Article

Human and Experimental Toxicology
1–11

DOI: 10.1177/0960327119881678
journals.sagepub.com/home/het

# Lipopolysaccharide induces epithelial–mesenchymal transition of alveolar epithelial cells cocultured with macrophages possibly via the JAK2/STAT3 signaling pathway

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## Abstract

Epithelial–mesenchymal transition (EMT) plays a key role in the process of pulmonary fibrosis (PF). Increasing evidences have shown that exaggerated EMT in recurrent pulmonary injury mediates the early pathogenesis of PF. This study aimed to evaluate EMT of human alveolar epithelial cells (A549) when cocultured with human macrophages Tohoku hospital pediatrics-1 (THP-1) induced by lipopolysaccharide (LPS) and investigate the role of Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling pathway. Firstly, we detected the inflammatory and EMT biomarkers in A549 cells monoculture and A549/THP-1 cells coculture in the presence or absence of LPS. Then, the activation of JAK2/STAT3 signaling pathway was determined in coculture. Interestingly, inflammatory markers, such as interleukin (IL)-6, matrix metalloproteinase (MMP)-9, transforming growth factor (TGF)-$\beta$, and collagen type I (COL-1), were enhanced in LPS treated coculture. Besides, the expression of E-cadherin decreased but $\alpha$-smooth muscle actin expression increased, indicating the presence of EMT in A549 cells when cocultured with THP-1 macrophages. However, these phenotypes could not be observed in LPS-treated A549 cells monoculture. Meanwhile, JAK2/STAT3 signaling pathway was activated, and the STAT3 DNA-binding and inflammatory markers were inhibited by Stattic. Together, these findings demonstrate the key role of JAK2/STAT3 signaling pathway in LPS promoted EMT of A549 in the presence of THP-1 macrophages as an in vitro PF model.

## Keywords

Alveolar epithelial cells, macrophages, coculture, lipopolysaccharide, epithelial–mesenchymal transition, pulmonary fibrosis, JAK2/STAT3

## Introduction

Pulmonary fibrosis (PF) is an irreversible and progressive lung disease characterized by lung parenchyma fibrogenesis, chronic inflammation, and exaggerated accumulation of extracellular matrix (ECM). Chronic inflammation drives the entire process of PF, which involves many pro-inflammatory factors. For example, interleukin (IL)-6 can promote collagen fiber hyperplasia and induce the accumulation of extracellular matrix and induces conversion of human lung fibroblasts to myofibroblasts.[1,2] Besides, damage induced by matrix metalloproteinases (MMPs), accumulation of collagen (COL-1 and

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COL-3), is also positively correlated with the degree of PF.[3,4]

Epithelial–mesenchymal cell transition (EMT) is a normal wound healing response occurring in most tissues. There distinct biological scenarios have been defined for occurrence of EMTs with varied physiological outcomes: (i) type 1 comprises implantation and development of embryo and does not cause fibrosis or tissue invasion; (ii) type 2 involves wound healing, regeneration of tissue, and fibrosis and is mostly in response to tissue repair; and (iii) type 3 occurs in transformation of neoplastic cells, leading to tissue invasion and metastasis.[5] The EMT of alveolar epithelial cells belongs to type 2 as a process of wound healing is a key point in the process of lung fibrogenesis.

Transforming growth factor (TGF)-$\beta$1 is a chief inducer of the EMT associated with pathological fibrosis.[6] TGF-$\beta$1 signaling elicited during pulmonary injury has been related to loss of E-cadherin, a protein responsible for assembling epithelial cell sheets and maintenance of the quiescence of the cells within these sheets.[7] The downregulation of E-cadherin discharges the cells from its basal membrane and becomes more phenotypically spindle-shaped and easy to move. The alveolar epithelial cells undergoing EMT will lose their cell polarity and adherens junction, downregulate epithelial signature like E-cadherin, and acquire mesenchymal characteristic like $\alpha$-smooth muscle actin ($\alpha$-SMA), and result in migrating easily and then acting as a source of fibroblasts.[8–10]

In the process of PF, macrophages activated early may orchestrate the inflammatory response, which in turn activate the bystander epithelial cells. Activated epithelial cells on the reverse can modify the macrophage phenotype via regulating the cytokine gene expression of monocytes and modulating the maturation of monocytes to macrophages.[11] Besides, inflammation also accelerates the process of activation of EMT and accumulation of ECM.[12] It has been well documented that macrophages can induce the EMT of epithelial cells under hypoxic conditions.[13] Increasing evidence has shown signal transducer and activator of transcription 3 (STAT3) signaling pathway is involved in EMT and tissue fibrosis.[14–16] However, the interaction between epithelial cells and macrophages and the effect of this interaction on the EMT of epithelial cells and the relationship between lipopolysaccharide (LPS)-induced EMT and Janus kinase 2 (JAK2)/STAT3 signaling pathway remain unclear.

In the present study, we evaluated the EMT of alveolar epithelial A549 cells when cocultured with THP-1 macrophages in the induction of LPS and investigated the action of JAK2/STAT3 signaling pathway in the EMT.

