthy Controls

	Patients (n=12)	Healthy control (n=8)	*p*
IFN-γ	30.17 ± 6.59	10.71 ± 2.13	0.0313
TNF-α	68.83 ± 2.33	55.63 ± 5.79	0.0269
CXCL-8	8.1 ± 0.7	4.02 ± 0.53	0.0005
IP-10	81.5 ± 7.23	161 ± 11.8	0<0.0001
TGF-β	10983 ± 1177	4438 ± 1023	0.001

Comparisons between the groups were performed using parametric Student's t-test. Values were reported as mean ± SEM.

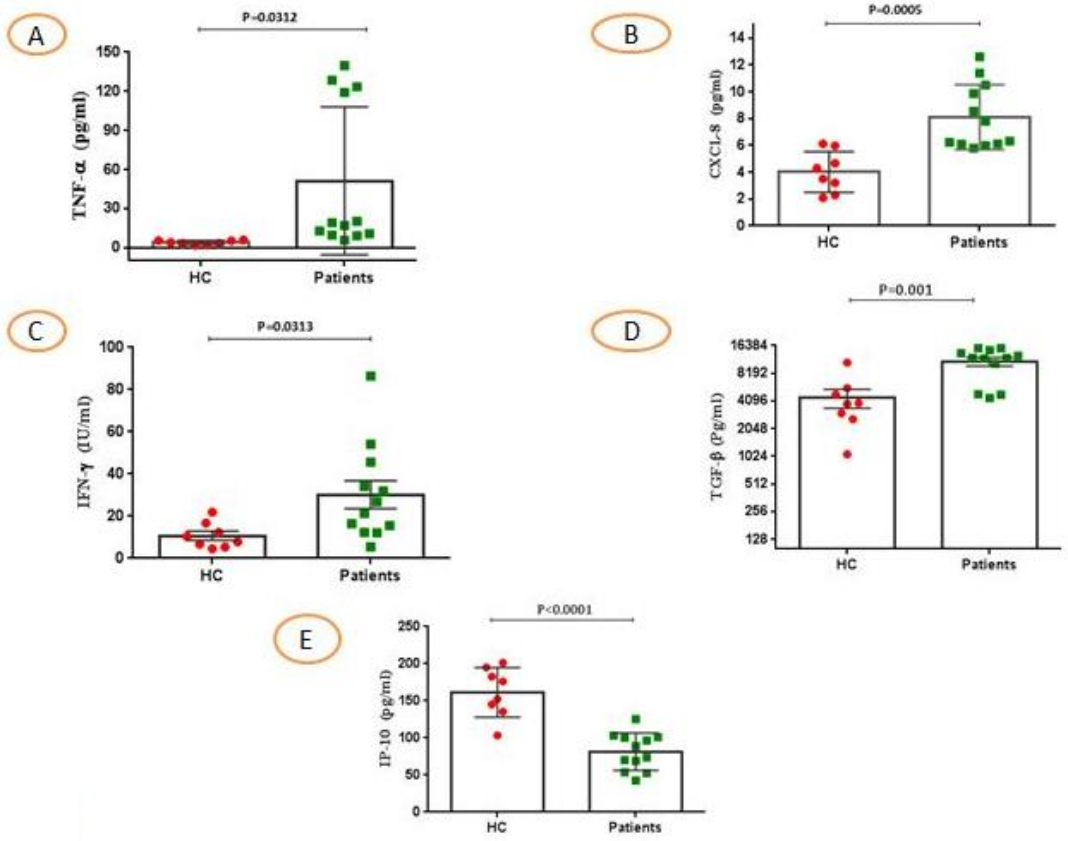

Figure 3. Serum cytokines levels in IPF patients and HCs. A. Dot blots of individual values and mean ± (SEM) of serum TNF-α level in IPF (N=12) and HC (N=8). B. Dot blots of individual values and mean ± (SEM) of serum CXCL-8 level in IPF (N=12) and HC (N=8). C. Dot blots of individual values and mean ± (SEM) of serum IFN-γ level in IPF (N=12) and HC (N=8). D. Dot blots of individual values and mean ± (SEM) of serum TGF-β level in IPF (N=12) and HC (N=8). E. Dot blots of individual values and mean ± (SEM) of serum IP-10 level in IPF (N=12) and HC (N=8). All values are presented as the mean ± SEM and comparisons made between IPF and HC group were performed using the Student's t-test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Table 5. Correlation between Variables

