Cigarette smoke-induced lung inflammation in COPD mediated via LTB4/BLT1/SOCS1 pathway

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Background: Evidence suggests that suppressor of cytokine signaling 1 (SOCS1) is crucial for the negative regulation of inflammation. We investigated the relationship between smoking, SOCS1, and leukotriene B4 (LTB4) in vitro and in clinical samples of COPD; besides which we detected the impact of LTB4 receptor 1 (BLT1) antagonist on inflammation.

Methods: SOCS1 expression in bronchial mucosa was determined by immunohistochemistry and real-time polymerase chain reaction. We also detect SOCS1 and BLT1 expression in alveolar macrophages from bronchoalveolar lavage fluid (BALF) by real time-PCR, in addition to measuring the level of cytokines in BALF using enzyme-linked immunosorbent assay. In vitro, we investigated the expression of SOCS1 in cigarette smoke extract-induced mouse macrophage cell line RAW264.7 by real-time polymerase chain reaction and Western blot, and detected the level of cytokines in the supernatant by enzyme-linked immunosorbent assay. Then, we investigated the effects of BLT1 antagonist U-75302 on SOCS1 expression in these cells.

Results: We obtained endobronchial biopsies (15 COPD patients and 12 non-COPD control subjects) and BALF (20 COPD patients and 20 non-COPD control subjects), and our results showed that SOCS1 expression significantly decreased in lung tissues from COPD patients. Inflammatory cytokines in BALF were higher in COPD and these inflammatory cytokines negatively correlate with SOCS1 levels. Further, the BLT1 antagonist restored SOCS1 expression and in turn inhibited inflammatory cytokine secretion in vitro.

Conclusion: Long-term cigarette smoke exposure induced SOCS1 degradation and LTB4 accumulation, which was associated with emphysema and inflammation. A BLT1 antagonist might be a potential therapeutic candidate for the treatment of COPD.

Keywords: treatment, LTBl antagonist, endobronchial biopsies, BALF, inflammatory cytokines

Introduction

COPD is a chronic inflammatory disease of the airways that is characterized by progressive and irreversible decline in lung function, excessive mucus secretion, and limited airflow.1,2 According to the World Health Organization, the annual mortality due to COPD is ~3 million people worldwide. It causes significant social and economic burden and is a serious public health issue.3 Cigarette smoking (CS) is the main risk factor for COPD and persistent airway inflammation, which contributes to irreversible pathological changes in the lung, such as airway remodeling and pulmonary emphysema.4 However, the mechanisms of pathogenesis are still not fully elucidated.

To our knowledge, cigarette smoke triggers immune dysregulation and pulmonary inflammation and activates innate immune cells such as macrophages, which play a crucial role in promoting inflammation and secretion of inflammatory cytokines by triggering pattern recognition receptors, either directly or indirectly via the release of damage-associated molecular patterns from stressed or dying cells.5,7 Inflammation is
a complex defense response. An uncontrolled and prolonged inflammatory response may cause lung tissue destruction and remodeling, as chronic inflammation continues even after smoking cessation. Therefore, elimination of inflammation caused by CS is a COPD treatment goal, which is difficult in clinical practice.

The suppressor of cytokine signaling 1 (SOCS1) mediated innate immune regulation with a negative feedback loop, which is important in inflammatory cytokine production. These studies suggest a novel regulatory mechanism prompting us to speculate that the absence of SOCS1, a negative regulatory protein, may lead to accumulation of proinflammatory cytokines and chronic inflammatory reaction in COPD. Therefore, we investigated the SOCS1 expression and inflammatory cytokine level in COPD patient’s lung tissues and bronchoalveolar lavage (BAL). The results may provide evidence supporting our hypothesis. However, the precise mechanism is still unclear.

