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Regulatory Effect of PD1/PD-Ligand 1 (PD-L1) on Treg Cells in Patients with Idiopathic Pulmonary Fibrosis

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Idiopathic pulmonary fibrosis (IPF) is a serious irreversible lung disease. The mechanism of immune checkpoint in idiopathic pulmonary fibrosis is still unknown.

Material/Methods: First, the expression levels of PD-1/PD-L1 on the surface of CD4+ T cells and the proportion of Treg cells in IPF or controls were detected by flow cytometry. Then, expression of TGF-β in blood samples was detected with ELISA. Moreover, a co-culture system was composed of fibroblasts stimulated by TGF-β and CD4+ T cells from healthy people. The proportions of Treg cells and PD-1 in the co-culture system were detected. In addition, we detected the proportion of Treg cells and the level of collagen-1 after adding PD-1 or PD-L1 protein antibody blocker to the co-culture system.

Results: Flow cytometry revealed the upregulated expression of PD-1/PD-L1 in CD4+ T cells of IPF patients. PD-1 appears to inhibit the differentiation of CD4+ T cells into Treg cells. Co-culture of myofibroblasts and CD4+ T cells induced the generation of collagen-1 and reduced the proliferation of CD4+ T cells. When PD-1 was blocked, the inhibition of Treg cell differentiation was reversed, accompanied by decreased collagen-1 production.

Conclusions: This work identified the molecular mechanism of PD-1 in patients with IPF. It may provide a new perspective on the therapeutic effect of PD-1.

MeSH Keywords: Idiopathic Pulmonary Fibrosis • Programmed Cell Death 1 Receptor • T-Lymphocytes • T-Lymphocytes, Regulatory

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Background

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive interstitial fibrosis pneumonia [1]. The healthy tissue of the lung is replaced by the changed extracellular matrix and the alveolar structure is destroyed, which decreases lung compliance, interrupts gas exchange, and finally leads to respiratory failure and death [2]. IPF affects about 3 million people around the world. With increased age, the incidence of IPF increases dramatically, which causes a huge economic and social burden [3,4]. The disease process of IPF is variable, some of which is unpredictable. Generally, after the diagnosis of symptoms, the time of progression to end-stage respiratory insufficiency and death is about 2–4 years [5].

International consensus guidelines suggested that IPF should be diagnosed at a multidisciplinary level, and known causes of intermittent lung diseases should be excluded by high-resolution computed tomography (HRCT) or surgical lung biopsy [6]. Despite the continuous development of diagnostic criteria, the process is still challenging. Usual interstitial pneumonitis (UIP) is a characteristic histopathological marker of IPF. It includes temporal lobe and spatial heterogeneous fibrosis, fibroblast and myofibroblast clusters, and disordered collagen and extracellular matrix (ECM) over-deposition, leading to the distortion of normal lung structure and the formation of honeycomb cysts [7]. The factors influencing IPF include telomere length, DNA methylation modification, and aging-related changes [8]. In patients with IPF, the pulmonary epithelium becomes dysfunctional. Many chemokines, such as active transforming growth factor-β (TGF-β), play an important regulatory role in this process [9,10].

On the inflammatory level, the level of TGF-β 1 in the lung tissues of patients with IPF is increased [11]. TGF-β 1 is a powerful fibrogenic medium that promotes epithelial cell apoptosis, epithelial mesenchymal transition (EMT), epithelial cell migration, production of other fibrogenic media, circulating fibrocyte recruitment and fibroblast activation, proliferation and transformation into myofibroblasts, and VEGF production [12]. At the cellular immunity level, the effect and mechanism of CD4+ CD25+ Foxp3+ regulatory T cells (Tregs) on IPF are not clear. In IPF patients, the proportion of Tregs is lower than in patients with other lung disease or healthy people. The increase of Tregs in peripheral blood is negatively correlated with disease severity [13]. Tregs significantly influence immune tolerance and prevention of autoimmunity, so their quantitative and functional defects may be related to the initial stage of IPF pathogenesis [14]. It had been reported that programmed cell death-1 (PD-1) in CD4+ T cells can promote the development of IPF through production of interleukin-17A (IL-17A) and TGF-β 1 [15]. However, there have been few studies on the relationship between PD-1 and Treg cell decrease in IPF.

