Original Paper

A STAT6 Inhibitor AS1517499 Reduces Preventive Effects of Apoptotic Cell Instillation on Bleomycin-Induced Lung Fibrosis by Suppressing PPAR γ

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Key Words

Stat6 • PPAR γ • Apoptotic cells • Macrophages • Lung fibrosis

Abstract

Background/Aims: The signal transducer and activator of transcription 6 (STAT6) transcription factor mediates PPAR γ -regulated gene expression in macrophages. However, it remains largely unknown how proximal membrane signaling events initiated by apoptotic cell recognition upregulate PPAR γ expression and activate the lung homeostatic program. **Methods:** The STAT6 inhibitor AS1517499 was used to determine the role of STAT6 in mediating PPAR γ activity, anti-inflammatory effects, and anti-fibrotic effects induced by apoptotic cell instillation after bleomycin treatment into C57BL/6 mice. Bronchoalveolar lavage fluid, alveolar macrophages and lungs were harvested at days 2, 7, and 14 and then analyzed by real-time PCR, immunoblotting, ELISA, immunocytochemistry and immunohistochemistry assays. **Results:** Our data demonstrate that apoptotic cell instillation after bleomycin results in prolonged enhancement of STAT6 phosphorylation in alveolar macrophages and lung. Co-administration of the STAT6 inhibitor, AS1517499, reversed the enhanced PPAR γ expression and activity induced by apoptotic cell instillation after bleomycin treatment. By reducing the expression of PPAR γ target genes, including CD36, macrophage mannose receptor, and arginase 1, AS1517499 inhibited efferocytosis and restored pro-inflammatory cytokine expression, neutrophil recruitment, protein levels, hydroxyproline content, and expression of fibrosis markers, including type 1 collagen α 2, fibronectin, and α -smooth muscle actin. STAT6 inhibition reversed the expression profile of hepatocyte growth factor and interleukin-10. **Conclusion:** These results indicate that prolonged STAT6 activation following one-time apoptotic cell instillation facilitates continuous PPAR γ activation, resulting in the resolution of bleomycin-induced lung inflammation and fibrosis.

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Introduction

Idiopathic pulmonary fibrosis (IPF) is characterized by continuous alveolar epithelial injury and dysregulated tissue repair, resulting in excessive accumulation of fibroblasts and myofibroblasts, deposition of extracellular matrix components and distortion of lung architecture, eventually leading to respiratory failure. IPF results in a life expectancy of 2-6 years after diagnosis [1]. Currently, no effective therapies have been developed to cure or even halt the progression of fibrosis [2]. Several studies have demonstrated that PPAR γ play important roles in regulating processes related to lung fibrosis and human IPF. In particular, Milam et al [3]. demonstrated that treatment of lung fibroblasts obtained from IPF patients or normal human controls with PPAR γ agonists results in inhibition of proliferative responses and induces cell cycle arrest. The PPAR γ agonists inhibited fibrosis, as assessed by measurement of lung hydroxyproline and/or collagen and by trichrome staining in the bleomycin-induced murine model [4, 5].

Several nuclear receptors, including peroxisome proliferator-activated receptor gamma (PPAR γ) and liver X receptor (LXR), have been linked to a dual role of macrophages in the rapid, efficient efferocytosis of dying cells and their alternative activation [6-10]. Previously, we demonstrated that apoptotic cell instillation resulted in immediate and prolonged enhancement of PPAR γ expression and activity in the lung over 21 days after bleomycin (BLM) treatment [11]. In contrast, viable cell instillation had no effect. Induction of PPAR γ alternatively programmed alveolar macrophages associated with enhanced efferocytic ability and coordinated the regulation of HGF, IL-10, and TGF- β in favor of resolving inflammation and preventing excessive fibrotic outcomes. The expression and activity of PPAR γ in various cell types are strictly regulated [12-14], and a variety of endogenous ligands can activate PPAR γ in different cell types [4, 15]. However, PPAR γ expression and the presence of appropriate ligands are usually not sufficient to elicit optimal or maximal responses [16].

Here we aimed to understand the impact of proximal signal transduction leading to PPAR γ activation in lung by apoptotic cell instillation after BLM treatment in an *in vivo* murine model. Signal transducer and activator of transcription 6 (STAT6) acts as a facilitating factor in macrophages for PPAR γ by promoting DNA binding and consequently increasing gene expression and the magnitude of responses [16]. However, it remains unknown how proximal membrane signaling events initiated by apoptotic cell recognition upregulate PPAR γ expression and activate the lung homeostatic program.