p	**CXCL-8**	**IP-10**	**TNF-α**	**IFN-γ**	**TGF-β**	**Age**	***t-bet***	***ROR-γt***	***GATA-3***	***FOXP3***	**IL-4**	**IL-17**
CXCL-8	-	0.764	0.196	0.871	0.782	0.416	0.861	0.546	0.695	0.398	0.082	0.326
IP-10	0.764	-	0.810	0.591	0.670	0.754	0.584	0.147	0.370	0.557	0.730	0.873
TNF-α	0.196	0.810	-	0.394	0.596	0.007	0.687	0.935	0.394	0.052	0.136	0.231
IFN-γ	0.871	0.591	0.394	-	0.389	0.602	0.915	0.498	0.937	0.385	0.921	0.087
TGF-β	0.782	0.670	0.596	0.389	-	0.222	0.865	0.596	0.907	0.811	0.535	0.857
Age	0.416	0.754	0.007	0.602	0.222	-	0.815	0.678	0.219	0.001	0.066	0.404
t-bet	0.861	0.584	0.687	0.915	0.865	0.815	-	0.067	0.006	0.620	0.158	0.113
ROR-γt	0.546	0.147	0.935	0.498	0.596	0.678	0.067	-	0.132	0.907	0.044	0.151
GATA-3	0.695	0.370	0.394	0.937	0.907	0.219	0.006	0.132	-	0.467	0.526	0.261
FOXP3	0.398	0.557	0.052	0.385	0.811	0.0017	0.620	0.907	0.467	-	0.488	0.514
IL-4	0.082	0.730	0.136	0.921	0.535	0.066	0.158	0.044	0.526	0.488	-	0.671
IL-17	0.326	0.873	0.231	0.087	0.857	0.404	0.113	0.151	0.261	0.514	0.671	-
Pearson r												
CXCL-8	-	-0.097	-0.401	0.052	-0.089	-0.259	-0.056	-0.193	0.126	0.268	-0.520	0.310
IP-10	-0.097	-	0.077	-0.172	-0.137	-0.101	0.176	0.444	0.284	0.188	0.111	0.051
TNF-α	-0.401	0.077	-	0.270	0.170	0.726	0.130	0.026	0.271	-0.571	0.455	-0.373
IFN-γ	0.052	-0.172	0.270	-	0.273	0.167	-0.034	-0.217	-0.025	0.275	0.031	-0.513
TGF-β	-0.089	-0.137	0.170	0.273	-	0.380	0.055	-0.170	-0.037	-0.077	0.199	-0.058
Age	-0.259	-0.101	0.726	0.167	0.380	-	0.075	0.133	0.382	-0.801	0.545	-0.265
t-bet	-0.056	0.176	0.130	-0.034	0.055	0.075	-	0.543	0.738	0.159	0.434	0.480
ROR-γt	-0.193	0.444	0.026	-0.217	-0.170	0.133	0.543	-	0.460	-0.037	0.586	0.440
GATA-3	0.126	0.284	0.271	-0.025	-0.037	0.382	0.738	0.460	-	-0.232	0.203	0.352
FOXP3	0.268	0.188	-0.571	0.275	-0.077	-0.801	0.159	-0.037	-0.232	-	-0.221	0.209
IL-4	-0.520	0.111	0.455	0.0318	0.199	0.545	0.434	0.586	0.203	-0.221	-	0.136
IL-17	0.310	0.051	-0.373	-0.513	-0.0581	-0.265	0.480	0.440	0.352	0.209	0.136	-

