Article

The Effects of Aerosolized STAT1 Antisense Oligodeoxynucleotides on Rat Pulmonary Fibrosis

Wenjun Wang[1], Bin Liao[2], Ming Zeng[1], Chen Zhu[1] and Xianming Fan[1, 3]

Previous study showed that aerosolized signal transducer and activator of transcription-1 (STAT1) antisense oligodeoxynucleotide (ASON) inhibited the expression of STAT1 and ICAM-1 mRNA and protein in alveolar macrophages (AMs) and decreased the concentrations of TGF-β, PDGF and TNF-α in bronchioalveolar lavage fluid (BALF) in bleomycin (BLM)-induced rat pulmonary fibrosis. Administration of STAT1 ASON ameliorated alveolitis in rat pulmonary fibrosis. However, further investigations are needed to determine whether there is an effect from administration of STAT1 ASON on fibrosis. This study investigated the effect of aerosolized STAT1 ASON on the expressions of inflammatory mediators, hydroxyproline and type I and type III collagen mRNA in BLM-induced rat pulmonary fibrosis. The results showed that STAT1 ASON applied by aerosolization could ameliorate alveolitis and fibrosis, inhibit the expressions of inflammatory mediators, decrease the content of hydroxyproline, and suppress the expressions of type I and type III collagen mRNA in lung tissue in BLM-induced rat pulmonary fibrosis. These results suggest that aerosolized STAT1 ASON might be considered as a promising new strategy in the treatment of pulmonary fibrosis. *Cellular & Molecular Immunology*. 2009;6(1):51-59.

Introduction

Idiopathic pulmonary fibrosis (IPF) is characterized by injury with loss of lung epithelial cells and abnormal tissue repair, resulting in abnormal accumulation of fibroblasts and myofibroblasts, deposition of extracellular matrix (ECM) and distortion of lung architecture, leading to respiratory failure (1). Clinically, IPF is characterized by progressive, exertional dyspnea and nonproductive cough, worsening of pulmonary function and radiographically-evident interstitial infiltrates. Due to the lack of a more effective alternative, the fundamental therapeutic approach has been the use of corticosteroids, alone or in combination with other immuno-suppressive agents; however, this has had little impact on long-term survival (2, 3).

[1]The Department of Respiratory Medicine, Affiliated Hospital of Luzhou Medical College, Luzhou 646000, China;

[2]The Department of Cardiothoracic Surgery, Affiliated Hospital of Luzhou Medical College, Luzhou 646000, China;

[3]Correspondence to: Dr. Xianming Fan, The Department of Respiratory Medicine, Affiliated Hospital of Luzhou Medical College, Luzhou 646000, China. Tel: +86-830-316-5321, Fax: +86-830-239-2753, E-mail: fxm129@ 163.com

With the development of cell biology and molecular biology, it has been demonstrated that alveolar macrophages (AMs) play a key role in the pathogenesis of IPF by virtue of their ability to release a variety of cytokines and inflammatory mediators (4, 5). Many of these cytokines exert their biological function through the janus kinase/signal transducers and activators of the transcription (JAK/STAT) pathway (6, 7). The importance of the JAK/STAT pathway has been established by the disruption of genes encoding STATs (8). The first discovered STAT protein was STAT1, which correlated with immunity and inflammation. Several studies showed that STAT1 correlated with the occurrence and development of inflammatory and immune diseases (9-12).

In our previous study, we found that there was abnormal STAT1 activation in the AMs of rats with bleomycin (BLM)-induced pulmonary fibrosis based on nuclear transportation of STAT1, which led to up-regulation of STAT1-dependent immune response gene ICAM-1 expression. The latter induced the accumulation and activation of several types of inflammatory cells in lung tissue. The cytokines released by inflammatory cells, in turn, activated STAT1 and increased further release of cytokines. This positive feedback may lead to alveolitis and pulmonary fibrosis. Furthermore, it has been demonstrated that STAT1 antisense oligodeoxynucleotide (ASON) targeting AMs *in vitro* inhibit the expression of STAT1 mRNA and protein in the AMs of BLM-induced rat pulmonary fibrosis. We also found that STAT1 ASON could attenuate the release of

proinflammatory cytokines, including TNF-α, IL-8 and NO in AMs, and can inhibit lung fibroblast proliferation and hydroxyproline secretion (13, 14). Our previous study proved that the STAT1 ASON delivered by aerosol to rat lungs could depress the expression of STAT1, ICAM-1 mRNA and protein in the AMs and could decrease the concentrations of TGF-β, PDGF and TNF-α in bronchoalveolar lavage fluid (BALF) in BLM-induced rat pulmonary fibrosis (15). In this study, our purpose was to determine the effect of STAT1 ASON on the expression of TNF-α, IFN-γ in BALF and TGF-β, PDGF-A, Smad4, STAT1, ICAM-1, and collagen in lung tissues in BLM-induced rat pulmonary fibrosis, and to evaluate its anti-fibrosis effect. Our data showed that (I) aerosolized STAT1 ASON could alleviate the extent of alveolitis and fibrosis; (II) STAT1 ASON did reduce the concentration of TNF-α, and did inhibit the decline of IFN-γ concentration in BALF; (III) STAT1 ASON prevented the increase of the expression of PDGF-A, TGF-β, Smad4, STAT1 and ICAM-1 in lung tissues; (IV) STAT1 ASON decreased the levels of hydroxyproline and type I and type III collagen mRNA in lung tissues.

Materials and Methods

Design and synthesis of STAT1 ASON
According to the theory of designing ASON and the analysis of STAT1 functional domain, we designed phosphoro-thioated STAT1 ASON, and then synthesized it by Sangon Biotechnology Engineering Company of Shanghai (Shanghai, China). The sequence for STAT1 ASON is 5'-GAAGCTCG TACCACTGCGACATCC-3'. The synthesized oligodeoxy-nucleotides were purified by HPLC, and stored at -20°C.

Incubation of liposome with STAT1 ASON
Complexes were formed by incubating a 1:1 ratio of the liposome (400 μg) with STAT1 ASON (400 μg) for 30 min at room temperature in sterile saline (final volume 3 ml for one rat).