In the present study, we investigated the role of STAT6 in enhancing PPAR γ expression and activity by apoptotic cell instillation after BLM treatment. Furthermore, we examined whether STAT6 inhibition reverses the anti-inflammatory and anti-fibrotic effects induced by these conditions in the mouse lung. To investigate the effect of the pharmacologic inhibition of the STAT6 pathway, we used the inhibitor AS1517499-previously used in several *in vivo* models including, antigen-induced bronchial hyper-reactivity [17], Graves' disease [18], and tumor growth and metastasis in a mammary carcinoma [19].

Materials and Methods

Reagents

BLM and paraformaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). The antibodies used for western blotting were as follows: anti-phospho STAT6 (Tyr-641), anti-STAT6, anti-PPAR γ , anti-type 1 collagen α 2, anti-fibronectin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho JAK3 (Tyr-980/981), anti-JAK3 (Cell signalling Technology, Danvers, MA), anti- α -smooth muscle actin (α -SMA) (Abcam, Cambridge, MA), and anti- α -tubulin (Sigma-Aldrich). AS1517499 (Axon medchem BV, Groningen, Netherlands), PD146176 (Cayman Chemical Co., Ann Arbor, MI) were used as supplied. Pierce BCA protein assay kit was purchased from Thermo Scientific (Rockford, IL).

Animal protocols

Specific pathogen-free male C57BL/6 mice (Orient Bio, Sungnam, Korea) weighing 20-25 g were used in all experiments. The Animal Care Committee of the Ewha Medical Research Institute approved the

experimental protocol. Mice were cared for and handled in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals. Mouse pharyngeal aspiration was used to administer the solution containing BLM (5 U/kg body weight in 30 μ l) [20, 21]. Two days after BLM treatment, saline alone, or 10×10^6 apoptotic or viable Jurkat cells in 50 μ l saline were administered intratracheally (*i.t.*) through pharyngeal aspiration [11, 22, 23].

For the immediate inhibition experiments, the specific STAT6 inhibitor AS1517499 (10 mg/kg, *i.p.*; dissolved in 20% DMSO in saline) [17] was administered one hour before *i.t.* instillation of 10×10^6 apoptotic cells at 2 days post- BLM treatment. After the initial dose, AS1517499 or its vehicle was administered once every two days. The 12/15-lipoxygenase (LO) inhibitor PD146176 (50 mg/kg, *i.p.*) [24] was administered with *i.t.* instillation of 10×10^6 apoptotic cells at 2 days post- BLM treatment. After the initial dose, the inhibitor was administered once a day.

BAL cells, lung tissue, and cell counts

Mice were euthanized at 2 h after apoptotic cell instillation or on days 7 and 14 after BLM treatment. Following BAL, the cell count was determined using an electronic Coulter Counter fitted with a cell sizing analyzer (Coulter Model ZBI with a channelizer 256; Coulter Electronics, Bedfordshire, UK) [25, 26]. BAL cells were isolated and then cytospun to assess phagocytic indices by immunocytochemistry [20, 22]. The phagocytic index was calculated using the following formula: ((number of apoptotic bodies)/(200 total macrophages) $\times$ 100). The After BAL, lungs were removed, immediately frozen in liquid nitrogen, and stored at -70°C .

Induction of apoptosis

Human T lymphocyte Jurkat cells were obtained from the American Type Culture Collection (Rockville, MD). Apoptosis was induced by UV irradiation at 254 nm for 10 min. The cells were then incubated for 2.5 h before use. They were approximately 70% apoptotic by evaluation of nuclear morphology by light microscopy [22, 27].

Quantitative real-time PCR (qPCR)

Gene expression was analyzed using real-time qPCR on a StepOnePlus system (Applied Biosystems, Life Technologies, Carlsbad, CA). For each qPCR assay, a total of 50 ng of cDNA was used. The primers used were as follows (name: forward primer, reverse primer). For mice, *PPAR γ* : 5'-GCCCTTTGGTGACTT-TATGG-3', 5'-CAGCAGGTTGTCTTGGATGT-3'; *CD36*: 5'-TTGTACCTATACTGTGGCTAAATGAGA-3', 5'-CTTGT-GTTTTGAACATTCTGCTT-3'; *MMR*: 5'-AGAAAATGCACAAGAGCAAGC-3', 5'-GGAACATGTGTTCTGCGTTG-3'; *Arg1*: 5'-GTGGGGAAAGCCAATGAAG-3', 5'-GCTTCCAAGTCCAGACTGT-3'; *TNF- α* : 5'-CCCCAAAGGGATGAGA-AGTT-3', 5'-CACTTGGTGGTTTGCTACGA-3'; *MIP-2*: 5'-AGACAGAAGTCATAGCCACTCTCAAG-3', 5'-CCTCCTTTC-CAGGTCAGTTAGC-3'; *HGF*: 5'-CACCCCTTGGGAGTATTGTG-3', 5'-GGGACATCAGTCTCATTACAG-3'; *IL-10*: 5'-GCTCTTACTGACTGGCATGAG-3', 5'-CGCAGCTCTAGGAGCATGTG-3'; *IL-4*: 5'-CGAGCTCACTCTGTGGTG-3', 5'-TGAACGAGGTCACAGGAGAA-3'; *IL-13*: 5'-CCTGGCTCTTGCTTGCCTT-3', 5'-GGTCTGTGTGATGTGCT-CA-3'; *HPRT*: 5'-CAGACTGAAGAGCTACTGTAATG-3', 5'-CCAGTGCAATTATATCTTCAAC-3'. mRNA levels were normalized on the basis of *HPRT* mRNA [28] and are reported as fold change in expression over the control group.