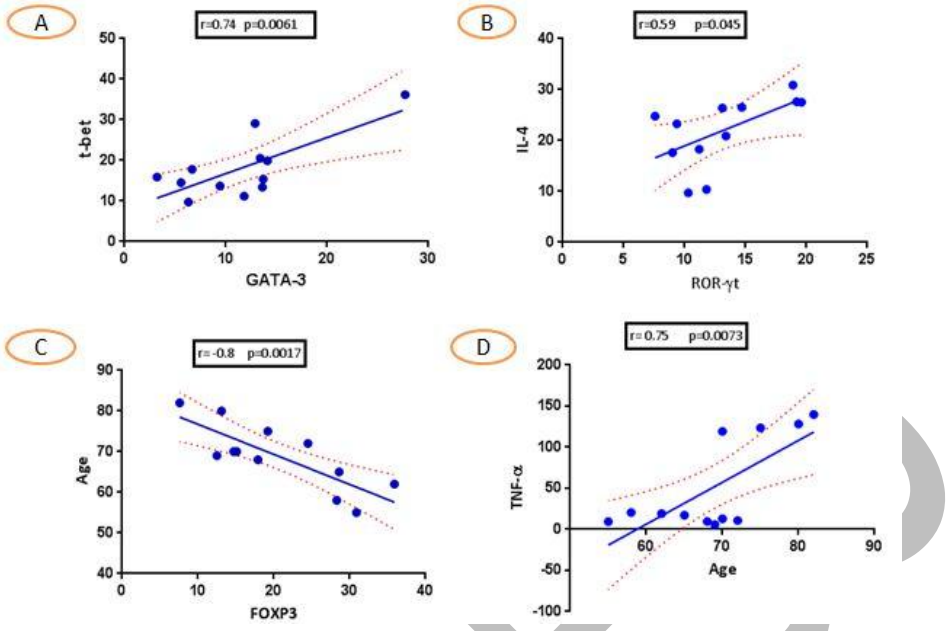

Figure 4. A. Correlations between *T-bet* transcription factors with *GATA3*. B. Correlations between IL-4 with *ROR-γt*. C. Correlations between age with *FOXP3*. D. Correlations between age and TNF-α.

DISCUSSION

The mechanisms underlying the relentless progression of fibrosis in IPF remain incompletely understood. This pilot study examined cytokine profiles and T-cell transcription factors in peripheral blood of IPF patients versus healthy controls. IPF patients showed elevated serum levels of CXCL-8, TGF-β, TNF-α, and IFN-γ, with reduced expression of IP-10. IL-17 expression was higher in $CD3^{+}CD4^{+}ROR\text{-}\gamma t^{+}$ cells, while *T-bet*, *GATA3*, *ROR-γt*, and *FOXP3* levels in PBMCs were similar. These findings highlight immune dysregulation in IPF.

Cytokine profiling in IPF has highlighted a shift from a Th1 to a Th2-dominant immune response, marked by increased IL-4, IL-13, and TGF-β levels, alongside a reduction in the antifibrotic cytokine IFN-γ.[25] However, our study revealed a significant increase in serum IFN-γ levels in IPF patients compared to healthy controls, contrasting with previous reports suggesting IFN-γ downregulation in IPF. This discrepancy may reflect a compensatory immune response aimed at counteracting fibrosis, as IFN-γ is known for its anti-fibrotic properties, including inhibition of fibroblast activation and extracellular matrix deposition. In addition, the small size of our single-centre study, treatment regimens, and the difference in age between patients and healthy controls may have affected the results. A similar mechanistic study performed in a larger multi-centre cohort is important. Furthermore, analysis of peripheral blood may have missed margination of key immune cells into the lung of IPF patients.

Elevated IL-8 levels are present in the bronchoalveolar lavage (BAL) fluid, sputum, and serum of IPF patients.[26,27] The elevated expression of IL-8 mRNA in alveolar macrophages isolated from patients with IPF is strongly correlated with increased IL-8 protein levels in BAL fluid, as well as enhanced neutrophil infiltration in the lungs.[28,29] Elevated IL-8 levels have been associated with disease activity and are considered a predictive biomarker for mortality in IPF.[30] These findings suggest a potential role for IL-8 in disease pathogenesis; however, the exact mechanisms by which IL-8 contributes to IPF remain incompletely understood.[31] Here we confirm that IPF patients display increased serum expression of IL-8 compared with controls.

Cytokine profiling studies in IPF have highlighted the pivotal role of TGF-β and IL-10 in disease progression. TGF-β is a key driver of fibrosis, promoting myofibroblast differentiation and extracellular matrix

deposition, whereas IL-10 exerts immunosuppressive effects that may contribute to an altered immune response in the lung microenvironment. The interplay between these cytokines and IL-8 could further modulate inflammation and fibrotic remodeling, necessitating further research to elucidate their combined impact on IPF pathophysiology.[32] Our results suggest that IPF patients have enhanced expression of TGF-β compared with controls. TGF-β plays a central role in IPF pathogenesis by driving fibrotic remodeling through the induction of insulin-like growth factor-1 (IGF-1). IGF-1 has been shown to enhance fibroblast proliferation and survival, contributing to excessive extracellular matrix deposition and tissue stiffening in the lung. Understanding the TGF-β–IGF-1 axis may provide crucial insights into novel therapeutic strategies aimed at mitigating fibrotic progression in IPF.[33]