Some progress has been made in understanding the pathogenesis of IPF and in developing treatment. However, there are still substantial treatment problems. Discovery of a treatment for IPF requires a comprehensive and in-depth understanding of the pathogenesis. The poor prognosis, limited clinical diagnosis and treatment, and high mortality all indicate the need for development of an effective IPF treatment strategy. The aim of this study was to explore the relationship between PD-1 and Treg cells in IPF patients, and to evaluate the effect of PD-1 on myofibroblast functions.

Material and Methods

Cell culture

Human lung fibroblasts (HFL1) from heathy people (Shanghai Zhongqiao Xinzhou, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Thermo, Waltham, USA) supplemented with 10% calf serum (GE, Logan, Australia) and 1% antibiotics. Antibiotics (10 000 units/ml penicillin, 10 000 μg/ml streptomycin, and 25 μg/ml amphotericin B) came from GIBCO Life Technologies (Waltham, USA). The fibroblast culture was maintained in T75 (Nest biotechnology, Rahway, USA) and cultured at 37°C in a humid environment containing 5% CO_2. In some of the HFL1 cells, we added 0.05 mg TGF-β (PeproTech, Cranbury, USA) and further cultured them for 12 h. The degree of fibrosis was observed and the subsequent experiments were carried out.

Sorting of CD4+ T cells

First, 5 ml of fresh whole blood of healthy controls was selected, and 5 ml of phosphate-buffered saline (PBS) was added into the whole blood, centrifuged for 10 min at 1200 rpm, and the supernatant was discarded, followed by washing again. A total of 150 μl of magnetic beads was added into the centrifuged precipitate to screen CD4+ T cells (BD, CA, USA), which were then kept in the dark for 30 min. Then, we added 2 ml PBS, blew it with a pipette, placed it on the magnetic frame, and kept it away from light for 10 min. We removed the supernatant from the tube, and did not absorb Brown magnetic beads. Subsequently, we added 1 ml PBS into the supernatant to suspend the magnetic beads, placed them on the magnetic frame, kept them away from light for 10 min, and discarded the supernatant. Finally, the culture medium was added, the magnetic beads were suspended, and we put them into the cell culture bottle for overnight culture. In addition, some cells were co-cultured with myofibroblasts.

Blocking PD-1 pathway *in vitro*

For the blocking experiment, CD4+ T cells and myofibroblasts were cultured with the method described above, and then

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Table 1. Patient demographics.

Characteristics	IPF patients (n=23)	Healthy controls (n=25)	χ^2/t	*P* value
Male sex, n (%)	18 (0.78)	19 (0.76)	χ^2=0.12	0.73
Age, yr, mean±SD	71.65±8.42	70.64±10.26	t=0.37	0.71
Smoking history, n (%)	19 (0.83)	11 (0.44)	χ^2=7.62	0.01
Pack/year, mean±SD	113.26±58.39	52.96±67.78	t=3.44	0.01
Antifibrotic therapy	0	0	0	0

incubated overnight with or without anti-PD-1 (5 μg/ml; eBioscience, Waltham, USA) or PD-Ligand 1 (PD-L1) (2 μg/ml; eBioscience, Waltham, USA) blocking antibody, then continued culturing for subsequent testing.

ELISA

The level of TGF-β in serum was measured using an enzyme-linked immunosorbent assay (ELISA) kit purchased from Abcam (Cambridge, UK). ELISA was performed according to the manufacturer's protocol to determine the concentration of TGF-β in serum. The supernatant of each sample was diluted in PBS, and 100 μl diluted sample was assessed using the kit. The optical density was measured at a wavelength of 450 nm using a microplate reader. All samples were assessed in triplicate.

Western blot (WB)

Protein expression and phosphorylation were detected by WB analysis. The cultured cells were harvested directly in 2×Laemmli buffer *in vitro*. The protein samples were separated by 10% acrylamide gel and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to their molecular weight. Then, the protein was imprinted on polyvinylidene fluoride (PVDF) membrane at 4°C and 150 mA. After being sealed with 5% skimmed milk powder (Sigma, Vienna, Austria) in PBS-Tween 20, the target protein of the membrane was detected. For PD-1 (Abcam, Cambridge, UK), collagen-1 (Abcam, Cambridge, UK) protein expression was detected. The HD2 and chemiluminescent imagers were used for photography and electrophoresis gel imaging.