Western blot analysis

Lung tissue homogenate samples and total cell lysates (50 μ g protein/lane) were separated on 10% SDS-polyacrylamide gels. Separated proteins were electrophoretically transferred onto nitrocellulose paper. The membranes were probed with specific antibodies to phospho-STAT6/SAT6, phospho-JAK3/JAK3, PPAR γ , type 1 collagen α 2, fibronectin, α -SMA, or α -tubulin and visualized by chemiluminescence.

Measurement of PPAR γ activity

PPAR γ activity was determined in nuclear extracts (8 μ g) isolated from lung tissue prepared using a kit and TRANSAM assay (Activ Motif, Carlsbad, CA) according to the manufacturer's instructions.

Measurement of total protein concentration

Protein concentrations of the BAL samples were used as indicators of blood-pulmonary epithelial cell barrier integrity. Total protein content in BAL fluid was measured according to the manufacturer's protocols.

Enzyme-Linked Immunosorbent Assay (ELISA)

BAL fluid samples were assayed using TNF- α , MIP-2, HGF, IL-10, 15-HETE, or lipoxin-A₄ ELISA kits as per the manufacturer's instructions (R&D Systems, Minneapolis, MN). The activity of myeloperoxidase (MPO) in lung homogenates was estimated by using a specific ELISA kit.

Immunocytochemistry

STAT6 phosphorylation and PPAR γ expression were evaluated in cytospin preparations from freshly isolated alveolar macrophages by immunocytochemistry. Cells were fixed with 4% paraformaldehyde, permeabilized with Triton X-100, and stained with mouse monoclonal anti-phospho-STAT6 or anti-PPAR γ antibody (Santa Cruz) overnight at 4°C. Subsequently, cells were washed with PBS three times and incubated with fluorescent isothiocyanate-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). The slides were mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Inc., Youngstown, OH). All slides were imaged using a confocal microscope (LSM5 PASCAL; Carl Zeiss, Jena, Germany) equipped with a filter set with excitation at 488 and 543 nm.

Measurement of hydroxyproline

Lung hydroxyproline content was measured using a hydroxyproline assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) [29]. The hydroxyproline content was expressed in micrograms per milligram of wet weight ($\mu\text{g}/\text{mg}$ wet lung tissue).

Lung histology

Lung tissue was fixed with 10% buffered formalin and embedded in paraffin. Sections 4 μm -thick were stained with hematoxylin-eosin (H&E) and with Masson's trichrome to evaluate inflammation and collagen deposition, respectively. Fibrosis was quantified using the entire lung by the Ashcroft scoring system [30]. The degree of fibrosis was graded from 0 (normal lung) to 8 (total fibrosis). The mean score from all fields at $\times 200$ magnification (more than 20 fields/lung section) was taken as the fibrosis score. To evaluate expression of phospho-STAT6 on macrophages and fibroblasts, immunofluorescent staining for the macrophage-specific marker Mac3 (BD Pharmingen, San Jose, CA), fibroblast-specific marker S100A4 (Thermo Fisher Scientific, Fremont, CA), and phospho-STAT6 was performed. The sections were incubated with Texas Red-conjugated anti-mouse IgG and FITC-conjugated goat anti-rat IgG (Vector Laboratories, Inc). The sections washed with TBS between all steps. The sections were mounted in Vectashield mounting medium with DAPI. All slides were imaged using a confocal microscope.

Statistical analysis

Values are expressed as the mean $\pm$ SEM. Analysis of variance (ANOVA) was performed for multiple comparisons and Tukey's post hoc test was applied where appropriate. Student's *t*-tests were performed for comparisons of two sample means. A *p*-value less than 0.05 was considered statistically significant. All data were analyzed using JMP software (SAS Institute, Cary, NC).