Aerosolization
The mode of aerosolization was performed as previously described (16), and was ameliorated in this study. In brief, in order to restrict rat movement, the animals were confined in plastic bottles with the heads extending from the tops of the bottles. The open extremity of the Ultrasonic nebulizer tube was aimed at the nose of the rat. In this manner, all STAT1 ASON/liposome particles were forced down the tube past the rat's nose for optimal inhalation effect during respiration. Three milliliters of STAT1 ASON/liposome complexes, or normal saline (NS), were respectively administered by nebulization for 45 min using a nebulizer, model WH-802 (Yue Hua, China), for every 3-5 min of aerosolization, with intervals of 1-2 min.

Animals and treatment
Forty-five adult female Wistar rats were randomly divided into three groups: the BLM group, the ASON group, and the NS group. The BLM group and the ASON group were intratracheally instilled with BLM, while the NS group was intratracheally instilled with a 0.9% NaCl solution. After the rats were anaesthetized (Ketamine anaesthesia) and fixed, tracheostomies were performed on them to facilitate the intratracheal instillation of 0.2-0.3 ml 0.9% NaCl solution or of BLM-A5 (0.5 mg/100 g body weight) in 0.2-0.3 ml of 0.9% NaCl solution while rotating the rats in an upright position to facilitate the thorough and even distribution of the drug in the lungs. After intratracheal administration, the NS group and the BLM group were aerosolized with NS, while the ASON group was aerosolized with phosphorothioated STAT1 ASON/liposome complexes on days 0, 2, 4 and 6. Each group was again divided into three subgroups, which were sacrificed on days 7, 14, and 28.

Bronchioalveolar lavage (BAL) and cell analysis
The rats were sacrificed by exsanguination from the right ventricle under ketamine anaesthesia, and then BAL was performed. The left lung was ligated at the hilus and a small plastic tube was inserted into the trachea and placed in the right main bronchus. The tube was attached to a 10 ml syringe, and 40 ml (in 5 ml, aliquots) of sterile phosphate buffer saline (PBS) at 37°C was instilled. The fluid was retrieved by gentle aspiration after each infusion, filtered through a double-layered sterile gauze on crushed ice, and then centrifuged at 4°C at 400 g for 15 min, and the supernatant was removed. The cell pellet was resuspended in PBS and the total number of lavage cells was counted using a hemocytometer and differential counts of lavage cells were made from Cytospin slides stained with Wright-Giemsa by viewing at least 200 cells using standard light microscopy. The results were presented as a percentage of cells.

ELISA for determining TNF-α and IFN-γ in BALF
BALF was thawed at room temperature and condensed ten times by lyophilization, and then added to wells of flat bottom microtiter plates coated with murine mAb to rat TNF-α. After incubation of the samples and thorough washing of the wells, HRP-conjugated anti-TNF-α antibody was added to the test wells. After a second incubation, the excess HRP-conjugated antibody was removed by washing. The HRP substrate was then added, and the color intensity was measured with a microtiter plate reader. An assay for IFN-γ was prepared in a similar way, following the manufacturer's instructions.

Lung histology
After BAL, the right lung was frozen immediately and stored in liquid nitrogen until needed and 4% polymethylaldehyde was instilled into the left lung trachea. The left lung tissues were then put into 4% polymethylaldehyde to be fixed, resected from three locations, paraffin-embedded, sectioned into 5 μm-thick slices, and stained with hematoxilin-eosin (HE) and Masson. The degree of alveolitis and fibrosis was further evaluated according to the standard described previously (17, 18).

Hydroxyproline measurement

To quantify collagen deposition, hydroxyproline content was measured according to the literature (19). In brief, a minced lung was homogenised in 6 N HCl and hydrolysed at 125°C for 16 h. The sample volume was adjusted to 10 ml with sterile water. Each 0.1 ml aliquot of the sample solution was added to 0.5 ml citric acid buffer and 1 ml 1.4% chloramine T, and then incubated at room temperature for 6 min. Then 1 ml 3.15 M perchloric acid was added and incubated for 5 min, followed by the addition of 1 ml 10% p-DMAB solution. After incubation at 80°C for 10 min, absorbance was measured at 560 nm with a spectrophotometer. The amount of hydroxyproline was determined by comparison with a standard curve prepared from known concentrations of the hydroxyproline reagent.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

The total RNA was isolated from the lung tissue using the Trizol reagent. Then an RT-PCR analysis was performed using a QuantiTect one-step RT-PCR Kit, following the manufacturer's instructions. One μg of RNA was mixed with 10 μl of 5× RT-PCR buffer, 0.6 mM of each primer, 2 μl dNTP, 2 μl RT/Taq Mix, in a final volume of 50 μl. The PCR reactions were as follows: cDNA synthesis: 30 min at 50°C; PCR: predenaturation, 95°C for 15 min; denaturation, 94°C for one minute; annealing, 62°C for one minute, extension: 72°C for 15 min for 30 cycles. Type I collagen sense primer: 5'-TGC CGT GAC CTC AAG ATG TG-3', antisense primer: 5'-CAC AAG CGT GCT GTA GGT GA-3'; type III collagen sense primer: 5'-CCA CCC TGA ACT CAA GAG C-3', antisense primer: 5'-TGA ACT GAA AGC CAC CAT T-3'; GAPDH sense primer: 5'-TCA ACG GCA CAG TCA AGG-3', antisense primer: 5'-GCA TCA AAG GTG GAA GAA T-3'. All samples were subjected to RT-PCR housekeeping gene GAPDH as a positive control and as an internal standard. PCR products were resolved on 2% agarose gels in 1× Tris-Acetic acid-EDTA (TAE) buffer, visualized by ethidium bromide, photographed using a gel labwork 4.5 system, and analyzed by densitometry.