The most recent study suggested that leukotriene B4 (LTB4) combined with its receptor, leukotriene B4 receptor 1 (BLT1), accelerates the degradation of SOCS1 mRNA. LTB4 is a key chemokine in the accumulation of granulocytes and macrophages via BLT1 and BLT2, a G protein-coupled cell surface receptor. It plays an important role in the COPD inflammatory response. Clinically, LTB4 promotes the survival of neutrophils in exhaled breath condensate (EBC) and sputum of COPD patients. The effects of SOCS1 on LTB4/BLT1 pathway in CS-induced airway inflammation are not well known. It is reasonable to hypothesize that accumulation of LTB4 in the CS-exposed lung might suppress SOCS1 transcription and expression, causing the release of a series of downstream proinflammatory cytokines. Thus, we chose the selective BLT1 receptor antagonist U-75302 to investigate if U-75302 can prevent inflammation in vitro. Our results may provide experimental evidence for the underlying mechanisms of COPD pathogenesis and a novel therapeutic approach for COPD via modulation of LTB4/BLT1 pathway.

**Materials and methods**

**Subjects**

We obtained endobronchial biopsies (15 COPD patients and 12 non-COPD control subjects) from the Respiratory Ward at Ruijin Hospital affiliated to the School of Medicine, Shanghai Jiao Tong University. In addition, BAL was performed in 20 COPD patients and 20 non-COPD control subjects in either the middle lobe or the lingula. All the samples are from clinically stable patients and the patients and controls for endobronchial biopsy and BAL were completely different, and the characteristics of subjects are listed in Table 1. All the COPD patients met the criteria proposed by the Global initiative for chronic Obstructive Lung Disease. All COPD patients were in a stable condition at the time of study. All control subjects had normal lung function without any history of asthma or chronic bronchitis and smoking history. No patients were diagnosed with current or recent (8 weeks) respiratory tract infection, or any exacerbations requiring oral steroid or antibiotic treatment. All subjects provided written informed consent to participate in the study, which was reviewed and approved by the Ruijin Hospital Ethics Committee Shanghai Jiao Tong University of Medicine. All measurements and experiments were performed in a blinded fashion.

**Preparation of alveolar macrophages from BALF**

By infusing 50 mL of sterile sodium chloride into either the middle lobe or the lingula, we obtained approximately 30 mL of bronchoalveolar lavage fluid (BALF) from each subject. BALF was centrifuged for 20 minutes at 1,500 rpm and the supernatant was discarded. The precipitate was resuspended in Dulbecco’s Modified Eagle’s Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum, supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were cultured on 3.5 cm plates for 3 hours, and washed three times by phosphate-buffered saline. The cells adhering to

<table>
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<tr>
<th>Characteristics</th>
<th>Control</th>
<th>COPD</th>
<th>P-value</th>
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<tr>
<td>Endobronchial biopsies</td>
<td>N=12</td>
<td>N=15</td>
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<tr>
<td>Age (year)</td>
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<tr>
<td>FEV1/FVC</td>
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<td>0.0004</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>12</td>
<td>0</td>
<td></td>
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<tr>
<td>Former</td>
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<td>3</td>
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<tr>
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<tr>
<td>Pack-years</td>
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</tr>
<tr>
<td>BALF</td>
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<td>65.15±1.661</td>
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<td>Sex (male:female)</td>
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<td>FEV1/FVC</td>
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<td>Smoking status</td>
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<td>Never</td>
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<tr>
<td>Former</td>
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<tr>
<td>Pack-years</td>
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<td>38.10±2.203</td>
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Abbreviations: BALF, bronchoalveolar lavage fluid; SD, standard deviation; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity.
the bottom were alveolar macrophages (AMs), which were verified by cellular staining.

**Immunohistochemical analysis**

Immunohistochemistry was used to analyze the protein levels and distribution of SOCS1 in bronchial tissues of COPD and control subjects. Briefly, we embedded lung tissues (endobronchial biopsies) in paraffin and placed 5 µm-thick sections on the slides, de waxed in xylene, and rehydrated. Endogenous peroxidases were inhibited with 0.5% hydrogen peroxide in methanol for 10 minutes, followed by overnight incubation at 4°C with a rabbit polyclonal IgG antibody against SOCS1 (Abcam, Cambridge, UK). Immunodetection was performed with diaminobenzidine, biotinylated goat anti-rabbit IgG reagent, and horseradish peroxidase reagent.