## Methods

### Regents

Lipopolysaccharide from *Escherichia coli* O55:B5 (L6529), phorbol 12-myristate 13-acetate (PMA) (P1585), and $\beta$-mercaptoethanol (M6250) were obtained from Sigma (St Louis, Missouri, USA). Stattic (S702402) was bought from Selleckchem (Houston, Texas, USA).

### Cell culture and treatment

Alveolar type II-like cell line (A549) and human monocyte/macrophage cell line (THP-1) cells were purchased from American Type Culture Collection (Manassas, Virginia, USA).

A549 cells and THP-1 cells were maintained in RPMI-1640 medium (Gibco, Waltham, Massachusetts, USA) supplemented with $\beta$-mercaptoethanol to a final concentration of 0.05 mM and 10% fetal bovine serum (Gibco, Waltham, Massachusetts, USA) and incubated at 37°C with 5% $CO_2$. The macrophage differentiation of the THP-1 cells was obtained by treating with 10 ng/ml PMA in RPMI-1640 medium without fetal bovine serum overnight.

The A549 and THP-1 cells with the ratio of 1:1 were cocultured using Transwell (Corning, New York, USA), in which A549 cells were seeded in bottom and THP-1 cells were cultured in Transwell insert and physically separated with a 0.4-μm-pore polyester filter. Firstly, the A549 monoculture in 24 well plate and A549/THP-1 coculture in Transwell were, respectively, stimulated with 2 μg/mL LPS, inflammatory, and EMT biomarkers were detected. Then, the activation of JAK2/STAT3 signaling pathway was determined in coculture. Subsequently, coculture cells were pretreated the Stattic, followed by LPS exposure, STAT3 DNA-binding and inflammatory markers were then detected.

### Measurement of cytokines

The supernatants from A549 monoculture and A549/THP-1 coculture for 48 h were isolated at 1000 r/min for 5 min. IL-6 (Boster, Wuhan, China), MMP-9 (Boster), TGF-$\beta$1 (Elabscience, Wuhan, China), and

COL-1 (Elabscience) levels were detected with enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions.

### Western blot assay

After being cocultured at the presence or absence of LPS for 24 h, cells were harvested and washed with cold PBS two times, following with adding radioimmunoprecipitation assay lysis buffer (Solarbio, Beijing, China). The protein concentration of nuclear extracts was determined by the bicinchoninic acid (BCA) method (Boster). Proteins were mixed with sodium dodecyl sulfate (SDS) sample buffer, boiled and separated at 100 voltage on 8% SDS-polyacrylamide gel electrophoresis, and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, Massachusetts, USA). The PVDF membranes were blocked with 5% skimmed milk powder in 1× Tris-buffered saline-Tween 20 (0.05%) (TBS-T) buffer for 1 h at room temperature and then incubated with primary antibodies overnight at 4°C. After being washed with TBS-T buffer for five times, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature and then washed with 1× TBS-T for five times. Finally, the bands were visualized by film exposure with ECL reagent (Solarbio). Antibodies used were as follows: Anti-E-cadherin (Abcam, ab15148, 1:1000), Anti-$\alpha$-SMA (Proteintech Group, 55135-1-AP, 1:500), Anti-p-STAT3 (Abcam, ab76315, 1:2000), Anti-STAT3 (Abcam, ab119352, 1:4000), Anti-GAPDH (Abcam, ab181602, 1:2000), and HRP-conjugated Affinipure Goat Anti-Rabbit IgG (H+L) (Proteintech Group, SA00001-2; 1:5000).

### Electrophoretic mobility shift assay

After being cocultured for 24 and 48 h, cells were harvested, and nuclear proteins were then extracted with nuclear protein extraction kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The protein concentration of nuclear extracts was determined by the BCA method. The DNA-binding of STAT3 transcription factor was detected with electrophoretic mobility shift assay (EMSA). STAT3 oligonucleotide probes were obtained from Beyotime Biotechnology as follow: 5′-GAT CCT TCT GGG AAT TCC TAG ATC-3′ and 3′-CTA GGA AGA CCC TTA AGG ATC TAG-5′. Binding reactions were performed at room temperature for 20 min in 10 μL of reaction buffer containing 10 μg of nuclear extracts, nuclease-free water, EMSA/gel-shift buffer, and oligonucleotide probes for STAT3. DNA-protein complexes were separated from free DNA probes at 120 voltage on 5% polyacrylamide gels with 0.5× Tris-borate-EDTA buffer, and then transferred to a nylon membrane. Finally, the bands were visualized by film exposure with enhanced chemiluminescence reagent (Millipore).

### Statistical analysis

Differences between groups were determined by one-way analysis of variance with the LSD and Dunnet's T3 post-hoc tests using the SPSS 21.0 software package (SPSS, Chicago, Illinois, USA). All results were presented as a mean ± standard error. $p$ Values less than 0.05 were considered statistically significant.