Sample collection

The whole blood of 25 healthy control subjects and 23 IPF subjects were collected for flow cytometry, ELISA, and western blot. IPF is diagnosed by multidisciplinary experts according to the criteria of the American Thoracic Society/European Respiratory Society [16]. The essential characteristics of samples are shown in **Table 1**. All patients provided informed consent. Blood samples were collected according to the International Code of Ethics for Biomedical Research Involving the Human Body and Subjects. This study was approved by the Ethics Committee of Xinjiang Medical University and carried out in accordance with the regulations of the Ethics Committee of Xinjiang Medical University. The study was approved by Xinjiang Medical University.

Flow cytometry

Anti-CD3, anti-CD4, anti-cd127, anti-CD25, anti-PD-1, anti-PD-L1, and anti-PE-A were obtained from BD Biosciences (CA, USA). The 10^6 cells in the suspension were washed with PBS and stained with fluorescent surface antibody for 30 min. Treg cells and PD-1/PD-L1 were identified after washing twice. CBA test kits were purchased from BD Biosciences. LSR-II flow cytometry (BD Biosciences, CA, USA) was used for flow cytometry experiments. FlowJo software (Tree Star, OR, USA) was used for data analysis.

Statistical analysis

SPSS for Windows V16.0 (Chicago, IL, USA) was used for all analyses. The values are expressed as mean±standard (SD). The chi-square test and t test were used to compare differences between groups. For all experiments, A statistically significant difference was defined as *P* value less than 0.05. (* $P<0.05$). Origin 8.0 software (OriginLab, MA, USA) was used to generate the diagram.

Results

PD-1+ CD4+ T cells increased in IPF subjects

Compared with controls, the expression levels of PD-1 and PD-L1 on the surface of CD4+ T cells in patients with IPF were significantly higher, as shown by flow cytometry (**Figure 1A**). It is generally believed that TGF-β signaling plays an important role in the development of IPF [17]. Then, we detected TGF-β expression in IPF subjects. Compared with the healthy control group, levels of TGF-β were significantly higher in the IPF group (**Figure 1B**). These results showed higher expression of both PD-1/PD-L1 and TGF-β in serum of patients with idiopathic pulmonary fibrosis.

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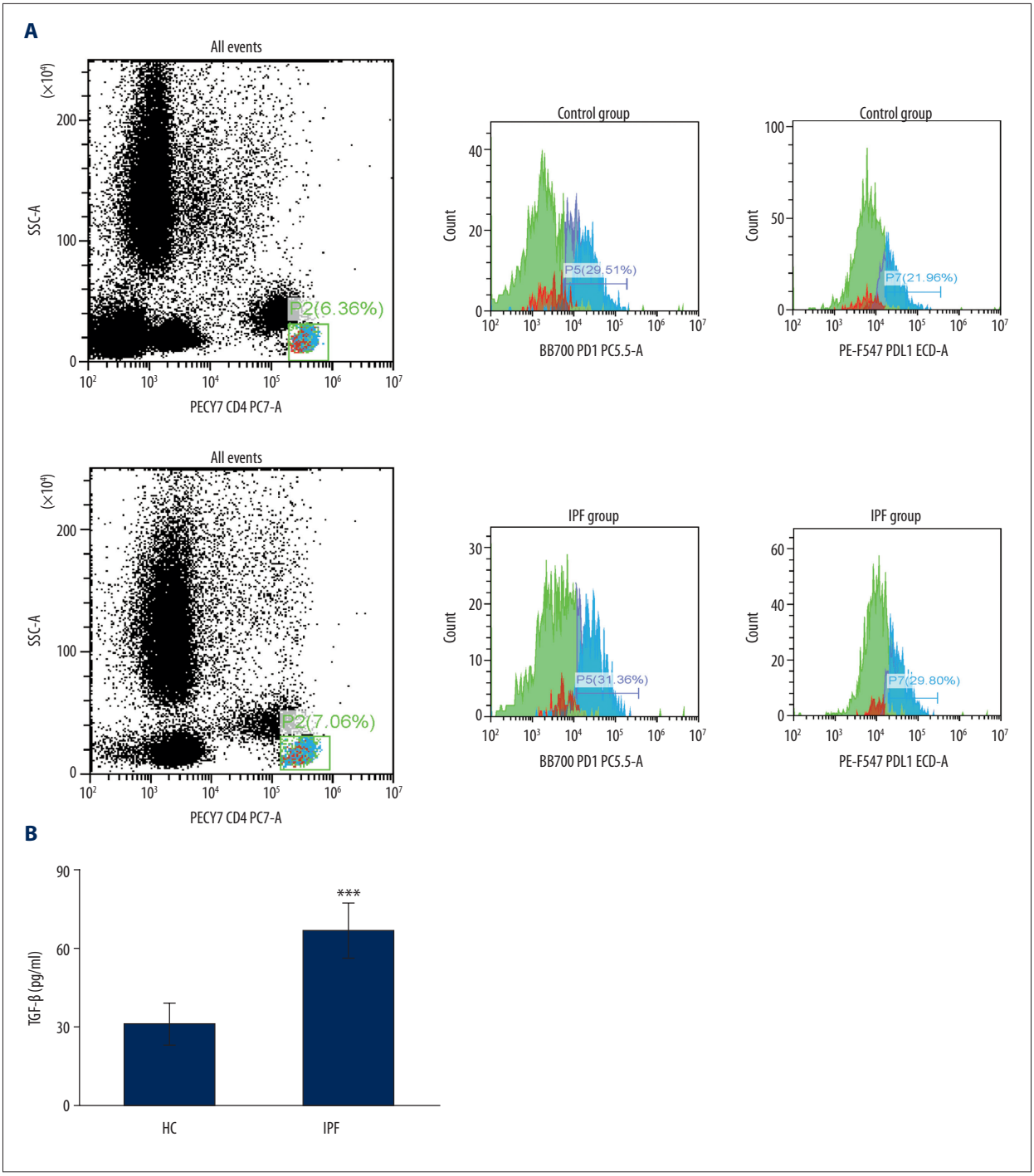