Results

Apoptotic cell instillation into BLM-stimulated lungs enhances STAT6 activation

Our data demonstrate that compared with animals in the BLM + saline (Sal) group, STAT6 phosphorylation in lung tissue was enhanced 2 h after apoptotic cell instillation and persisted until 14 days after BLM treatment (Fig. 1A). In addition, confocal microscopy revealed enhanced STAT6 phosphorylation in alveolar macrophages in the BLM + apoptotic Jurkat cells (ApoJ) group on days 2 and 7 after BLM treatment, compared to the BLM + Sal group (Fig. 1B). However, viable cell instillation had no effect (for all online suppl. material, see www.karger.com/doi/10.1159/000487877, Suppl. Fig. 1). Co-administration of the specific STAT6 inhibitor AS1517499 with apoptotic cells reversed the enhanced STAT6 phosphorylation on days 2, 7, and 14 after BLM treatment. However, the inhibitor administered with buffer had no effects. STAT6 phosphorylation was barely detectable in mice treated with saline, DMSO (vehicle) or AS1517499 in lung tissue and/or alveolar

Fig. 2. Phospho-STAT6-positive staining in alveolar macrophages and lung fibroblasts after apoptotic cell instillation. Two days after bleomycin (BLM) treatment, lungs were instilled with saline alone (Sal) or apoptotic Jurkat cells (ApoJ) intratracheally. Where indicated, the first dose of 10 mg/kg AS1517499 (AS, i.p.) was administered 1 h before ApoJ instillation into BLM-treated lungs (2 days) and once every two days thereafter. (A, B) Immunofluorescence staining for phospho-STAT6 (red) in macrophage-specific marker (Mac3, green) or fibroblast-specific marker (S100A4, green) was performed in lung sections on day 14 following BLM treatment. Arrowheads indicate colocalization of phospho-STAT6 in alveolar macrophages or lung fibroblasts. The imaging medium was Vecta-shield fluorescence mounting medium containing DAPI. Scale bars = 10 μ m. Representative images were obtained from three mice in each group.

macrophages and lung tissue and lung activity PPAR γ activity on days 2, 7, and 14 after BLM compared to the BLM group (Fig. 3A-D). Treatment of mice with vehicle or AS1517499 had no effect compared to the saline group (Fig. 3A-D; see online suppl. material, Suppl. Fig. 2B). AS1517499 co-administration with apoptotic cells significantly reversed the increase in PPAR γ mRNA and protein level in alveolar macrophages and lung tissue as well as PPAR γ activity in lung tissue (Fig. 3A-D). Confocal microscopy confirmed this reduction in PPAR γ expression within the nuclei of alveolar macrophages on days 2 and 7 after BLM treatment (Fig. 3E). On days 2-14 after BLM treatment, AS1517499 also reversed apoptotic cell-induced enhancement of PPAR γ target molecules involved in alternative macrophage programming [9, 31, 32], including CD36, MMR (Fig. 4A-C), Arg1 (see online suppl. material, Suppl. Fig. 3A, B), in alveolar macrophages and lung tissue.

We also assessed whether STAT6 activation contributes to increasing alveolar macrophage efferocytosis following apoptotic cell instillation. AS1517499 co-administration significantly inhibited the enhanced efferocytic ability of alveolar macrophages by apoptotic cell instillation throughout days 2-14 after BLM treatment (Fig. 4D, E). Treatment with the inhibitor and buffer produced no effect on PPAR γ , its target molecules, and efferocytosis.

12/15-lipoxygenase inhibition does not reduce enhanced expression of PPAR γ and its target genes

12/15-LO is involved in IL-4-dependent enhancement of PPAR γ expression and activation via production of PPAR γ ligands, such as 15-hydroxyeicosatetraenoic (HETE) and lipoxin-A4 [33, 34]. Thus, we examined whether 12/15-LO also participates in enhancing PPAR γ expression and functional activation by apoptotic cell instillation after BLM treatment using PD146176, a specific inhibitor of 12/15-LO. PD146176 co-administration did not alter apoptotic cell-induced expression of PPAR γ mRNA and target genes on days 2 and 7