## Results

### Effect of LPS on inflammatory markers secretion in A549 monoculture and A549/THP-1 coculture

To investigate the effect of LPS on inflammatory markers, monocultured A549 cells and cocultured A549/THP-1 cells were respectively stimulated with 2 μg/mL LPS for 48 h. The supernatants were collected for ELISA. The data of Figure 1 exhibited a slight increase in the levels of MMP-9 and COL-1 in A549 monoculture in LPS-treated group. In the coculture, large increases were observed for IL-6, MMP-9, TGF-$\beta$1, and COL-1.

### Effect of LPS on EMT markers of A549 cells cocultured with THP-1 macrophages

To determine whether LPS could induce EMT of A549 cells, and whether the presence of THP-1 macrophages affected EMT of A549 cells, we evaluated the expression of EMT markers E-cadherin and $\alpha$-SMA of A549 cells when single cultured or cocultured with THP-1 macrophages. As shown in Figure 2, significant decrease of E-cadherin and increase of $\alpha$-SMA in A549 cells were observed after being cocultured with THP-1 in the presence of LPS compared to control group and LPS-treated A549 monoculture group.

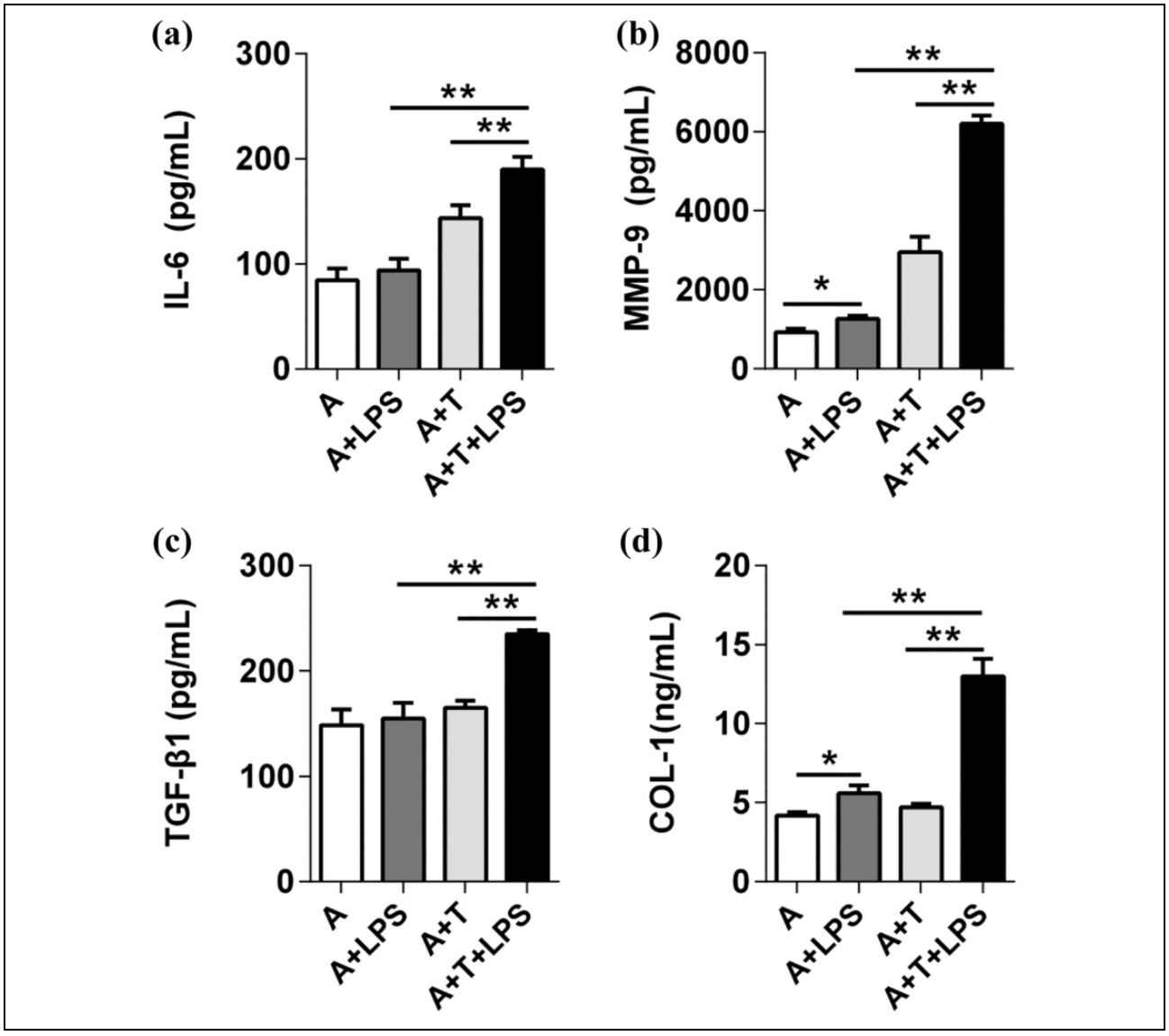


**Figure 1.** The effect of LPS on proinflammatory markers secretion in A549 monoculture and A549/THP-1 coculture. The monocultured A549 cells and cocultured A549/THP-1 cells, were, respectively stimulated with 2 μg/mL LPS for 48 h. Then, the levels of proinflammatory markers in supernatants were detected with ELISA. (a) IL-6, (b) MMP-9, (c) TGF-$\beta$1, and (d) COL-1 levels were enhanced in coculture in the presence of LPS. A: A549, A + LPS: LPS-treated A549, A + T: A549/THP-1 coculture, and A + T + LPS: LPS-treated coculture. The values are presented as mean ± standard deviation of three independent experiments ($n = 3$), $^{**}p < 0.01$. LPS: lipopolysaccharide; ELISA: enzyme-linked immunosorbent assay; TGF-$\beta$1: transforming growth factor-$\beta$1; IL-6: interleukin-6; MMP-9: matrix metalloproteinase-9.