Figure 1. Changes of PD1 pathway and TGF-β in IPF patients. (**A**) Compared with the controls, the proportion of CD4+ PD1+ and CD4+ PDL1+ cells in IPF patients increased. (**B**) Compared with the controls, TGF was expressed at significantly higher levels in blood of IPF patients as detected by ELISA. *** $P<0.001$.

The proportion of Treg cells was lower in IPF patients

TGF-β could induce CD4+ T cells to differentiate into Treg by transcription factor Foxp3 [18]. Flow cytometry showed that the proportion of Treg cells in patients with IPF was significantly lower than in controls. However, there was no significant difference in the other immune cell subtypes (**Figure 2A**). Subsequently, we detected the levels of cytokines in the blood by CBA method. We found that the level of IL-10 was significantly upregulated, as was the level of IL-4 (**Figure 2B**).

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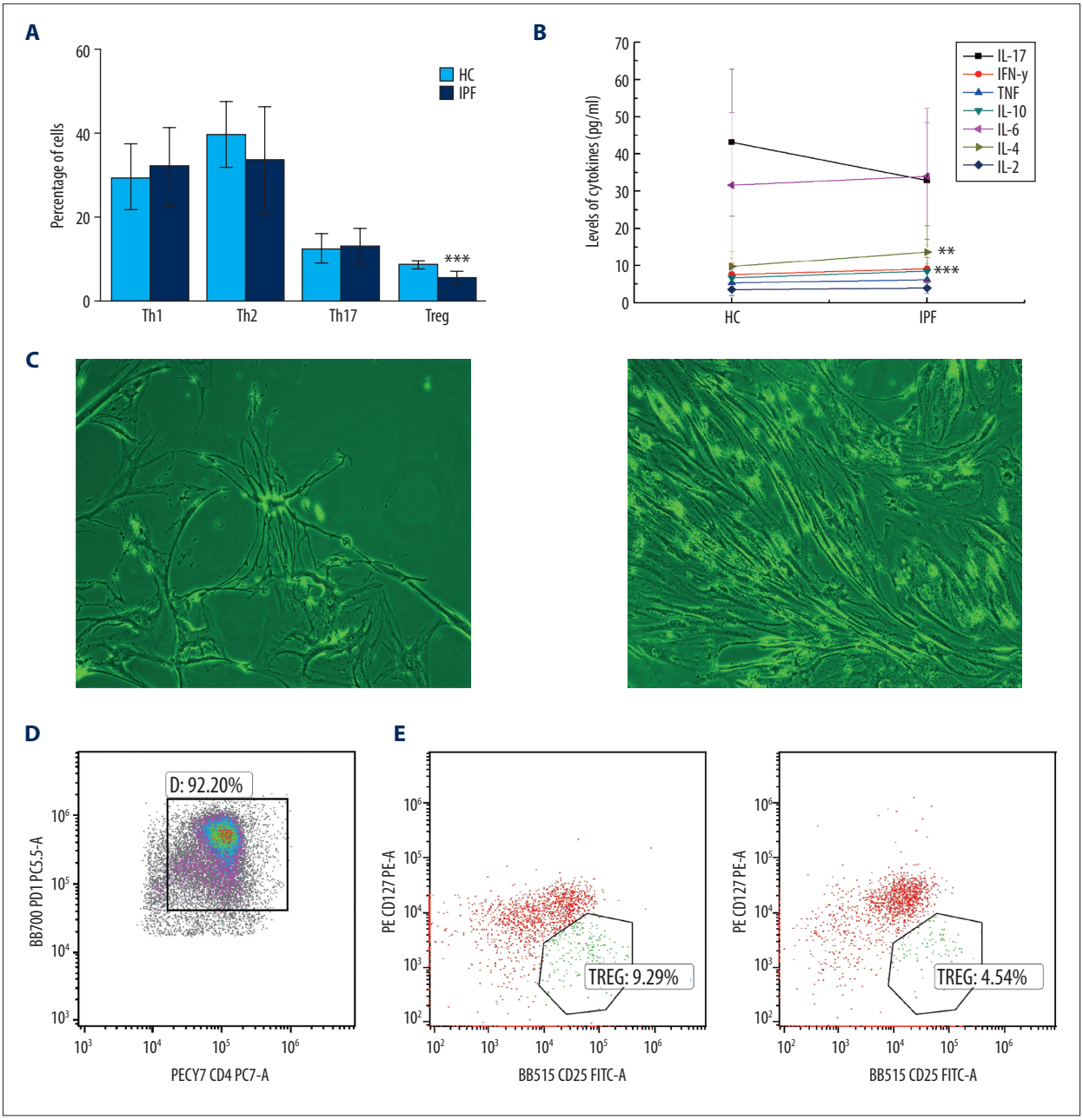