Fig. 3. Inhibition of STAT6 reverses enhanced PPAR γ expression and activity by apoptotic cell instillation. After intratracheal instillation of saline alone (Sal), viable Jurkat cells (ViaJ), or apoptotic Jurkat cells (ApoJ) at 2 days post-bleomycin (BLM) treatment, lavage supernatant was collected at 2 h after ApoJ instillation and on days 7 and 14 after BLM treatment. Where indicated, the first dose of 10 mg/kg AS1517499 (AS, i.p.) or its vehicle (Veh; 20% DMSO in saline) was administered 1 h before ApoJ instillation into saline or BLM-treated lungs (2 days) and once every two days thereafter. (A, B) 2–14 days after BLM treatment, PPAR γ mRNA levels in alveolar macrophages and lung tissue were analyzed by real-time PCR and normalized to HPRT mRNA levels. Results are reported as fold change from the control group. (C) Western blot analysis of PPAR γ protein in lung tissue homogenates at 2 h after ApoJ instillation and on days 7 and 14 after BLM treatment. The relative densitometric intensity was determined for each band and normalized to α -tubulin. (D) PPAR γ activity in nuclear extracts from lung tissue was analyzed at 2 h after ApoJ instillation and on days 7 and 14 after BLM treatment as described in Materials and Methods. (A–D) Values represent the mean $\pm$ SEM from five mice in each group. * $P < 0.05$ compared with saline control; * $P < 0.05$ for BLM + ApoJ vs. BLM + Sal; # $P < 0.05$ for BLM + ApoJ vs. BLM + ApoJ + AS. (E) Immunofluorescence staining (red) of PPAR γ in alveolar macrophages at 2 h after ApoJ instillation and at 7 days after BLM treatment. The imaging medium was Vectashield fluorescence mounting medium containing DAPI. Scale bars = 20 μ m. Representative results from three mice per group are shown.

after BLM treatment (see online suppl. material, Suppl. Fig. 4). Moreover, apoptotic cells did not increase 15-HETE and lipoxin-A4 production in bronchoalveolar lavage (BAL) fluid compared to the BLM + Sal group (see online suppl. material, Suppl. Fig. 5A, B). These data suggest that 12/15 LO-derived 15-HETE and lipoxin-A4 are not involved in enhancing PPAR γ expression and activation by apoptotic cell instillation into BLM-stimulated lung.

Fig. 4. Inhibition of STAT6 reverses enhanced expression of PPAR γ target genes and efferocytic ability induced by apoptotic cell instillation. After intratracheal instillation of saline alone (Sal) or apoptotic Jurkat cells (ApoJ) at 2 days post-bleomycin (BLM) treatment, lavage supernatant was collected at 2 h after ApoJ instillation and on days 7 and 14 after BLM treatment. Where indicated, the first dose of 10 mg/kg AS1517499 (AS, i.p.) or its vehicle (Veh; 20% DMSO in saline) was administered 1 h before ApoJ instillation into saline or BLM-treated lungs (2 days) and once every two days thereafter. (A, B) 2–14 days after BLM treatment, mRNA levels of PPAR γ target molecules, including CD36 and MMR in alveolar macrophages and lung tissue, were analyzed by real-time PCR and normalized to HPRT mRNA levels. Results are reported as fold change from the control group. (C) Western blot analysis of CD36 and MMR protein in lung tissue homogenates at 2 h after ApoJ instillation and on days 7 and 14 after BLM treatment. The relative densitometric intensity was determined for each band and normalized to α -tubulin. (D) Phagocytic indices were measured in BAL alveolar macrophages. (E) Representative photomicrographs showing cytopsin-stained BAL cells at 2 h after apoptotic cell instillation into the BLM-stimulated lung. Arrowheads indicate alveolar macrophages with engulfed apoptotic cells or fragments. Scale bars = 50 μ m. Values represent the mean $\pm$ SEM of results from five mice in each group. * P <0.05 compared with saline control; * P <0.05 for BLM + ApoJ vs. BLM + Sal; # P <0.05 for BLM + ApoJ vs. BLM + ApoJ + AS.

homogenates at 2 h after ApoJ instillation and on days 7 and 14 after BLM treatment. The relative densitometric intensity was determined for each band and normalized to α -tubulin. (D) Phagocytic indices were measured in BAL alveolar macrophages. (E) Representative photomicrographs showing cytopsin-stained BAL cells at 2 h after apoptotic cell instillation into the BLM-stimulated lung. Arrowheads indicate alveolar macrophages with engulfed apoptotic cells or fragments. Scale bars = 50 μ m. Values represent the mean $\pm$ SEM of results from five mice in each group. * P <0.05 compared with saline control; * P <0.05 for BLM + ApoJ vs. BLM + Sal; # P <0.05 for BLM + ApoJ vs. BLM + ApoJ + AS.

STAT6 inhibition attenuates the anti-inflammatory effects of apoptotic cell instillation and reverses the expression profiles of pro-resolving cytokines

We investigated whether inhibition of apoptotic cell-enhanced STAT6 activation affects TNF- α and MIP-2 production and other inflammatory responses, which we examined by assessing neutrophil numbers and total protein levels in BAL fluid. Our data demonstrate that AS1517499 co-administration reversed the down-regulation of TNF- α and MIP-2 mRNA