### *Activation of nuclear transcription factor STAT3 in A549/THP-1 coculture*

The transcription factors are the main signal-transduction molecules activated in response to LPS-induced inflammatory response. STAT3 is involved in PF and inflammation.[17,18] In this study, LPS treatment markedly increased the STAT3 DNA-binding of coculture A549 and THP-1 cells (Figure 3).

### *Effect of LPS exposure on the JAK2/STAT3 signaling pathway*

STAT pathway is connected upstream with JAK family protein and capable of integrating inputs from different signaling pathways. JAK2/STAT3 was reported to relate to EMT.[14] In this study, we detected the expression of JAK2 and STAT3 and also their phosphorylation. As shown in Figure 4, the results indicated that LPS remarkably increased the

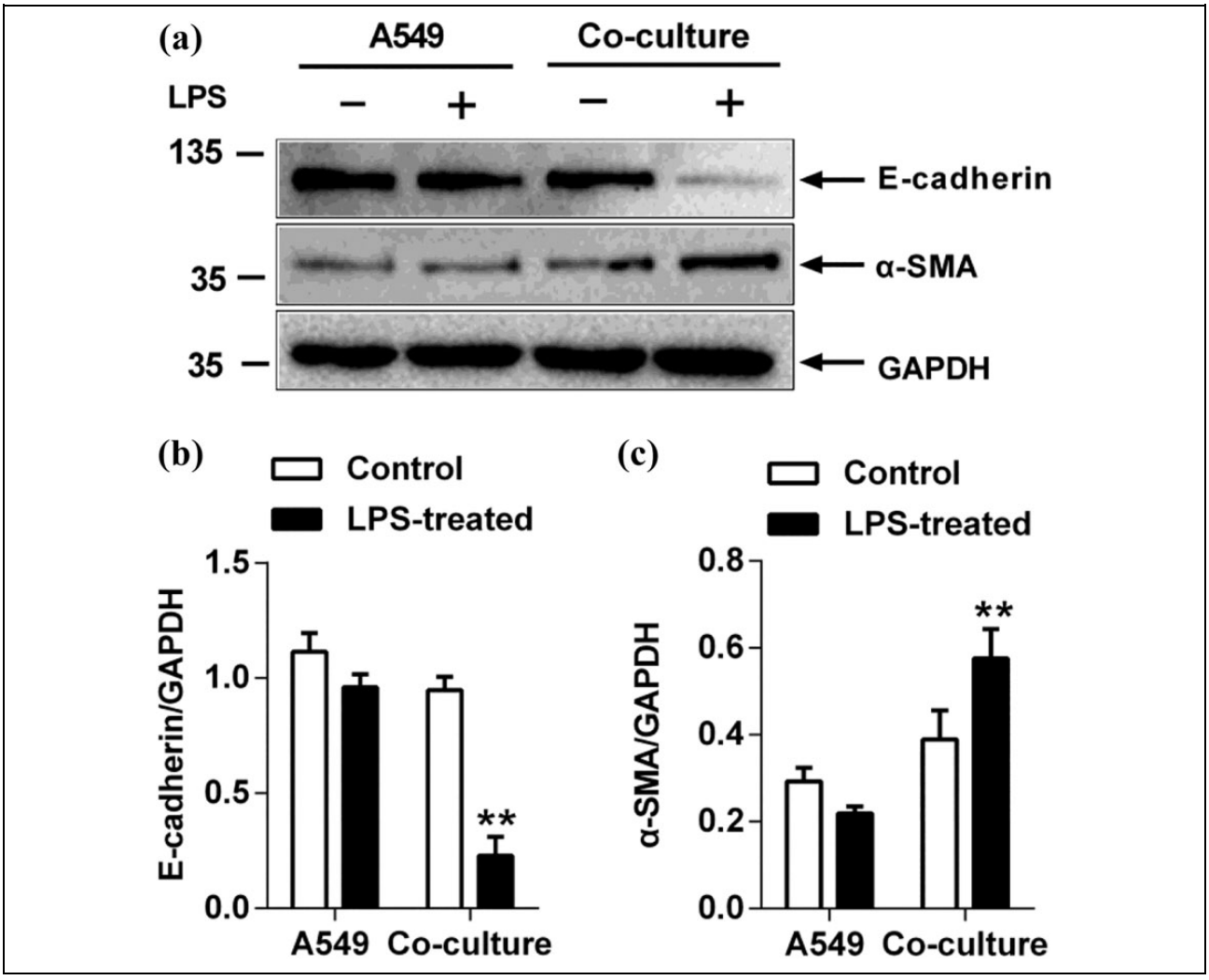


**Figure 2.** Effect of LPS on the expression of EMT markers of A549 cells. After being cocultured with THP-1 cells for 48 h in the presence or absence of LPS, E-cadherin and $\alpha$-SMA were analyzed by Western blot. When cocultured with THP-1 macrophages, the E-cad (a and b) expression decreases while $\alpha$-SMA (a and c) expression increases in the presence of LPS. Densitometric analysis was performed for quantitative