Immunohistochemical analysis of STAT1, Smad4, PDGF-A, TGF-β and ICAM-1 protein in lung tissues

The lungs were obtained from each group of animals on days 7, 14, and 28 under ketamine anesthesia. Tissue samples were fixed immediately in 4% paraformaldehyde at 4°C overnight, embedded in paraffin, and cut into 5 μm sections. Specimens were heated for 1 h at 60°C and rehydrated. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 and sodium azide (1 mg/ml) for 10 min. Non-specific protein binding was blocked by incubation in 10% goat serum for 10 min. Sections were respectively incubated with anti-rat STAT1 monoclonal antibody (1:100 dilution), anti-rat Smad4 mAb (1:100 dilution), anti-rat TGF-β mAb (1:100 dilution), anti-rat PDGF-A mAb (1:150 dilution) or anti-rat ICAM-1 mAb (1:150 dilution) overnight at 4°C. Sections were rinsed three times for 5 min each in PBS before incubation with horseradish peroxidase (HRP)-labeled anti-mouse IgG or

anti-rabbit IgG (1:100 dilution) for 1 h, and then rinsed again with PBS three times for 5 min each. Slides were incubated for 10 min in a medium containing 50 mmol/L Tris-HCl buffer, 0.02% (wt/vol) DAB, and 0.01% H_2O_2. Chromogenic reaction was terminated with water followed by hematoxylin counter staining. The sections were examined under the Leica imaging microscope (Leica, Germany) using ×20 to ×40 objectives. Images were acquired with Leica camera (Leica). Immunohistochemical staining was analyzed by the image Pro-Plus 5.1 microscope image analysis system, using integrated optical density (IOD).

Statistical analysis

Data were presented as mean ± SEM. One-way analysis of variance followed by a Dunnett test was used to determine significant differences between two groups. A probability value of $p < 0.05$ was considered statistically significant.

Results

Histopathological features of lung tissues

In the NS group, there were no signs of septal oedema, inflammation, or fibrosis in the alveolar structure. In the BLM group, on day 7, the alveolar septa were markedly oedematous and thickened, accompanied by infiltration of inflammatory cells, fibrotic changes appeared, and alveolar spaces became smaller in the focal region of the lesions. On day 14, cellular infiltration was still present, along with proliferating fibroblasts, thickened alveolar walls, increasing deposition of collagen. The destruction of alveolar structure and patchy fibrosis was apparent. On day 28, inflammatory changes had gradually subsided; remarkable destruction of alveolar structure had appeared; and most alveolar spaces were reduced due to the accumulation of collagen and fibroblasts. Histopathological changes in the ASON group revealed minimal amount of collagen content in the lung parenchyma and a reduced number of cells. According to the standard described previously (17, 18), in comparison with the BLM group, the alveolitis and fibrosis scores in the ASON group indicated that these conditions were significantly reduced (Figures 1 and 2).

The total number of cells and differential counts of cells in BALF

The total number of cells in BALF obtained from the NS group was about 10×10^4/ml. There was a significant increase in the total number of cells in the BLM group and in the ASON group on days 7, 14, and 28. Peaked cell counts were obtained on day 7. After 7 days, the cell counts gradually declined. More than 90% of the cells in BALF obtained from the NS group were AMs, while AM proportion dropped after BLM administration. A greater amount of the polymorphonuclear leucocytes (PMNs) and lymphocytes (LYMs) were detectable in the BLM group on day 7 and day 14. The number then declined on day 28. The total cell counts in the ASON group were lower than those in the BLM group. On day 7 and day 14, the percentage of AMs in the
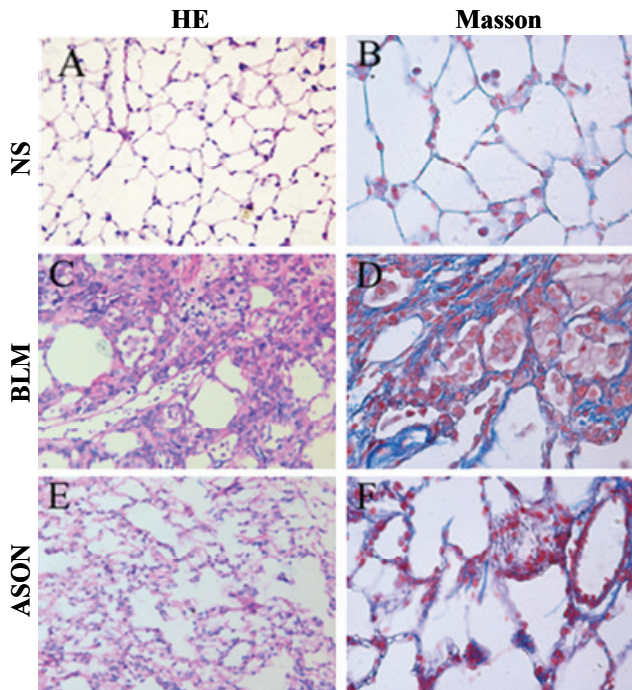
Figure 1. Histopathological examination of lung tissues on day 28 in the NS group (A: HE, ×200; B: Masson, ×400), the BLM group (C: HE, ×200; D: Masson, ×400) and the ASON group (E: HE, ×200; F: Masson, ×400). In the NS group (A and B), alveolar structure showed no signs of septal oedema, inflammation or fibrosis. In the BLM group (C and D), inflammatory changes and remarkable destruction of alveolar structure had occurred. Most alveolar spaces disappeared and were replaced by collagen and fibroblasts. However, after rats were treated with ASON (E and F), destruction of alveolar structure was less, accompanied by minimal amounts of collagen deposition in the lung parenchyma and less infiltration of inflammatory cells.
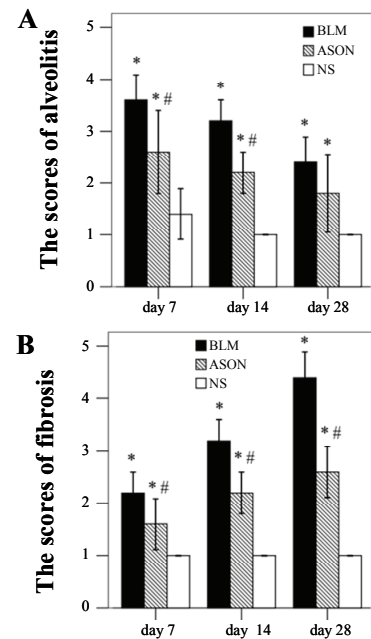

Figure 2. Scores of alveolitis and fibrosis. Lung tissues that were obtained from the NS group, the BLM group and the ASON group on days 7, 14 and 28 were used for HE and Masson staining. The scores shown are expressed as the mean ± SEM (n = 5). (A) Compared with the NS group, the scores of alveolitis in both the BLM group and the ASON group at different time points showed a significant increase (*$p < 0.05$). However, compared with the BLM group, the scores of alveolitis in ASON group on days 7 and 14 showed a significant decrease ($^{\#}p < 0.05$). Hence, comparison of the scores of alveolitis revealed that STAT1 ASON ameliorated alveolitis that occurred on days 7 and 14. (B) Compared with the NS group, the scores of fibrosis in both the BLM group and the ASON group at different time points showed a significant increase (*$p < 0.05$). However, compared with the BLM group, the scores of fibrosis in ASON group at different time points showed a significant decrease ($^{\#}p < 0.05$). Hence, comparison of the scores of fibrosis revealed that STAT1 ASON ameliorated fibrosis.