Figure 2. The effect of cell fibrosis on Treg cells differentiation. (**A**) Changes of Treg cells proportion in IPF patients was the most obvious. (**B**) Detection of cytokines in blood of IPF patients. Among them, the changes of IL-4 and IL-10 were the most significant. (**C**) TGF-beta stimulates pulmonary fibroblasts to obtain myofibroblasts. (TGF-beta concentration: 1 ug/ml) (100×). (**D**) The purity of CD4+ T cells isolated from peripheral blood by magnetic beads was more than 90%. (**E**) The proportion of Treg cells decreased significantly after CD4+ T cells co-culture with myofibroblasts for 24 h. ** $P<0.01$, *** $P<0.001$.

In addition, TGF-β is a cytokine that plays an essential role in induction of fibrosis [19]. Therefore, we added TGF-β to human lung fibroblasts (HLF) to stimulate cell fibrosis and transform them into adult myofibroblasts (**Figure 2C**). The CD4+ T cells were isolated from healthy human peripheral blood by magnetic beads, and the cell purity was more than 90% (**Figure 2D**). Two kinds of cells were co-cultured, and the proportion of Treg cells decreased significantly after 24 h (**Figure 2E**). The results suggested that elevated TGF-β in patients with IPF does not ultimately lead to increased Treg cells.