evaluation. The values are presented as mean $\pm$ standard deviation of three independent experiments ($n = 3$), $^{**}p < 0.01$ versus control group. LPS: lipopolysaccharide; EMT: epithelial–mesenchymal transition; $\alpha$-SMA: $\alpha$-smooth muscle actin.

phosphorylation of JAK2 and STAT3 in coculture A549/THP-1 cells, however, had no effect on JAK2 and STAT3 expression.

### *Stattic treatment reduced LPS-induced STAT3 DNA-binding and inflammatory markers secretion of coculture A549/THP-1 cells*

Stattic is an effective STAT3 inhibitor that can selectively inhibit the activation and nuclear translocation of STAT3.[19] To determine whether the LPS induced the EMT of A549 cocultured with THP-1 macrophages probably through the JAK2/STAT3 signaling pathway, we investigated the effect of Stattic on the STAT3 DNA-binding and cytokines expression related to EMT. The data from Figures 5 and 6 indicated that Stattic treatment reduced STAT3 DNA-binding and IL-6, MMP-9, TGF-$\beta$1, and COL-1 secretion.

## Discussion

EMT plays a pivotal role in PF, and its initiation needs a variety of distinct molecular processes engaged in progression of inflammation and deterioration of ECM.[5] LPS, a constituent of the outer cell membrane