ASON group was higher than that in the BLM group, and at various times, the percentage of PMNs in the ASON group was lower than that in the BLM group. However, there were no significant differences in the LYM analysis between the ASON group and the BLM group (Figure 3).

Expression of type I and type III collagen mRNA in lung tissue

The mRNA levels of type I and type III collagen were measured by RT-PCR on days 7, 14, and 28. Minimal levels of type I and type III collagen were detected in the NS group. But compared with the NS group, the mRNA levels of type I and type III collagen in the BLM group were markedly increased. They peaked on day 7, and then gradually declined on days 14-28. Compared with the NS group, expression levels of type I and type III collagen mRNA in the ASON group were also increased; however, they had increased to a lesser extent than those of the BLM group (Figure 4).

Hydroxyproline content in lung tissues

Collagen content of lung tissues was assessed by the hydroxyproline assay. The concentrations of hydroxyproline in lung tissue in BLM group were significantly higher than those in NS group. Compared with the BLM group, hydroxyproline content in ASON group was significantly reduced. However, compared with the NS group, hydroxyproline content in ASON group was higher (Figure 5).

The effect of STAT1 ASON on IFN-γ and TNF-α in BALF

The concentrations of IFN-γ and TNF-α in BALF were measured using ELISA. As shown in Figure 6, the concentration of IFN-γ in BALF in the BLM group was lower than that in the NS group, while the concentration of IFN-γ in BALF in the ASON group on day 14 and 28 was lower than that in the NS group. The concentration of TNF-α in BALF in the ASON group and in the BLM group was higher than that in the NS group. Compared with the BLM group, the concentration of TNF-α in BALF in the ASON
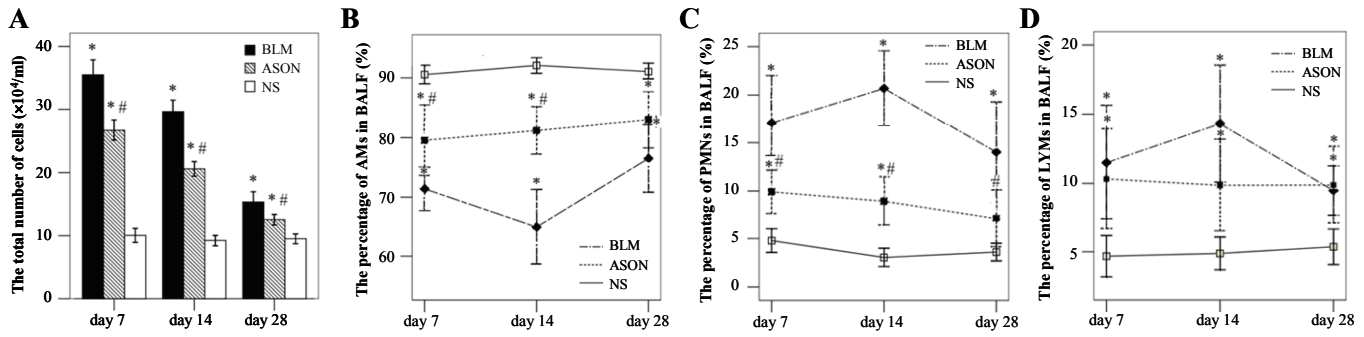
Figure 3. Effects of STAT1 ASON on the total number of cells and differential counts of cells in BALF in BLM-induced rat pulmonary fibrosis. Data were expressed as the mean ± SEM (n = 5). (A) Compared with the NS group, the total cells in both the BLM group and the ASON group at different time points significantly increased (*$p < 0.05$). Compared with the BLM group, the total cells in the ASON group showed a significant decease ($^{\#}p < 0.05$). (B) Compared with the NS group, the percentage of AMs in both the BLM group and the ASON group at different time points significantly decreased (*$p < 0.05$). Compared with the BLM group on days 7 and 14, the percentage of AMs in the ASON group was higher ($^{\#}p < 0.05$). (C) Compared with the NS group, the percentage of PMNs at different time points in the BLM group was higher, while the percentage of PMNs on days 7 and 14 in the ASON group was also higher (*$p < 0.05$). Compared with the BLM group at different time points, the percentage of PMNs in the ASON group was decreased ($^{\#}p < 0.05$). (D) Compared with the NS group, the percentage of LYMs in both the BLM group and the ASON group at different time points significantly increased (*$p < 0.05$). But there was no difference between the BLM group and the ASON group.
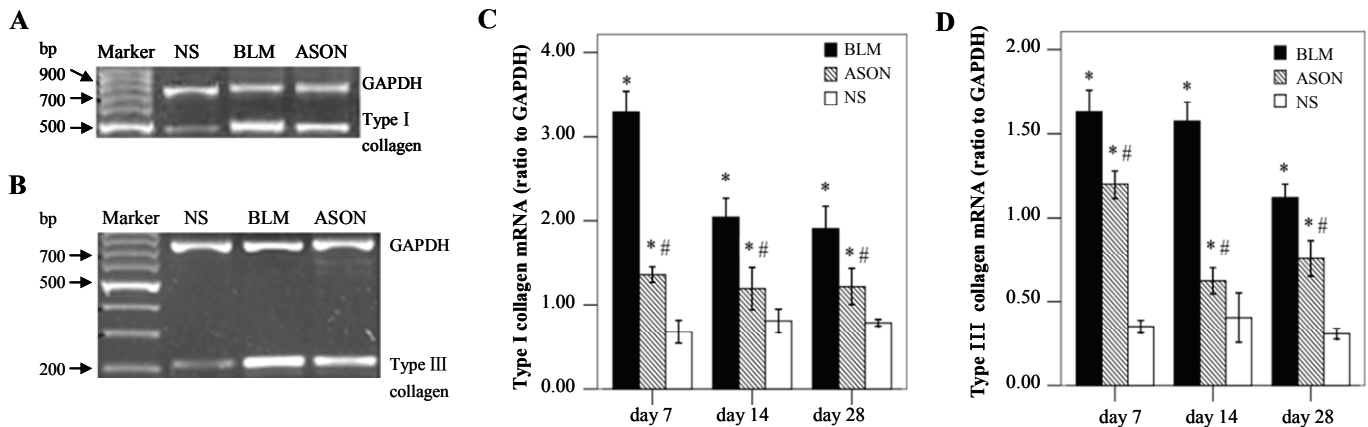