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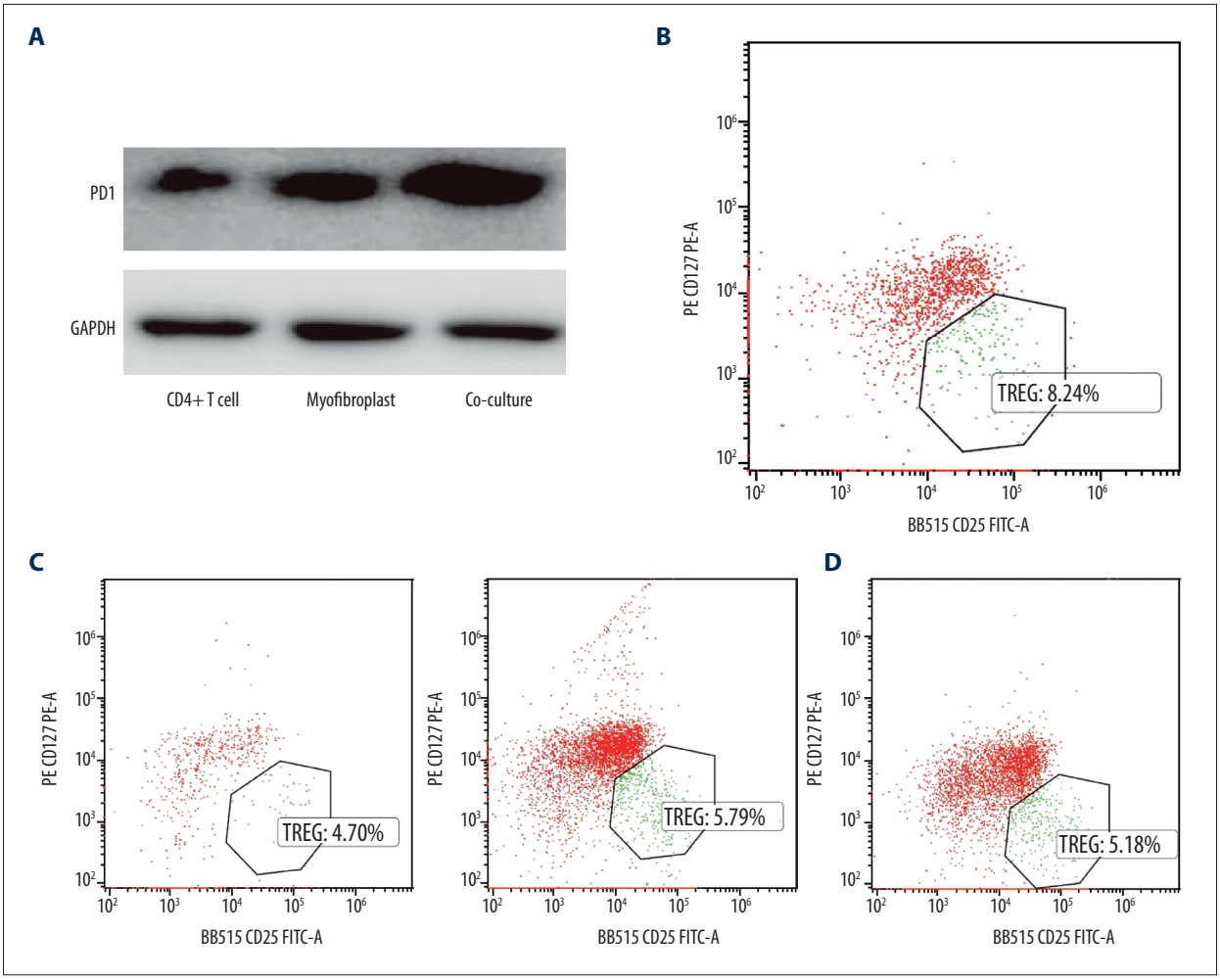

Figure 3. PD-1/PD-L1 pathway inhibited CD4+T cell differentiation into Treg cells. (**A**) The expression of PD1 in the co-culture system of myofibroblasts and CD4+ T cells increased significantly. GAPDH protein was used as negative standard. (**B**) After blocking PD1, the numbers of Treg cells increased in the co-culture system. (**C**) When myofibroblasts were blocked by PD1, the numbers of Treg cells in the co-culture system increased. Blocking PD1 in CD4+ T cells did not significantly change the proportion of Treg cells. (**D**) After blocking PDL1 in the myofibroblasts, the numbers of Treg cells in the co-culture system also increased.

PD-1 overexpression inhibits differentiation of CD4+ T cells into Treg cells

Compared with myofibroblasts or CD4+ T cells, the expression of PD-1 was increased in the co-culture system (**Figure 3A**). PD-1 protein antibody blocker was added to the co-culture system and the proportion of Treg cells was assessed 12 h later. It was found that the proportion was significantly increased (**Figure 3B**). The results showed that PD-1 blocking can reduce the inhibition of Treg cells differentiation. Then, we added PD-1 protein antibody blocker to the selected CD4+ T cells or myofibroblasts for 12 h, and then co-cultured them. The decrease of Treg cell differentiation was reversed only when myofibroblasts were blocked by PD-1 (**Figure 3C**).

Further, we added PD-L1 protein antibody blocker to myofibroblasts for 12 h and then co-cultured them with CD4+ T cells. The results suggest that the inhibition of Treg cell differentiation was also reversed in a few parts (**Figure 3D**).