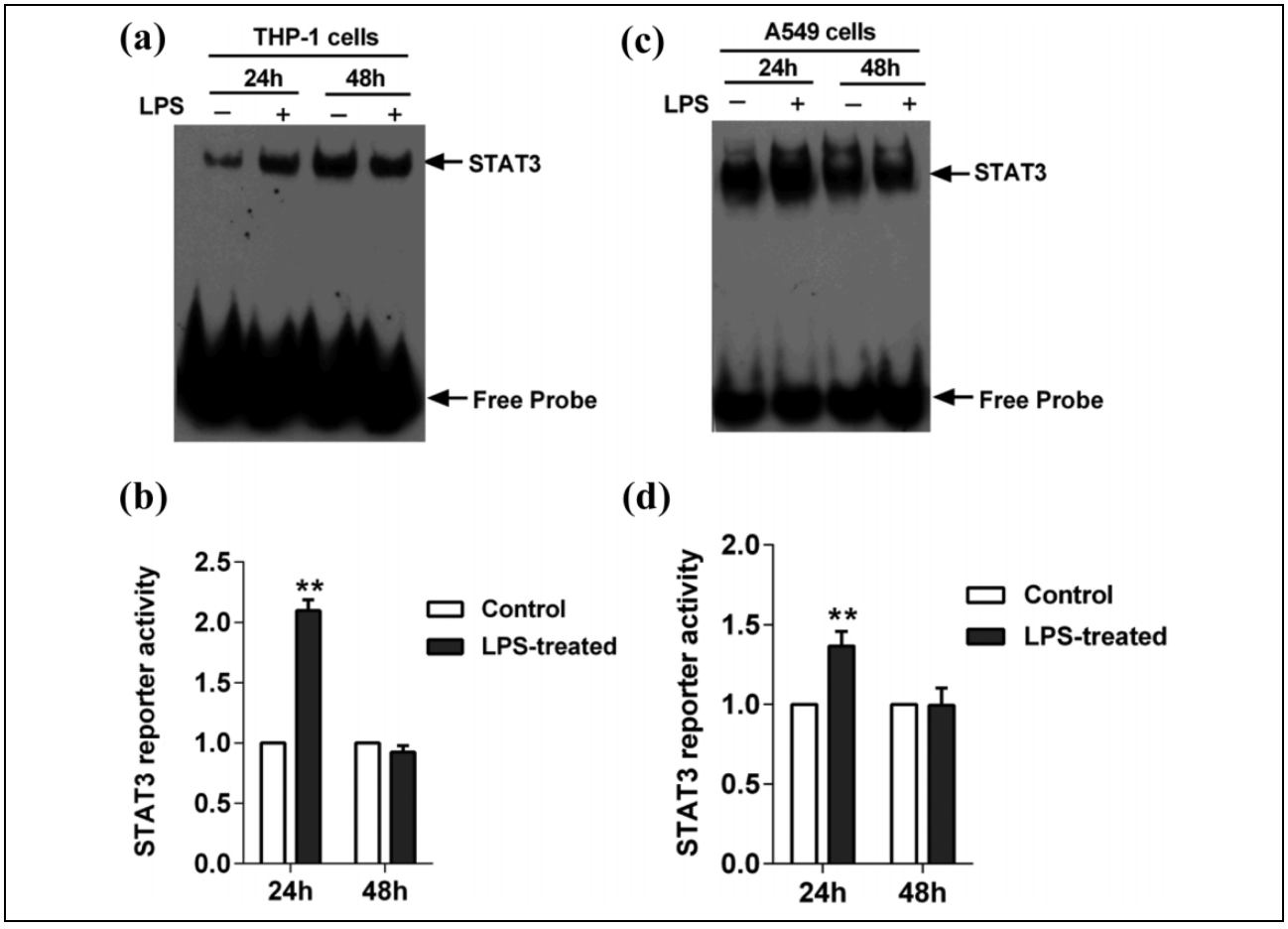


**Figure 3.** Activation of nuclear transcription factor STAT3 of THP-1 cells and A549 cells. After being treated with LPS for 24 and 48 h, the nucleoprotein was isolated for EMSA to determine the DNA-binding of STAT3. LPS could significantly increase the STAT3 DNA-binding of coculture THP-1 cells (a and b) and A549 cells (c and d). The values are presented as mean $\pm$ standard deviation of three independent experiments ($n = 3$), $^{**}p < 0.01$ versus control group. LPS: lipopolysaccharide; STAT3: signal transducer and activator of transcription 3; EMSA: electrophoretic mobility shift assay.

of gram-negative bacteria, which is an inducer of inflammation, can be also used to trigger EMT in many epithelial cell types. In vitro data showed that LPS induced the EMT of peritoneal mesothelial cells by decreasing the expression of E-cadherin and upregulating the $\alpha$-SMA expression.[20] LPS also induced EMT of intrahepatic biliary epithelial cells.[21] But there is insufficient evidence to confirm the role of LPS in EMT of cocultured cells. In this study, LPS induced higher expression levels of cytokines, MMP, and collagen in A549 and THP-1 coculture as compared to the respective control. Subsequently, LPS downregulated the expression of E-cadherin and upregulated $\alpha$-SMA expression in A549 and THP-1 coculture. Besides, LPS exposure could rapidly activate JAK2/STAT3 signaling pathway of cocultured A549 cells and THP-1 macrophages. On the contrary, pretreatment with STAT3 inhibitor resulted in lowered the levels of cytokines and collagen. Hence, we speculated that JAK2/STAT3 signaling pathway might play a crucial role in the LPS-induced EMT of A549 cells in the presence of THP-1 macrophages.

Previous studies have documented that inflammation and ECM were involved in EMT and PF.[22] To further evaluate the LPS-induced inflammatory response and ECM, we firstly detected the levels of cytokines, MMP, and collagen in the A549 monoculture and A549/THP-1 coculture. The data showed that A549 cells exhibited a slight increase in the levels of MMP-9 and COL-1 without THP-1 macrophages as evident from ELISA. In the coculture, however, large increases were observed for IL-6, MMP-9, TGF-$\beta$1, and COL-1. IL-6 was identified as important pleiotropic cytokine that participates in inflammation, and