Fig. 5. Inhibition of STAT6 abolishes the anti-inflammatory effects of apoptotic cell instillation. After intratracheal instillation of saline alone (Sal) or apoptotic Jurkat cells (ApoJ) at 2 days post-bleomycin (BLM) treatment, lavage supernatant was collected at 2 h after ApoJ instillation and on days 7 and 14 after BLM treatment. Where indicated, the first dose of 10 mg/kg AS1517499 (AS, i.p.) or its vehicle (Veh; 20% DMSO in saline) was administered 1 h before ApoJ instillation into saline or BLM-treated lungs (2 days) and once every two days thereafter. (A, B, D, E) TNF- α and MIP-2 mRNA levels in alveolar macrophages and lung homogenates were analyzed by real-time PCR at 2 h after ApoJ instillation and normalized to HPRT mRNA levels. Results are reported as fold change from the control group. (C, F) TNF- α and MIP-2 protein levels in the BAL fluid were quantified by ELISA at 2 h after ApoJ instillation. (G) Neutrophil numbers, (H) alveolar macrophage numbers, (I) total protein levels in the BAL fluid, and (J) MPO activity in lung tissue were quantified. Values represent the mean $\pm$ SEM from five to ten mice in each group. * P <0.05 compared with saline control; * P <0.05 for BLM + ApoJ vs. BLM + Sal; # P <0.05 for BLM + ApoJ vs. BLM + ApoJ + AS. (K) Hematoxylin and eosin stains of lung sections at 7 days post-BLM treatment. Scale bars = 20 μ m. Representative results from three mice are shown for each group.

in BLM-treated alveolar macrophages and lung tissue at 2 h after apoptotic cell instillation (Fig. 5A, B, D, E). AS1517499 also restored the protein level of these cytokines in BAL fluid (Fig. 5C, F) at 2 days after BLM treatment. Moreover, inhibition of STAT6 signaling restored the numbers of neutrophils and alveolar macrophage, total protein level in BAL fluid, and MPO activity in lung tissue on days 7 and/or 14 after BLM treatment (Fig. 5G-J). However, co-administration of AS1517499 with buffer had no effect throughout the time period

Fig. 6. STAT6 plays a role in altering the expression profile of pro-resolving cytokines following apoptotic cell instillation. After intratracheal instillation of saline alone (Sal) or apoptotic Jurkat cells (ApoJ) at 2 days post-bleomycin (BLM) treatment, lavage supernatant was collected at 2 h after ApoJ instillation and on days 7 and 14 after BLM treatment. Where indicated, the first dose of 10 mg/kg AS1517499 (AS, i.p.) or its vehicle (Veh; 20% DMSO in saline) was administered 1 h before ApoJ instillation into saline or BLM-treated lungs (2 days) and once every two days thereafter. HGF and IL-10 mRNA levels in alveolar macrophages (A, D) and lung homogenates (B, E)

were analyzed by real time-PCR and normalized to HPRT mRNA levels. Results are reported as fold change from the control group. HGF and IL-10 protein levels in BAL fluid (C, F) were quantified by ELISA. Values represent the mean $\pm$ SEM from five mice in each group. * P <0.05 compared with saline control; * P <0.05 for BLM + ApoJ vs. BLM + Sal; * P <0.05 for BLM + ApoJ vs. BLM + ApoJ + AS.

examined. Histology revealed that long-term STAT6 inhibition reversed the decrease in inflammatory cells residing within alveolar spaces and lung parenchyma, as well as alveolar structure degradation, in lung tissue instilled with apoptotic cells (Fig. 5K).

AS1517499 co-administration reversed the immediate and prolonged enhancement of HGF and IL-10 mRNA in alveolar macrophages and lung tissue and protein in BAL fluid following apoptotic cell instillation after BLM treatment (Fig. 6A-F). However, AS1517499 co-administration with buffer yielded no effect.

STAT6 inhibition attenuates anti-fibrotic effects of apoptotic cell instillation

We next examined the role of STAT6 in mediating the anti-fibrotic effects of apoptotic cell instillation by assessing apoptotic activity and the expression of fibrosis markers in experimental lung samples. AS1517499 co-administration reversed the reduction of matrix markers of fibroproliferation, such as type 1 collagen α 2 and fibronectin, and a marker of myofibroblast differentiation, α -SMA, at day 14 after BLM treatment (Fig. 7A; see online suppl. material, Suppl. Fig. 6). Hydroxyproline, a marker of collagen content in lung tissue, was also restored by this inhibitor (Fig. 7B). Treatment with inhibitor alone produced no effect. Furthermore, trichrome staining revealed that long-term STAT6 inhibition restored collagen-positive interstitial areas within damaged alveolar structures at day 14 after BLM treatment (Fig. 7C). Histopathological evaluation of lung fibrosis was further performed using an established Ashcroft scoring method [30]. AS1517499 co-administration reversed the decreased score by apoptotic cell instillation after BLM treatment (Fig. 7D).