**RNA extraction and real-time PCR**

Total RNA from AMs and bronchial tissues were extracted using an RNasea kit (Qiagen, Valencia, CA, USA), and total RNA from RAW264.7 cells was extracted using TRIzol reagents (Thermo Fisher Scientific), according to the manufacturer’s instructions. Single-stranded cDNA was synthesized for each sample with oligo (dT) primers using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Waltham, MA, USA), according to the manufacturer’s protocol. Total RNA (500 ng) was amplified with a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific) with FastStart Universal SYBR Green (Hoffman-La Roche Ltd., Basel, Switzerland) after cDNA synthesis. The standard curve for quantification was created using a modified reference gene (glyceraldehyde-3-phosphate dehydrogenase; R, reverse; SOCS1, suppressor of cytokine signaling 1).

**Cell line and CS extract**

The RAW264.7 mouse macrophage cell line was obtained from the Cell Collection and Research Center of Chinese Academy of Science. CS was collected and treated with DMEM by continuous suction in a chamber connected to a vacuum. Treatment with 500 mL of DMEM required mainstream smoke from ten cigarettes and 5 minutes for each cigarette. The resultant CS extract (CSE)–DMEM was considered as 100% stock solution and ready for use after adjusting the pH to 7.4 and sterile filtration (0.2 µm). Serial dilutions of 50%, 30%, 20%, and 10% CSE solution were prepared for cell culture. DMEM only was considered as 0% CSE.

**Exposure of RAW264.7 culture to CSE and U-75302 treatment**

The most appropriate condition was determined using a combination of different concentrations of CSE stimulation at different exposure times of 3, 6, 12, 24, and 48 hours. Basically, 30% CSE was the maximal dilution without excessive cell mortality after 24 hours exposure. For treatment with U-75302, RAW264.7 cells were seeded overnight and treated with 20% CSE with 10 nmol/mL U-75302 (Enzo, Telluride, CO, USA) for 24 hours. Supernatant was then harvested and total cellular RNA and protein were extracted.

**Cytokine enzyme-linked immunosorbent assays**

The concentrations of interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) in the BALF, and the mouse IL-6 and TNF-α levels in the cell culture supernatant were determined using DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. LTB4 concentration in BALF and cell culture supernatants were measured using a specific enzyme immunoassay with a commercial kit (Cayman Chemical Co, Ann Arbor, MI, USA).

**Western blot**

Cells were lysed with 100 µL radio immunoprecipitation assay lysis buffer (Sigma-Aldrich Co., St Louis, MO, USA). Protein concentrations were measured with bicinchoninic acid assay kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. Equal amounts of proteins (30 µg) were subjected to a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gels were run at 80 V for 30 minutes followed by 120 V for 1 hour before transferring onto a polyvinylidene fluoride

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**Table 2 Primer sequences for real-time PCR**

<table>
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<th>Gene</th>
<th>Sequence</th>
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<td>Human-SOCS1</td>
<td>F: GTCCCCCTGGTGGTGTAG</td>
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<tr>
<td></td>
<td>R: AAGAGTTAGAGTGCGAG</td>
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<tr>
<td>Human-GAPDH</td>
<td>F: GGGAAAGGTAAGGTCCGAG</td>
</tr>
<tr>
<td></td>
<td>R: GGGCTGATTGCGCAAACA</td>
</tr>
<tr>
<td>Human-BLT1</td>
<td>F: TATGCTGCGGATCAGCATGC</td>
</tr>
<tr>
<td></td>
<td>R: CCGTGAAGGGCCAGGCCCTATGCG</td>
</tr>
<tr>
<td>Mouse-SOCS1</td>
<td>F: GTGTCGAGGAGGTGGTTG</td>
</tr>
<tr>
<td></td>
<td>R: CTGAGAGTGGAGTGGAGT</td>
</tr>
<tr>
<td>Mouse-GAPDH</td>
<td>F: AAATTGGAAGGTCGGTTG</td>
</tr>
<tr>
<td></td>
<td>R: AGGTCAATGAAGGGTGCTG</td>
</tr>
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</table>