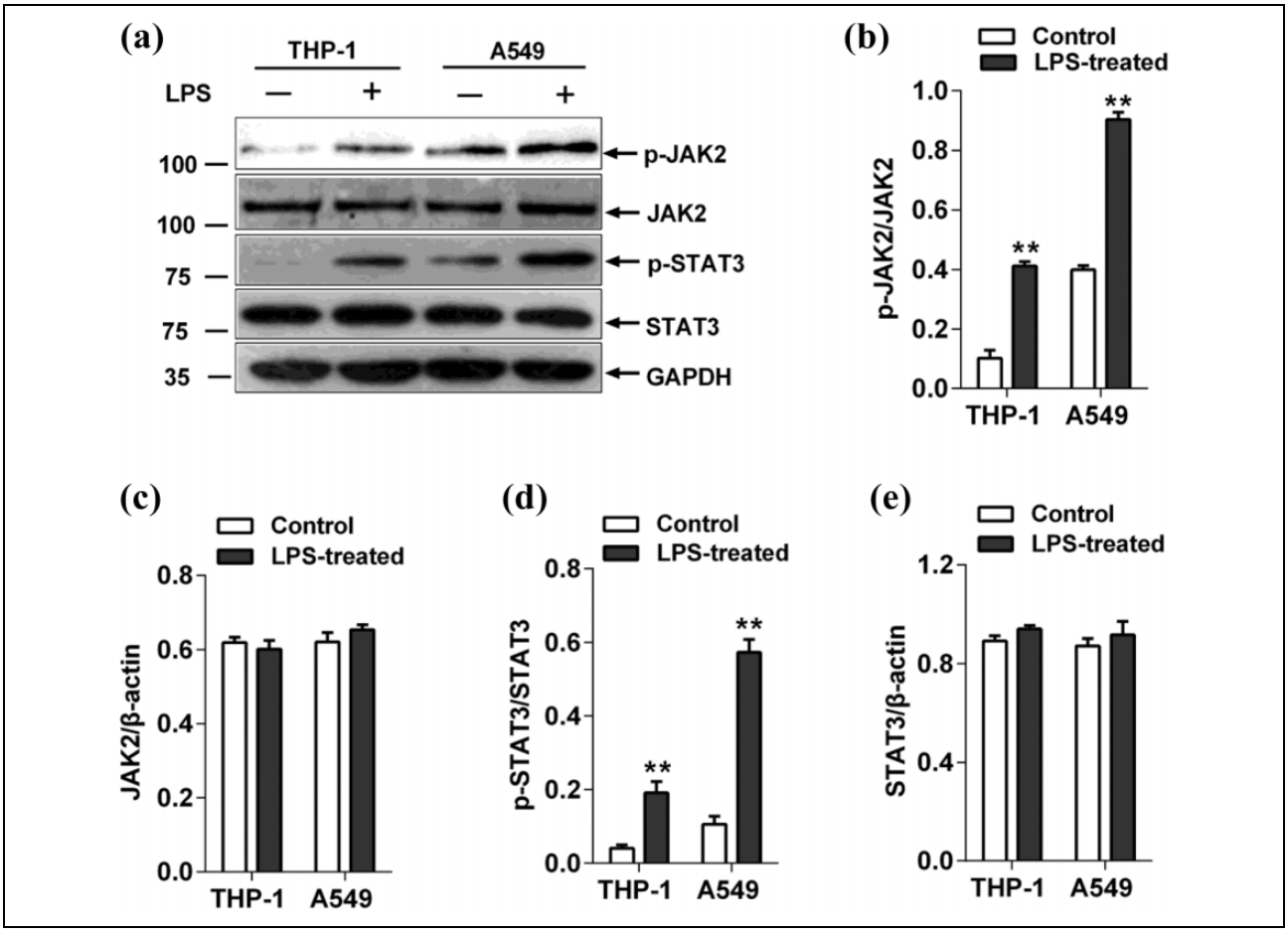


**Figure 4.** The expression of proteins of JAK2/STAT3 pathway of cocultured A549 and THP-1 cells. A549 and THP-1 cells were cocultured for 24 h at the presence or absence of LPS, and cell lysates were then collected for Western blot to detect the expression of JAK2, STAT3, and their phosphorylation. The phosphorylation of JAK2 (a) and (b) and STAT3 (a) and (c) increased in coculture A549 and THP-1 cells at the presence of LPS. Densitometric analysis was performed for quantitative evaluation. The values are presented as mean $\pm$ standard deviation of three independent experiments ($n = 3$), $^{**}p < 0.01$ versus control group. LPS: lipopolysaccharide; STAT3: signal transducer and activator of transcription 3; JAK2: Janus kinase 2.

it was also reported to be a potent inducer of the EMT. Reports indicated that IL-6 induced and aggravated the EMT and subsequent PF[1] and blocking IL-6 could attenuate PF,[2] which correlates with our findings. TGF-$\beta$1, an important inducer of EMT, was reported to downregulate E-cadherin expression and induce epithelial cells to lose cell–cell contact.[23] The production of endogenous TGF-$\beta$1 was regulated by many factors. A study by Tian et al. demonstrated that MMP-9 induces TGF-$\beta$1 activation by cleaving latent TGF-$\beta$-binding protein-1, thus stimulating and enhancing EMT.[24] The inhibitor of MMP-9 was shown to attenuate TGF-$\beta$1-induced EMT.[25] In addition, IL-6 could enhance the effect of TGF-$\beta$ on fibrosis by promoting upregulation of the TGF-$\beta$ receptor.[26] As a synthesis of ECM molecule, COL-1 was also regarded as characteristic of mesenchymal cells, which is known to be essential for EMT and directly stimulates the EMT of lung.[27] TGF-$\beta$1 also controls the function of collagen by upregulating its expression. Walsh et al. in their study have shown that TGF-$\beta$1 increased the expression of COL-1 in A549 cells, and knocking-down COL-1 was sufficient to inhibit TGF-$\beta$1-induced EMT.[28] Together, these data could probably be attributed to the conversion of cells from epithelial cells to mesothelial cells when cocultured with THP-1 macrophages.

To further confirm the above findings, we determined the presence of EMT in A549 cells by detecting the hallmarks of EMT. It is now well accepted that