STAT6 inhibition reduces survival rate following apoptotic cell instillation

Of the mice treated with BLM, ~50% died within 14 days after BLM treatment (Fig. 7E). Our data show that apoptotic cell instillation reduced the death rate (20% mortality). However, AS1517499 co-administration with apoptotic cells reversed this reduction (40% mortality).

Fig. 7. Inhibition of STAT6 reverses the reduced fibrotic response and enhanced survival rate following apoptotic cell instillation. After intratracheal instillation of saline alone (Sal) or apoptotic Jurkat cells (ApoJ) at 2 days post-bleomycin (BLM) treatment, lavage supernatant was collected at 2 h after ApoJ instillation and on day 14 after BLM treatment. Where indicated, the first dose of 10 mg/kg AS1517499 (AS, i.p.) or its vehicle (Veh; 20% DMSO in saline) was administered 1 h before ApoJ instillation into saline or BLM-treated lungs (2 days) and once every two days thereafter. (A) Western blot analysis using anti-type 1 collagen $\alpha 2$ (Col1), anti-fibronectin (Fn), or anti- α -smooth muscle actin (SMA) antibodies was performed to assess the level of Col1, Fn, and α -SMA protein in lung tissue homogenates. The relative densitometric intensity was determined for each band and normalized to α -tubulin. (B) Collagen deposition in the whole lung was determined by measuring hydroxyproline content on day 14. Values represent the mean $\pm$ SEM from five mice in each group. * $P < 0.05$ compared with saline control; * $P < 0.05$ for BLM + ApoJ vs. BLM + Sal; # $P < 0.05$ for BLM + ApoJ vs. BLM + ApoJ + AS. (C) Lung sections were visualized with Masson's trichrome staining on day 14. Scale bars = 50 μ m. Representative results from three mice per group are shown. (D) Ashcroft scoring of the lung sections. (E) The percentage of surviving mice was plotted over a 14-day period. Data were collected from 20 mice in each group.

Discussion

Recent *in vivo* studies suggest that the modulation of PPAR γ activity in the lung can influence inflammatory and immune response in some pulmonary diseases, such as COPD and asthma. Additionally, treatment with PPAR γ ligands in a variety of mouse models ameliorated lung fibrosis [4, 5]. Of interest, the importance of PPAR γ signaling pathway in lung fibrosis was demonstrated in a very recent study dealing with the anti-fibrotic effectiveness of a synthetic analogue of tetrahydrocannabinol (Ajulemic acid). Like very few other compounds this drug exerts potent anti-fibrotic effect when given during the fibrogenic phase of BLM lung [35]. Our previous studies demonstrated that anti-fibrotic effects of apoptotic cell instillation after bleomycin treatment were significant on days 14 and 21 after BLM treatment [11, 20]. In addition, anti-EMT effect of apoptotic cell instillation on primary type II alveolar epithelial cells was determined at 14 days after BLM treatment [36]. Although fibrotic response including hydroxyproline levels at 21 days after bleomycin is greater than those at 14 days, fibrotic response day 14 after bleomycin is prominent. Thus, in the present study, we decided the experimental plan up to 14 days after bleomycin and examined whether pharmacologic inhibition of STAT6 reverses the anti-inflammatory and anti-fibrotic effects induced by these conditions in the mouse lung.

Many endogenous PPAR γ ligands, such as fatty acids, prostaglandin D₂ (PGD₂)-derived 15deoxy-12, 14 PGJ₂, and lysophosphatidic acid, can activate PPAR γ in a variety of cell types

[4, 15]. Indeed, PPAR γ and its ligands, including 15-HETE and lipoxin-A4, are elevated in alveolar macrophages isolated from patients with acute lung injury [37, 38]. In our studies, we found that 15-HETE and lipoxin-A4 production were not further increased by apoptotic cell instillation relative to BLM + Sal. Moreover, 12/15 LO inhibition by PD146174 did not significantly alter the expression of PPAR γ and its direct targets in lung cells following apoptotic cell instillation after BLM treatment. These data indicate that membrane-associated signaling pathways, rather than PPAR γ ligands, may be major contributors to the enhanced PPAR γ signaling observed after apoptotic cell instillation. Consistent with this, Kiss et al [32] demonstrated that a homeostatic program in phagocytes includes a proximal membrane signaling event initiated by phosphatidylserine recognition, a downstream signaling event occurring through nuclear receptors.