**Abbreviations:** BLT1, leukotriene B4 receptor 1; F, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; R, reverse; SOCS1, suppressor of cytokine signaling 1.
membrane (EMD Millipore, Billerica, MA, USA). After blocking with phosphate-buffered saline containing 5% nonfat milk for 2 hours at room temperature, we incubated overnight at 4°C with rabbit polyclonal anti-SOCS1 antibody (1:1,000; Abcam), or a β-actin antibody (1:1,000; Cell Signaling, Danvers, MA, USA). The membrane was washed for 5 minutes with 15 mL tris-buffered saline and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2,000; Sigma) for 1 hour at room temperature. After washing, we added 1 mL chemiluminescent substrate (Thermo Fisher Scientific) to the membrane. The signal was detected and quantified with an enhanced chemiluminescence system (ImageQuant LAS-4000 MINI; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). All samples were normalized to β-actin.

Statistical analysis
Results are presented as mean ± standard error of the mean if not stated otherwise. A two-tailed t-test was performed in all results’ statistical analysis for comparisons of baseline characteristic. A linear regression was adopted for association between SOCS1 mRNA levels, LTB4 expression level, and FEV1 (forced expiratory volume in 1 second) %predicted using Spearman’s rank correlation test. Statistical analyses were performed as described in each figure legend, using GraphPad Prism. P-values ≤0.05 were considered statistically significant.

Results
Immunohistochemistry of SOCS1 in bronchial mucosa of COPD patients
Immunohistochemistry staining showed the presence of SOCS1 protein mainly in the airway epithelial cells and AMs (Figure 1). We found that the expression level of SOCS1 in bronchial mucosa was significantly lower in patients with COPD than in control subjects (808.17±697.06 vs 15,944±7,181.53; Figure 1A–D).

SOCS1 mRNA expression in the bronchial mucosa
The SOCS1 mRNA level in bronchial biopsies was compared between 12 non-COPD and 15 COPD subjects by real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR). The SOCS1 expression relative to the housekeeping gene GAPDH was significantly lower in COPD subjects than in control subjects (0.6587±0.1045 vs 2.786±0.4748; shown in Figure 2). These results were consistent with immunohistochemistry results illustrated in Figure 1, indicating that SOCS1 expression was significantly decreased in lung tissues of COPD patients.

SOCS1 and BLT1 mRNA expression in the AMs
Total RNA of AMs in BALF was isolated and the mRNA of SOCS1 and LTB4 receptor BLT1 was then determined by RT-qPCR. The SOCS1 expression relative to GAPDH was significantly lower in COPD subjects than in control subjects (3.583±0.5430 vs 1.173±0.1867), whereas the BLT1 mRNA expression was higher (0.7000±0.1168 vs 2.670±0.6158) than that of SOCS1 (Figure 3) suggesting increased BLT1 expression after long-term cigarette smoke exposure.

Cytokine levels in alveolar BALF
The IL-8 and TNF-α levels were higher in BALF of COPD patients than in control subjects (Figure 4A–C). These results indicated persistent airway inflammation in COPD patients.

SOCS1 and LTB4 expression and clinical outcomes
In this study, a negative correlation existed between SOCS1 mRNA level in lung tissue and LTB4 concentration in BALF (Figure 5A). Significant positive correlation was seen between SOCS1 mRNA level in lung tissues and FEV1 %predicted (Figure 5B). These data indicated that SOCS1/LTB4 pathway was crucial for the progression of COPD.

Expression of SOCS1 and cytokines in CSE-induced RAW264.7 cells
To detect the potential effects of CSE on the expression of SOCS1, we used 20% CSE to treat RAW264.7 cells for the indicated period (6, 12, and 24 hours). The cells were harvested, and total RNA was isolated to analyze the mRNA expression by RT-qPCR. In addition, concentrations of various cytokines in supernatants were measured by enzyme-linked immunosorbent assay. The results showed that the level of LTB4 was significantly upregulated in CSE-treated RAW264.7 cells than in control groups. However, the mRNA level of SOCS1 reached its peak 6 hours later, after treatment, and decreased subsequently at 12 and 24 hours (Figure 6A and B). Next, we assessed the proinflammatory cytokines in CSE-stimulated RAW264.7 cells, as shown in Figure 6C and D. Western blot analyses suggest that the expression of SOCS1 protein was increased in the early stages of CSE treatment and remarkably decreased compared with the control group at 24 hours (Figure 6E). Collectively, these
data indicated that SOCS1 plays an important role in the process of inflammation.