Figure 4. RT-PCR results of type I collagen (A and C) and type III collagen (B and D). The expressions of type I collagen and type III collagen mRNA in lung tissues were detected by RT-PCR. Both type I collagen and type III collagen expression data were normalized to GAPDH expression. Data are expressed as the mean ± SEM (n = 5). The expressions of type I collagen and type III collagen mRNA in the ASON group were higher than those in the NS group (*$p < 0.05$), but lower than those in the BLM group ($^{\#}p < 0.05$).

group was significantly reduced, while the concentration of IFN-γ in BALF in the ASON group was higher than that in the BLM group (Figure 6).

Expression of PDGF-A, ICAM-1, STAT1, TGF-β and Smad4 in lung tissues

In the NS group, TGF-β, STAT1, PDGF-A, ICAM-1, and Smad4 in lung tissues were weakly expressed by the alveolar epithelium and the AMs. The presence of the above proteins was remarkably up-regulated in the BLM group on day 7, and then gradually declined on days 14 and 28. However, after the rats were treated with ASON, the expressions of TGF-β, STAT1, PDGF-A, ICAM-1, and Smad4 in lung

tissues were much less prominent in intensity than those present in the rats in the BLM group at days 7, 14, and 28. Compared with the NS group, the expressions of TGF-β, STAT1, PDGF-A, ICAM-1, and Smad4 in lung tissues in the ASON group were also increased (Figures 7 and 8)

Discussion

IPF is an incurable disorder with a poor prognosis. The molecular mechanisms that lead to pulmonary fibrosis are poorly understood. AMs have been demonstrated to play a key role in the pathogenesis of IPF by releasing a variety of
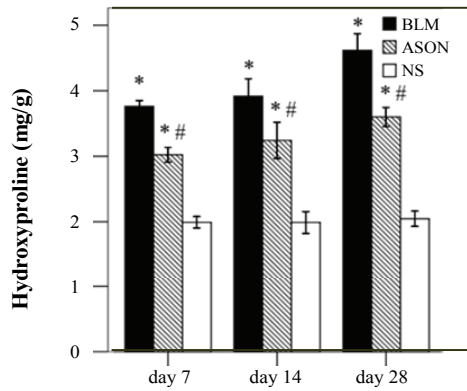
Figure 5. Hydroxyproline content of lung tissues (mg/g of wet tissue) on days 7, 14 and 28. Data are expressed as the mean ± SEM (n = 5). Compared with the NS group, hydroxyproline content at different time points in both the BLM group and the ASON group significantly increased (*p < 0.05). Compared with the BLM group, hydroxyproline content in the ASON group significantly reduced ($^{\#}p$ < 0.05).

cytokines and inflammatory mediators (4, 5). Our previous study indicated that abnormal STAT1 activation in AMs might play a pivotal role in the process of alveolitis and pulmonary fibrosis. In the present study, we found that STAT1 ASON could alleviate alveolitis and fibrosis, attenuate the expression of cytokines, including TGF-β, PDGF, STAT1, Smad4, and ICAM-1 in lung tissues, attenuate the concentration of TNF-α in BALF, suppress the decrease of the concentration of IFN-γ in BALF, and inhibit the expressions of type I and type III collagen mRNA in lung tissue in BLM-induced rat pulmonary fibrosis.

ICAM-1 is one of the STAT1-dependent immune-response genes. The mechanism of increasing ICAM-1 expression by STAT1 activation is apparent. STAT1 ASON decreased STAT1 expression, and the inhibition of STAT1 led to the down-regulation of STAT1-dependent immune-response gene ICAM-1 expression. The decreased ICAM-1 expression abated the accumulation and activation of several types of inflammatory cells in lung tissues and also decreased the release of inflammatory mediators in these inflammatory cells. Hence, reduction of TGF-β, PDGF in lung tissues and TNF-α in BALF is possibly seen as a result of the reduction of the infiltration of inflammatory cells and as a result of the decreased release by these inflammatory cells. It is also possibly seen as a result of the down-regulation of the STAT1 signaling pathway, which has been shown to be involved in cytokine effects on immunity and inflammation (9-12). Accordingly, the decreased inflammatory mediators, in turn, decreased infiltration of inflammatory cells. Such positive feedback effect may lead to the lessening of alveolitis. However, some studies indicated that STAT1-deficient (STAT1$^{-/-}$) mice exhibited more severe pulmonary fibrosis compared with wild-type STAT$^{+/-}$ mice (20). STAT1 is considered to protect the lungs against the progression of fibrosis after BLM injury, which is due to the
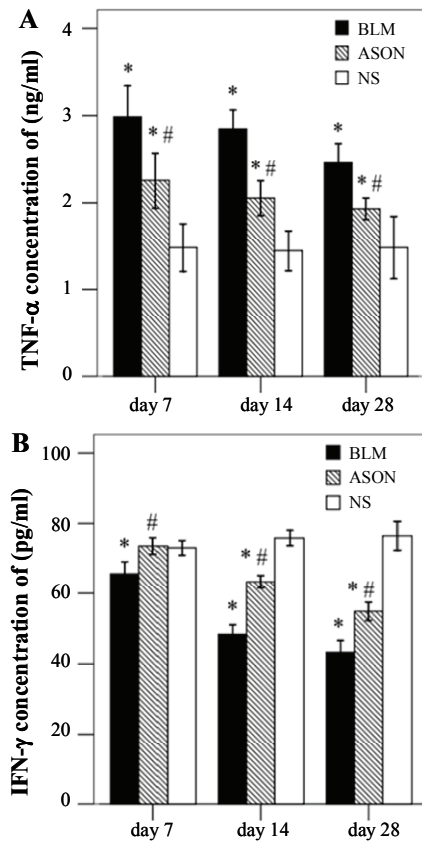