PD-1 inhibits myofibroblast proliferation and collagen-1 production *in vitro*

Then, we evaluated the collagen-1 produced by co-culture of myofibroblasts and CD4+ T cells. We first observed a slight decrease compared with the single myofibroblasts, but, compared with the single CD4+ T cells, the proliferation ratio of co-cultured cells decreased significantly (**Figure 4A**). Western blot analysis showed that the level of collagen-1 in supernatant of the co-culture system was significantly higher than

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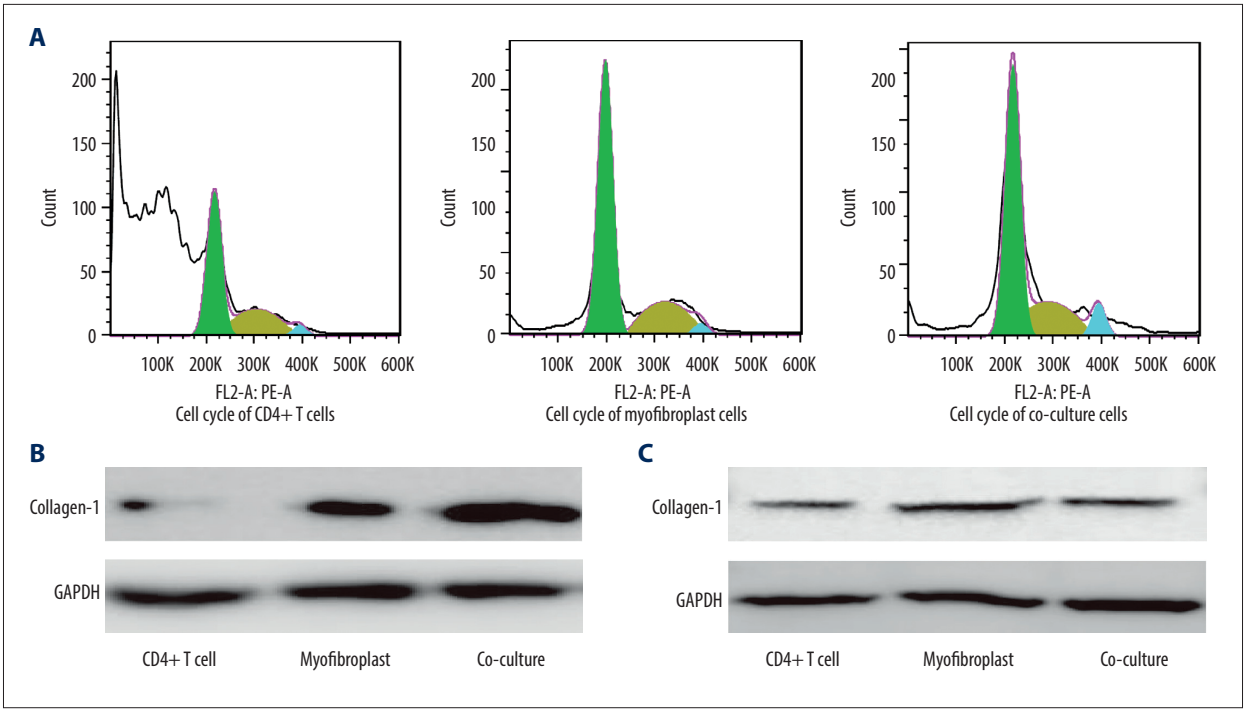

Figure 4. PD-1 promotes collagen-1 production in myofibroblasts. (**A**) The proliferation rate in co-cultured cells was lower than that in CD4+ T cells, but there was no significant difference compared with myofibroblasts. The S phase of CD4+ T cells, myofibroblasts, and co-cultured cells were 8.99, 19.12, and 21.49, respectively. (**B**) The content of collagen-1 in the co-culture system was higher than that in the single-culture system. (**C**) The addition of PD-1 blocker in the co-culture system can reduce the content of collagen-1. GAPDH protein was used as negative standard.

that of single myofibroblasts or CD4+ T cells (**Figure 4B**). In the co-culture system of myofibroblasts blocked by PD-1, the level of collagen-1 decreased significantly. It is worth noting that there was no significant difference in the level of collagen-1 compared with CD4+ T cells. Compared with myofibroblasts alone, the level of collagen-1 was reduced (**Figure 4C**).