**Effect of U-75302 on SOCS1 expression in RAW 264.7 cells**

In order to analyze the role of LTB4 in the degeneration of SOCS1 expression, U-75302, a BLT1 antagonist, was used to treat CSE-stimulated RAW264.7 cells. As shown in Figure 7A, addition of U-75302 enhanced SOCS1 mRNA and protein expression (Figure 6F). U-75302 also abolished the increase in TNF-α and IL-6 mRNA and protein expression in CSE-stimulated cells (shown in Figure 7B–D). These data supported the hypothesis correlating SOCS1 expression with LTB4 accumulation.
Discussion

Our study demonstrated lower expression of SOCS1 and higher levels of LTB4 in COPD patients; importantly, BLT1 antagonist could restore SOCS1 expression and prevent inflammation in vitro, thus, our results revealed a novel therapeutic target in COPD smoke-induced airway inflammation. As the primary mediator of innate immune response, AMs are initially activated by CS. AMs are directly involved in the secretion of cytokines, including IL-8, TNF-α, and LTB4 resulting in chronic inflammatory response and tissue destruction, exacerating emphysema in COPD patients. Therefore, inhibition of inflammatory cytokine secretion in macrophages is a potential therapeutic strategy in inflammation and autoimmune diseases. Similarly, our study showed high levels of IL-8, TNF-α, and LTB4 in AMs, BALF, and lung tissue in COPD patients. For the first time, we showed that the basal level of SOCS1 decreased while the expression of BLT1 increased in bronchial mucosa and AMs from patients with COPD. Our data suggest that the exaggerated inflammatory response may be associated with poor SOCS1 expression.

SOCS1 mediates the negative regulation of inflammatory response. Previous studies reported an increased level of inflammatory cytokines, including TNF-α, IL-6, and IL-8 in SOCS1-deficient macrophages. Wang et al and Serezani et al showed that LTB4/BLT1 pathway enhanced degradation of SOCS1 mRNA via miR-155, consistent with other studies. Some studies showed that LTB4 was involved in the pathogenesis of pulmonary emphysema. We observed higher levels of LTB4 in the BALF of COPD patients and in the EBC, consistent with previous studies. LTB4 is a key neutrophil chemotactic factor, causing neutrophil infiltration in COPD patients. We found for the first time a significant negative correlation between the LTB4 levels in BALF and SOCS1 expression in bronchial mucosa from patients with COPD, suggesting that the accumulation of LTB4 contributes negatively to inflammation in vivo. We found that membrane injury in airway epithelial and AMs

Figure 2 SOCS1 mRNA expression levels in lung tissue.
Notes: RT-qPCR detection of SOCS1 mRNA expression of lung tissue. COPD patients showed a significant low level of SOCS1 mRNA compared with the control sample. The results are presented as mean ± SEM (n=12–15; ****P<0.0001 vs the control group).
Abbreviations: RT-qPCR, real-time reverse transcription quantitative polymerase chain reaction; SOCS1, suppressor of cytokine signaling 1; SEM, standard error of the mean.

Figure 3 mRNA expression of SOCS1 and BLT1 in alveolar macrophages.
Notes: RT-qPCR detection of mRNA expression of alveolar macrophages. COPD patients showed a significant low level of SOCS1 mRNA compared with control sample (A). In contrast, BLT1 had an opposite result (B). The results are presented as mean ± SEM (P<0.05 vs the control group).
Abbreviations: BLT1, leukotriene B4 receptor 1; RT-qPCR, real-time reverse transcription quantitative polymerase chain reaction; SOCS1, suppressor of cytokine signaling 1; SEM, standard error of the mean.
caused by longtime CS exposure exaggerated the LTB4 levels in the BALF of COPD patients and then activated LTB4/BLT1 signaling. Once triggered, the SOCS1 mRNA was degraded resulting in uncontrolled inflammatory response. We, therefore, hypothesized that BLT1 antagonist might prevent these events.