Figure 6. Effects of STAT1 ASON on the concentrations of TNF-α and IFN-γ in BALF in BLM-induced rat pulmonary fibrosis. The concentrations of TNF-α and IFN-γ in BALF were measured by ELISA. Data were expressed as the mean ± SEM (n = 5). (A) Compared with the NS group, the concentration of TNF-α in BALF in both the BLM group and the ASON group significantly increased (*p < 0.05). Compared with the BLM group, the concentration of TNF-α in the ASON group was significantly reduced ($^{\#}p$ < 0.05). (B) Compared with the NS group, the concentration of IFN-γ in the BLM group was significantly reduced at various time points (*p < 0.05). However, the data in the ASON group showed that only on days 14 and 28 the concentration of IFN-γ in the ASON group was lower than that in the NS group (*p < 0.05) and at various time points the concentration of IFN-γ was higher than that in the BLM group ($^{\#}p$ < 0.05). Hence, STAT1 ASON inhibited the increase of the concentration of TNF-α and the decline of the concentration of IFN-γ in BALF.

growth arrest/apoptosis function of this transcription factor. Moreover, IFN-γ exerts a promitogenic effect on fibroblasts in the absence of STAT1. Unlike STAT1-deficiency (STAT1$^{-/-}$) that completely prevents the expression of STAT1, STAT1 ASON could only partly inhibit the expression of STAT1. Taken together, we suggest that STAT1 has dual effects in pulmonary fibrosis: STAT1 can induce the expression of STAT1 itself, and the over-expression of STAT1 could aggravate acute injury and inflammation during initial lung injury stages and could thereby worsen fibrosis; but the proper expression of STAT1 is necessary to
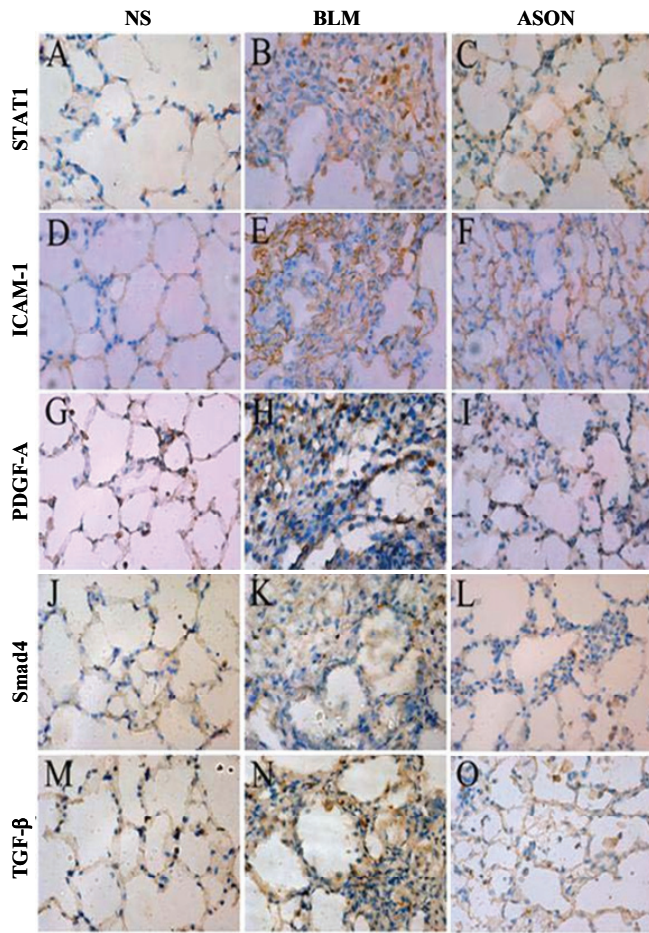
Figure 7. Immunohistochemical staining for STAT1 (A, B, C), ICAM-1 (D, E, F), PDGF-A (G, H, I), Smad4 (J, K, L), and TGF-β1 (M, N, O) in lung tissue on day 28 (×400). Weak staining was observed in the NS group (A, D, G, J, M). Positive staining was significantly enhanced in the BLM group (B, E, H, K, N). However, compared with the BLM group, the level of positive staining in the ASON group (C, F, I, L, O) was prominently attenuated.

Figure 8. Effect of STAT1 ASON on the expressions of STAT1, ICAM-1, PDGF-A, Smad4, TGF-β1 in lung tissues on days 7 (A), 14 (B) and 28 (C). Immunohistochemical results were quantified by image Pro-Plus 5.1 using integrated optical density (IOD). Data were expressed as the means ± SEM (n = 5). Compared with the NS group, the expressions of STAT1, ICAM-1, PDGF-A, Smad4 and TGF-β1 in both the BLM group and the ASON group were significantly increased (*$p < 0.05$). At different time points, the expressions of TGF-β, STAT1, PDGF-A, ICAM-1 and Smad4 in the ASON group were much less than those in the BLM group ([#]$p < 0.05$).

play a biological anti-fibrotic role for cytokine signal and normal immune responses. In the present study, on the one hand, STAT1 ASON inhibited the over-expression of STAT1. This effect was beneficial to alleviate acute injury and inflammation, so as to ameliorate pulmonary fibrosis. On the other hand, the normal function of STAT1 was not inhibited, which was enough to exert a biological anti-fibrotic role.