STAT6 has been reported to facilitate PPAR γ expression and its transcriptional activity in macrophages [7]. It will be important to determine the relevance of PPAR γ -STAT6 interaction in disease pathogenesis, immunological responses, and therapeutic intervention [39]. Here, we investigated whether STAT6 signaling plays a critical role in enhancing PPAR γ expression and activation in alveolar macrophages and lung cells induced by apoptotic cell instillation following BLM treatment. We first demonstrated further enhancement of STAT6 phosphorylation following apoptotic cell instillation. Immunocytochemical analysis showed STAT6 phosphorylation and nuclear translocation in alveolar macrophages, which was substantial and sustained after apoptotic cell instillation. However, STAT6 phosphorylation was not enhanced following viable cell instillation. These data demonstrate the specificity of STAT6 activation for apoptotic cell recognition systems.

Within the lung, PPAR γ is expressed by the epithelium, smooth muscle cells, fibroblasts, endothelium, macrophages, eosinophils, and dendritic cells [40]. Our previous studies demonstrated that apoptotic cell instillation enhances persistently PPAR γ expression and activity in alveolar macrophages and/or lung tissue over 21 days after BLM treatment [11]. However, the presence of viable Jurkat cells had no effect. Moreover, immunohistochemistry findings indicated that apoptotic cell-induced PPAR γ expression was observed mainly in macrophages and, to some extent, fibroblasts at 14 days after BLM treatment [11]. Here using double immunofluorescence staining, we found strong phospho-STAT6 expression in these cells following introduction of BLM + ApoJ at this time point.

A previous study using an *in vitro* model demonstrated that STAT6 may regulate PPAR γ target genes by binding to their regulatory sequences directly [13]. Our *in vivo* model demonstrated that co-administration of the STAT6 inhibitor AS1517499 reversed apoptotic cell-induced enhancement of PPAR γ mRNA and protein expression as well as its activity. Indeed, confocal microscopy confirmed the involvement of STAT6 signaling in enhancing nuclear PPAR γ staining in alveolar macrophages by apoptotic cell instillation. Moreover, similar to the PPAR γ antagonist GW8662 [11], AS1517499 co-administration reversed apoptotic cell-induced expression of PPAR γ target genes, namely CD36, MMR, and Arg1, in alveolar macrophages and lung tissue. Furthermore, STAT6 inhibition reversed the enhanced phagocytic index in alveolar macrophages. Collectively, these data support the hypothesis that STAT6 and PPAR γ interaction promotes the transcriptional program for anti-inflammatory and anti-fibrogenic responses of alveolar macrophages, which is associated with enhanced efferocytic ability.

Our previous data showed that GW9662 inhibited anti-inflammatory and anti-fibrotic effects of apoptotic cell instillation over 21 days after BLM treatment [11]. Similarly, AS1517499 co-administration restored pro-inflammatory mediators, including TNF- α and MIP-2, and other inflammatory responses, including neutrophil number, total protein levels in BAL fluid, and MPO activity in lung tissue. Moreover, AS1517499 co-administration reversed the effects of apoptotic cells on HGF and IL-10 expression. Long-term STAT6 inhibition reversed the reduction in hydroxyproline content, type 1 collagen α 2, fibronectin, and α -SMA expression in lung tissue. The involvement of STAT6 in the anti-inflammatory and anti-fibrotic effects of apoptotic cell instillation was also confirmed by lung histology. The

similar effects observed between AS1517499 and GW8662 indicate that STAT6 signaling mediates apoptotic cell-induced anti-inflammatory and anti-fibrotic effects via PPAR γ .

STAT proteins are critical mediators of cytokine signaling. Among the seven family members, STAT6 is activated by IL-4 and IL-13 and plays a predominant role in immunity by switching M1 to M2 macrophages and stimulating the Th2 response. Other cytokines, including IFN- α , platelet-derived growth factor, angiotensin II, leptin, and both Ig and CD44 crosslinking activate STAT6 in different cell types [41-45]. Our data suggest that IL-4 and IL-13 do not stimulate STAT6 phosphorylation following apoptotic cell instillation since apoptotic cell instillation did not affect or even reduce BLM-induced IL-4 and IL-13 mRNA and protein in lung and BAL fluid at 7 days after BLM treatment (see online suppl. material, Suppl. Fig. 7A, B, respectively) [11].

Our findings suggest that activation of STAT6 signaling by apoptotic cell instillation facilitates PPAR γ expression and activation, leading to expression of targets such as *CD36*, *MMR*, and *Arg1* in alveolar macrophages and lung tissue. Furthermore, STAT6 functions to mediate the anti-inflammatory and anti-fibrotic effects of apoptotic cell instillation via enhanced PPAR γ activation. Although the molecular details of the proximal membrane signaling events initiated by apoptotic cell recognition remain unknown, the prolonged STAT6 phosphorylation may facilitate PPAR γ activation following apoptotic cell instillation, leading to alterations in inflammatory and fibrogenic programs in alveolar macrophages and lung to control BLM-induced inflammation and fibrosis.

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Disclosure Statement

The authors have nothing to disclose.

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