To test this hypothesis, we identified SOCS1 expression in CSE-stimulated macrophages in vitro, for the first time.
Figure 6 Expression of SOCS1, LT&B4, TNF-α, and IL-6 in CSE-treated RAW264.7 cells.

Notes: Total RNA isolated from RAW264.7 cells after 0, 6, 12, and 24 hours treated with CSE was examined. The expression of mRNA was assessed by real-time PCR (A). The supernatant was collected at 0, 6, 12, and 24 hours, and the amount of TNF-α, LTB4, and IL-6 released was measured by ELISA (B–D). Whole-cell extracts from 0, 12, and 24 hours cultures were isolated and subjected to Western blot to detect the expression of SOCS1 (E). The SOCS1 expression had increased when treated with U-75302 at 24 h (F). Data were expressed as mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs control group.

Abbreviations: CSE, cigarette smoke extract; ELISA, enzyme-linked immunosorbent assay; IL-6, interleukin-6; LTB4, leukotriene B4; SOCS1, suppressor of cytokine signaling 1; TNF-α, tumor necrosis factor-α; SEM, standard error of the mean.

Figure 7 (Continued)
Interestingly, we found that SOCS1 mRNA level was significantly upregulated in CSE-stimulated RAW264.7 during the early period. To our knowledge, JAK/STAT signal pathway plays a vital role in various conditions, including immune reaction, cell growth, and differentiation. Studies showed that CSE induced the release of damage-associated molecular pattern molecules and activated pattern recognition receptors, and downstream signaling pathways that trigger a negative feedback loop and enhance SOCS1 expression to control inflammatory response. Increased inflammatory cytokines such as IL-8, TNF-α, and LTB4 were also secreted in clinical samples and in vitro. Further, our data showed that SOCS1 expression significantly decreased with increased LTB4 in the supernatants after 24 hours CSE treatment. For the first time, we showed that BLT1 antagonist U-75302 restored SOCS1 expression induced by LTB4 and reduced the levels of inflammatory cytokines IL-6 and TNF-α in vitro. The results showed that BLT1 antagonist may control chronic airway inflammation and prevent the progress of COPD pathology. Our results were consistent with other findings. Watanabe et al found that the pathogenesis of airway remodeling was inhibited by the BLT1 specific antagonist, U-75302. Further, U-75302 reduced neutrophil chemotactic activity in samples of EBC and induced sputum from patients with COPD. More recently, animal experiments showed that U-75302 attenuated lung injury and the level of systemic cytokines in polymicrobial sepsis-induced mice. However, previous studies showed that LTB4 antagonist did not have any benefit in COPD patients, and these results may be associated with the uncontrolled clinical trials with novel measures of pharmacodynamic effect, patient selection, end points, and disease indications; furthermore, the LTB4 antagonist in these studies was not specific and dual BLT1 and BLT2 inhibition may exert a lack of effect if the functions of these two receptors are opposing. Thus, selective BLT1 inhibition could reduce inflammation, although simply BLT1 antagonist therapeutic may not be effective in COPD patients, because BLT1 receptor should have other protective roles in cell survival, etc, however, we might at least discover a target to further study.

There were also some limitations in our study; we utilized murine macrophages for the in vitro study and whether the results can be extrapolated to humans is unknown. However, our study indicated the effect of CS-induced lesions on SOCS1 expression and LTB4/BLT1 pathway. Further investigation of U-75302 in an animal model will be interesting.

In summary, this study demonstrated lower levels of SOCS1 and higher levels of LTB4 in COPD patients when compared with control groups. Importantly, SOCS1 expression was negatively correlated with that of LTB4, which indicated that LTB4/BLT1 pathway might play a critical role in airway inflammation in COPD. Thus, BLT1 antagonist could be a new therapeutic candidate in COPD treatment targeting smoke-induced inflammation.

Acknowledgment
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Disclosure
The authors report no conflicts of interest in this work.

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