As we all know, TGF-β and IFN-γ exert antagonistic effects upon collagen synthesis. TGF-β is well known to be the principal factor that induces collagen gene expression, especially type I and type III collagen, and leads to tissue fibrosis through the cellular Smad signal transduction pathway (21-23). Smad2 and Smad3 are direct substrates of the TGF-β receptor kinase, and interact with Smad4, which serves as a common signal mediator. In contrast, it has been suggested that IFN-γ-induced STAT1 signal negatively regulates collagen gene transcription (24). IFN-γ abrogates
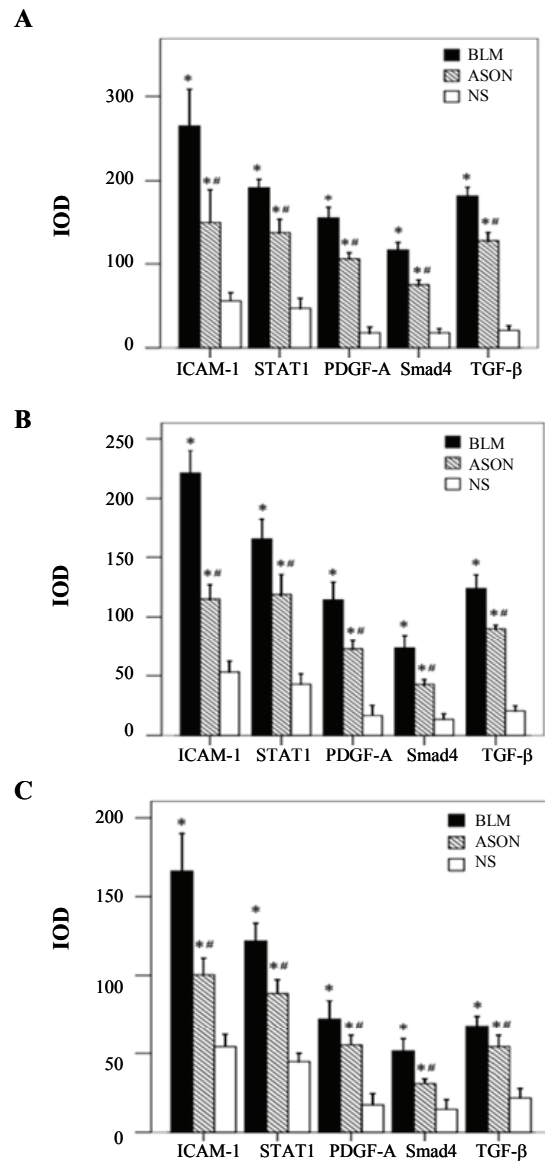
the TGF-β/Smad signaling by inducting the level of Smad7. Induction of Smad7 by IFN-γ is JAK/STAT1-dependent. At the same time, several studies have revealed the existence of JAK- or STAT1-independent IFN-γ signal pathway (25-27). Recent studies reported a novel mechanism for IFN-γ/CK2-mediated transcription regulation (28, 29). In the present

study, it is difficult to explain that IFN-γ inhibited collagen deposition resulted from the inhibition of the JAK/STAT signaling pathway. We consider that although the over-expression of STAT1 is suppressed by STAT1 ASON, the unsuppressed expression of STAT1 is also enough to exert its biological function. Recent studies have proven that the inhibition of the STAT1 signaling pathway prevented the high glucose-induced increase in TGF-β and fibronectin synthesis (30).

Accumulating evidence suggest that pulmonary fibrosis is a consequence of TNF-α expression in the lungs. Most TNF-α is produced by activated macrophages after completion of a series of reactions: precursor differentiation, priming, and a trigger reaction. The cytokine priming and trigger signals from the basis of a regulatory system that sets the threshold and determines the onset of macrophage effect or activity. Therefore, TNF-α could trigger not only an initial acute inflammatory response to tissue damage, but could provide chronic inflammatory signals that would exceed the de-activation of thresholds of fibrotic mechanisms and lead to uncontrolled wound healing. TNF-α exerts its effects through activation of TNFR1/2, which leads to a rapid activation of the JAK/STAT pathway. Some studies show macrophages produce TNF-α, depending on IFN-γ, which activates macrophages, and which has been believed to use the JAK-STAT pathway (31). In the present study, the concentration of TNF-α in BALF in rat pulmonary fibrosis treated by STAT1 ASON was decreased, and less expression of TNF-α at an early stage would cause less acute inflammation and prevent chronic inflammation.

Another important fibrogenic mediator, PDGF, induces fibroblast chemotaxis, and fibroblast proliferation, and promotes fibroblast-mediated tissue matrix contraction (32). It can also stimulate the production of ECM components, such as fibronectin and type I and type III collagen. A link between STAT signaling and PDGF gene expression has not been established. Some authors suggested that the JAK-STAT pathway contributes to PDGF-induced mitogenesis, because inhibition of the STAT kinases Src and the JAK kinases blocked PDGF-induced proliferation (33). However, in the present study, we observed that STAT1 ASON inhibited the expression of PDGF-A in lung tissues, which may have resulted from down-regulation of the STAT1 signaling pathway and lessened alveolitis. In BLM-induced pulmonary fibrosis, collagen synthesis increases and its degradation decreases. In our previous study, STAT1 ASON was shown to reduce lung fibroblast proliferation and hydroxyproline secretion *in vitro*. In the present study, after rats were aerosolized with ASON targeting STAT1, expression of type I and type III collagen mRNA and hydroxyproline secretions were decreased, and fibrotic lesions and collagen deposition in lung tissue were significantly attenuated, which resulted from the reduced alveolitis and the reduced release of inflammatory cytokines because of the inhibition of the STAT1 signaling pathway.

In conclusion, our research demonstrated that aerosolized STAT1 ASON could alleviate the extent of alveolitis and fibrosis, reduce the release of inflammatory cytokines and fibrogenic mediators such as TNF-α, PDGF-A, TGF-β, and decrease the expression of type I and type III collagen mRNA and hydroxyproline secretion. All results are due to down-regulation of the STAT1 signaling pathway and the interaction of cytokines. Aerosolized STAT1 ASON might be considered as a promising new strategy in the treatment of pulmonary fibrosis.