TNF has been implicated in the transition from pulmonary inflammation to fibrosis. While TNF signaling is essential for initiating immune responses, persistent TNF activation can drive fibroblast activation and extracellular matrix deposition, leading to irreversible lung remodeling. Targeting soluble TNF and its downstream signaling pathways may represent a potential therapeutic avenue for preventing or slowing fibrotic progression in IPF.[34] Evidence suggests that chronic immune activation contributes to disease initiation and progression. Pro-inflammatory cytokines, including IL-8 and TNF, are implicated in sustaining an inflammatory milieu that facilitates fibroblast activation and extracellular matrix deposition. A deeper understanding of the inflammatory pathways involved in IPF may lead to novel anti-inflammatory strategies aimed at mitigating disease progression and improving patient outcomes.[31] We confirm here that IPF patients have increased TNF-α expression compared to controls.

Specific blockage of the IP-10 receptor CXCR3 significantly decreased the severity of chronic pulmonary inflammation by decreasing the recruitment of inflammatory cells. IP-10 is proposed as a key player responsible for the development of tree shrew-based collagen-induced arthritis (TsCIA)-CPI.[35] In the BML model of lung fibrosis, IP-10 lung levels correlated with a higher angiogenic response. Systemic administration of IP-10 significantly reduced BLM-induced pulmonary fibrosis without any changes in lung lymphocytes or NK cell numbers and reduced angiogenesis.[36] No direct effects of IP-10 on pulmonary fibroblasts have been reported although IP-10 inhibits fibroplasia and deposition of extracellular matrix via modulating angiogenesis.[36] Furthermore, CXC chemokines such as IL-8 and IP-10 regulate angiogenic activity in IPF, potentially influencing disease progression. While IL-8 primarily promotes angiogenesis, IP-10 exerts anti-angiogenic effects, creating a complex regulatory balance that may contribute to aberrant vascular remodeling in fibrotic lungs. Investigating the interplay between these chemokines and their effects on endothelial cell function may provide new insights into therapeutic interventions targeting vascular abnormalities in IPF.[37] We demonstrated reduced levels of IP-10 in IPF patients.

Our findings suggest a significant upregulation of IL-17 expression in $CD3^+CD4^+ROR\text{-}\gamma t^+$ cells, while the expression levels of key transcription factors associated with T helper (Th) cell differentiation—*T-bet* (Th1), *GATA3* (Th2), *ROR-γt* (Th17), and *FOXP3* (Treg)—remained unchanged in PBMCs. This selective increase in IL-17 suggests an imbalance favoring Th17 cell activity, which could contribute to the chronic inflammation observed in IPF. Previous studies have implicated Th17 cells in IPF pathogenesis, as IL-17 can promote fibroblast activation, collagen deposition, and neutrophilic inflammation, leading to progressive fibrosis.[38] While the lack of significant changes in transcription factor expression might indicate a preserved overall balance among Th cell subsets in PBMCs, the elevated IL-17 in $ROR\text{-}\gamma t^+$ cells suggests a functional dysregulation rather than a numerical expansion of Th17 cells. Similar findings have been reported in other fibrotic diseases, where Th17-related cytokines exacerbate tissue remodeling and disease progression.[39] Future analysis should look at nuclear localization rather than intracellular expression of these transcription factors.

In conclusion, our pilot study highlights the potential role of IL-17-producing Th17 cells and their associated cytokine signaling in the pathogenesis of IPF. The elevated IL-17 levels, coupled with other pro-inflammatory cytokines such as IL-8, TNF, and TGF-β, suggest a complex, interconnected inflammatory environment that drives fibrotic progression in IPF. Targeting the IL-17 pathway, along with other key cytokines involved in the inflammatory cascade, may offer novel therapeutic approaches to mitigate the chronic inflammation and fibrosis in IPF. In addition, we note a significant reduction in the expression of IP-10 in these IPF patients. Further investigations are needed to

better understand the interactions between these cytokines and their direct effects on lung tissue remodeling, ultimately leading to more effective treatments for IPF.

STATEMENT OF ETHICS

The study was approved by the institutional ethics board of the Masih Daneshvari Hospital (Ethics number IR.SBMU.MSP.REC.1398.568).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

Upon reasonable request (specify contact method).

AI ASSISTANCE DISCLOSURE

Not applicable.

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