Discussion

IPF is one of the most common and severe forms of induced interstitial lung disease (ILD). Cancer is also a major complication of the disease. Although there have been many approved drugs and other interventions, IPF still is the most challenging interstitial lung disease in clinical treatment [20]. As we all know, PD-1 has become a new biomarker in ILD, and its antibody therapy has been widely used in ILD [21–23]. However, there have been few studies on the mechanism and therapeutic value of PD-1 in IPF. In the present study, molecular detection was used to explore the regulatory effect of PD-1 in IPF on Treg cells. Mechanistically, inhibition of the PD-1 pathway can reverse the Treg cells differentiation, then reduce the generation of collagen-1.

In fact, experimental evidence and histological analysis indicated the presence of innate and adaptive immune cells, especially lymphocytes, which may contribute to alveolar destruction and progressive lung remodeling [24]. The regulation of T cell function was coordinated through co-stimulation and co-inhibition. Inhibitory receptors, including PD-1, have a negative effect on CD4+ T cell function [25]. Blocking the PD-1 pathway can restore T cell function in granulomatous diseases, such as chronic beryllium disease (CBD) and granulomatous disease in polyangiitis (GPA) [26,27]. Blocking the PD-1 pathway can also improve the clinical symptoms of lung cancer [28]. However, the effect of PD-1 on Treg cells in IPF patients has not been previously described. In our results, the PD-1 was highly expressed in IPF patients, accompanied by a decrease in the proportion of Treg cells and an increase in TGF-β content. It has been noted that the increase of TGF-β can promote CD4+ T cells differentiating into Treg cells to some extent [29]. However, the increased TGF-β in the results of this study did not result in increased Treg cells, while Treg cells have plasticity, allowing them to differentiate into Th17 cells under the condition of increased IL-6/PSTAT3 expression [30]. Remarkably, the high level of PD-1/PD-L1 expression resulted in the intrinsic damage of Treg function and the reduction of reactivity of effector clone, which ultimately failed to control HIV infection [31]. However, some studies showed that Treg

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infiltration was significantly related to the infiltration of PD1-positive cells, and it was reported that 81% of tumor patients with PD1-positive lymphocyte infiltration were also in the high Treg subgroup [32]. In the present study, smoking history was significantly different between IPF patients and controls, and smoking seems to be the most relevant risk factor for IPF [33]. However, there are no studies that have found whether smoking affects the expression of PD1/PD-L1 in IPF patients, which needs further exploration.

It is difficult to characterize the phenotype of T cells of IPF. A few studies have demonstrated that T cells in peripheral blood of IPF have specific gene expression characteristics. Based on previous research reports, flow cytometric analysis of explained lung cell suspensions showed the increase of CD8 + cd28null T cells in IPF relative to normal lung explants [34]. Compared with the controls, IPF patients had a higher proportion of lung CD4+ T cells, a higher proportion of CCR4+ CD4+ T cells, and a lower proportion of CCR6+ CD4+ T cells [35]. The present study was different from previous reports, which found that the increased proportion of activated Treg cells was related to the severity of idiopathic pulmonary fibrosis [13]. However, our work showed that the proportion of Treg cells in IPF patients or myofibroblasts decreased significantly, which was related to PD-1/PD-L1 signaling. The decrease numbers of Treg cells may be related to the smoking characteristics of IPF patients, which needs further research [36].

The limitation of this study is that other immunological and non-immunological pathogenic factors of IPF were not examined. For example, lung CCR2+ CD4+ T cells are immunomodulatory and attenuate the development of pulmonary fibrosis [37].

Another issue we did not explore is how TGF-β and the tissue microenvironment cooperate to promote the multiple effects of regulating cell responses of different cell types in IPF [19]. Mesenchymal stem cells can make TGF-β1 downstream signal transduction sensitive, thus regulating IL-6/STAT3 activation, stimulating Treg expansion, and promoting anti-fibrosis [38].

Despite years of intense research, the exact causes of IPF remain unknown. The results of the present study show that PD-1 overexpression inhibits CD4+ T cells differentiating into Treg cells, leading to increased collagen-1 production. Our exploration of the effect of PD-1 in IPF may help discover effect targets.

Conclusions

The levels of PD-1, PD-L1, and TGF-β in IPF patients were increased. The increased PD-1/PD-L further inhibited the differentiation of CD4+ T cells into Treg cells, which can reverse the effect of TGF-β on the differentiation of Treg cells. PD-1 also decreased the proliferation of myofibroblasts co-cultured with CD4+ T cells, but promoted the generation of collagen-1 in myofibroblasts.

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Conflicts of interest

None.

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