Acknowledgement

References

1. Gharaee-kermani M, Hu B, Thannickal VJ, Phan SH, Gyetko MR. Current and emerging drugs for idiopathic pulmonary fibrosis. Expert Opinion on Emerging Drugs. 2007;12:627-646.
2. Gross TJ, Hunninghake GW. Idiopathic pulmonary fibrosis. N Engl J Med. 2001;345:517-525.
3. Selman M, King TE, Pardo A. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. Ann Intern Med. 2001;134:136-151.
4. Scheule RK, Perkins RC, Hamilton R, Holian A. Bleomycin stimulation of cytokine secretion by the human alveolar macrophages. Am J Physiol. 1992;262:L386-L391.
5. Prasse A, Pechkovsky DV, Toews GB, et al. A vicious circle of alveolar macrophages and fibroblasts perpetuates pulmonary fibrosis *via* CCL18. Am J Respir Crit Care Med. 2006;173: 781-792.
6. O'Shea JJ, Gadina M, Schreiber RD. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. Cell. 2002;109:S121-131.
7. Levy DE, Darnell JEJ. Stats: transcription control and biological impact. Nat Rev Mol Cell Bio. 2002;3:651-662.
8. Takeda K, Akira S. STAT family of transcription factor in cytokine-mediated biological responses. Cytokine Growth Factor Rev. 2000;11:199-207.
9. Sampath D, Castro M, Look DC, Holtzman MJ. Constitutive activation of an epithelial signal transducer and activator of transcription (STAT) pathway in asthma. J Clin Invest. 1999;103: 1353-1361.
10. Ramana CV, Chatterjee-Kishore M, Nguyen H, et al. Complex roles of stat1 in regulating gene expression. Oncogene. 2000;19: 2619-2627.
11. Gharavi NM, Alva JA, Mouillesseaux KP, et al. Role of the JAK/STAT pathway in the regulation of interleukin-8 transcription by oxidized phospholipids *in vitro* and in atherosclerosis *in vivo*. J Biol Chem. 2007;282:31460-31468.
12. Fan XM, Wang ZL, Li ZH. STAT1 activation and STAT1-dependent immune-response gene ICAM-1 expression in alveolar macrophages of rats suffering from interstitial pulmonary fibrosis. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi. 2003;19:3-6.
13. Fan XM, Wang ZL. STAT1 antisense oligonucleotides attenuate the proinflammatory cytokines release by alveolar macrophages in bleomycin-induced fibrosis. Cell Mol Immunol. 2005;2: 211-217.
14. Fan XM, Liu CT, Zhan XQ, Xiong Y, Feng YL, Wang ZL. The effect of STAT1 antisense oligonucleotides on lung fibroblast

proliferation and hydroxyproline secretion. Chin J Tuberc Respir Dis. 2005;28:709-713.

15. Zeng M, Liao B, Zhu C. Wang WJ, Zhan XQ, Fan XM. Aerosolized STAT1 antisense oligodeoxynucleo-tides attenuates inflammatory mediators in bronchoalveolar lavage fluid in bleomycin-induced rat pulmonary fibrosis. Cell Mol Immunol. 2008;5:219-224.

16. Carpenter M, Epperly MW, Agarwal A,et al. Inhalation delivery of manganese superoxide dismutase-plasmid/liposomes protects the murine lung from irradiation damage. Gene Ther. 2005;12: 685-693.

17. Szapiel SV, Elson NA, Fulmer JD, Hunninghake GW, Crystal RG. Bleomycin-induced interstitial pulmonary disease in the nude athymic mouse. Am Rev Respir Dis. 1979;120:893-899.

18. Fulmer JD, Bienkowski RS, Cowan MJ, et al. Collagen concen-tration and rates of synthesis in idiopathic pulmonary fibrosis. Am Rev Respir Dis. 1980;122:289-301.

19. Yang ZH, Zhu MX, Gong YF, et al. Assay of lung hydr-oxyproline on rats and its application. Bull Acad Mil Med Sci. 1995;l9:44-46.

20. Kulozik M, Hogg A, Lankat-Buttgereit B, Krieg T. Co-localization of transforming growth factor beta 2 with alpha 1(I) procollagen mRNA in tissue sections of patients with systemic sclerosis. J Clin Invest. 1990;86:917-924.

21. Peltonen J, Kahari L, Jaakkola S, et al. Evaluation of trans-forming growth factor β and type I procollagen gene expression in fibrotic skin diseases by *in situ* hybridization. J Invest Dermatol. 1990;94:365-371.

22. Jacob G, Roulot D, Koteliansky V E, et al. *In vivo* inhibition of rat stellate cell activation by soluble transforming growth factor β type II receptor: A potential new therapy for hepatic fibrosis. Proc Natl Acad Sci U S A.1999;96:12719-12724.

23. Walters DM, Antao-Menezes A, Ingram JL, et al. Susceptibility of signal transducer and activator of transcription-1-deficient mice to pulmonary fibrogenesis. Am J Pathol. 2005;167:1221-1229.

24. Ghosh AK, Yuan WH, Mori Y, Chen Sj, Varga J. Antagonistic regulation of type I collagen gene expression by interferon-γ and transforming growth factor-β. Integration at the level of p300/CBP transcriptional coactivators. J Biol Chem. 2001;276: 11041-11048.

25. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. Annu Rev Biochem. 1998;67: 227-264.

26. Remana CV, Gill MP, Han Y, Ransohoff RM, Schreiber RD, Stark GR. Stat1-independent regulation of gene expression in response to IFN-γ. Proc Natl Acad Sci U S A. 2001;98:6674-6679.

27. Hu J, Roy SK, Shapiro PS, et al. ERK1 and ERK2 activate CCAAAT/enhancer binding protein-β dependent gene trans-cription in response to interferon-γ. J Biol Chem. 2001;276: 287-297.

28. Mead JR, Hughes TR, Irvine SA , Singh NN, Ramji DP. Interferon-γ stimulates the expression of the inducible cAMP early repressor in macrophages through the activation of casein kinase 2. A potentially novel pathway for interferon-γ-mediated inhibition of gene transcription. J Biol Chem. 2003;278:17741-17751.

29. Hughes TR, Tengku-Muhammad TS, Irvine SA, Ramji DP. A novel role of Sp1 and Sp3 in the interferon-γ-mediated suppression of macrophage lipoprotein lipase gene transcription. J Biol Chem. 2002;277:11097-11106.

30. Wang XD, Shaw S, Amiri F, Eaton DC, Marrero MB. Inhibition of the JAK/STAT signaling pathway prevents the high glucose-induced increase in TGF-β and fibronectin synthesis in mesangial cells. Diabetes. 2002;51:3505-3509.

31. Rendon-Mitchell B, Ochani M, Li J, et al. IFN-γ induces high mobility group box 1 protein release partly through a TNF-α dependent mechanism. J Immunol. 2003;170:3890-3897.

32. Bonner JC. Regulation of PDGF and its receptors in fibrotic diseases. Cytokine Growth Factor Rev. 2004;15:255-273.

33. Simon AR, Takahashi S, Severgnini M, Fanburg BL, Cochran BH. Role of the JAK-STAT pathway in PDGF-stimulated proliferation of human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol. 2002;282:L1296-L1304.