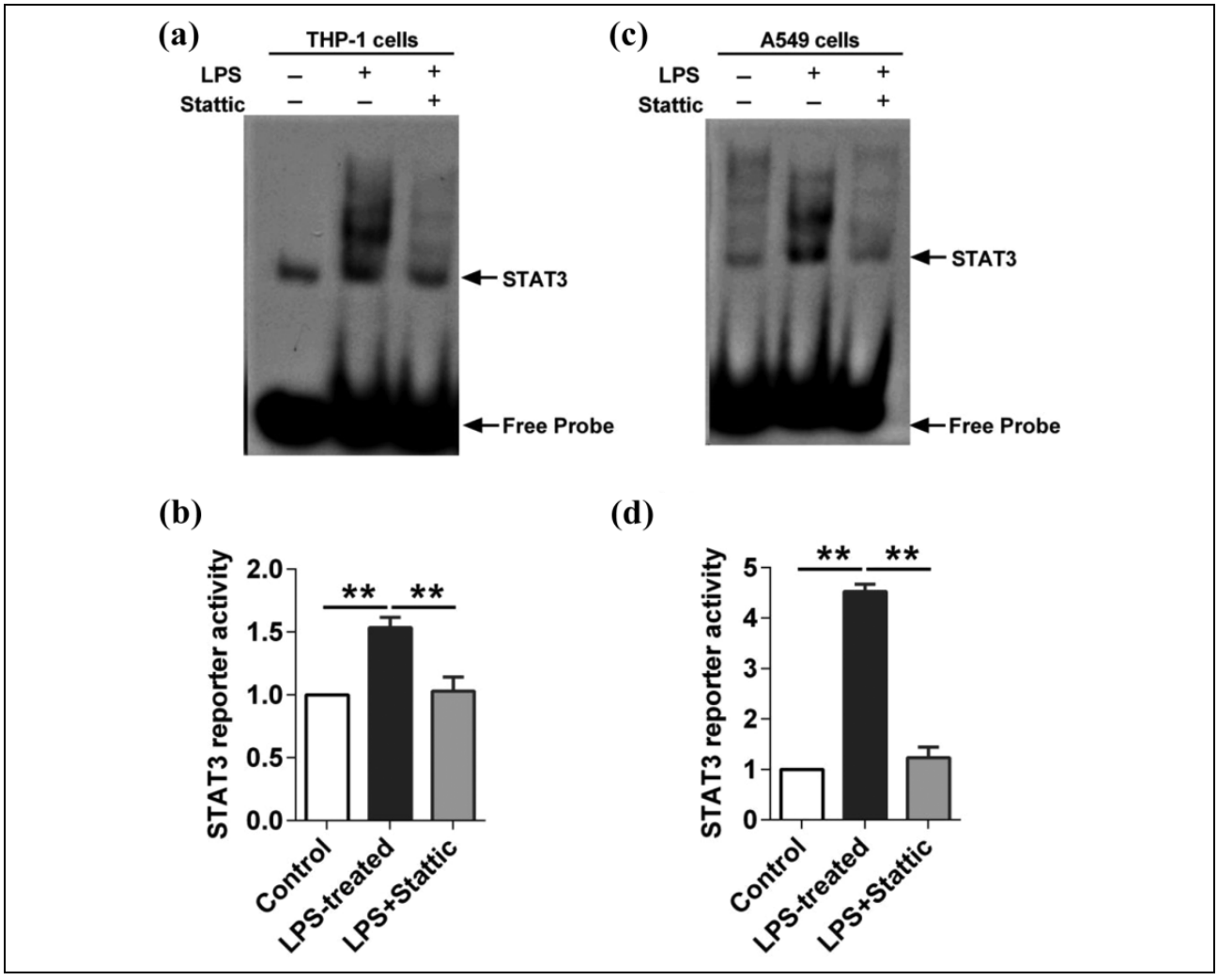


**Figure 5.** Stattic treatment reduced STAT3 DNA-binding of coculture A549/THP-1 cells. Coculture A549/THP-1 cells were pretreated with Stattic for 2 h, following LPS for 24 h, the nucleoprotein was isolated for EMSA to determine the DNA-binding of STAT3. Stattic treatment could reduce STAT3 DNA binding of A549 (a and b) and THP-1 cells (c and d). The values are presented as mean $\pm$ standard deviation of three independent experiments ($n = 3$), $^{**}p < 0.01$. LPS: lipopolysaccharide; STAT3: signal transducer and activator of transcription 3; EMSA: electrophoretic mobility shift assay.

the expression of E-cadherin and $\alpha$-SMA was regarded as the epithelial and mesenchymal markers and often assessed in EMT. Increasing number of in vitro and in vivo studies have shown decrease of E-cadherin and increase of $\alpha$-SMA in EMT.[12,24,27] E-cadherin is cell–cell adhesion molecule, helping to assemble epithelial cell sheets and to maintain the epithelial phenotype. Its downregulation discharges the cells from their basal membrane, which become phenotypically more spindle-shaped and easy to dislodge.[23] Interestingly, in our study, we observed significant downregulation of E-cadherin and upregulation of $\alpha$-SMA in A549 cells, which suggested the presence of EMT in LPS treated A549 cells cocultured with THP-1 macrophages.

However, the exact molecular mechanism that LPS-induced EMT of A549 cells cocultured with THP-1 macrophages remains unclear. Increasing evidences have shown that JAK/STAT signaling pathway activated by pro-inflammatory cytokines may be responsible for initiating and maintaining the EMT.[14,29,30] As a transcription factor, the transcriptional activity of STAT3 is positively regulated by phosphorylation at the tyrosine residue (Y705) by upstream kinases such as JAK2.[31] Consistent with these studies, we observed the upregulation of STAT3 DNA-binding and remarkably increased phosphorylation of JAK2 and STAT3 in the coculture A549 cells and THP-1 macrophages in LPS-treated group. To further explore the relationship

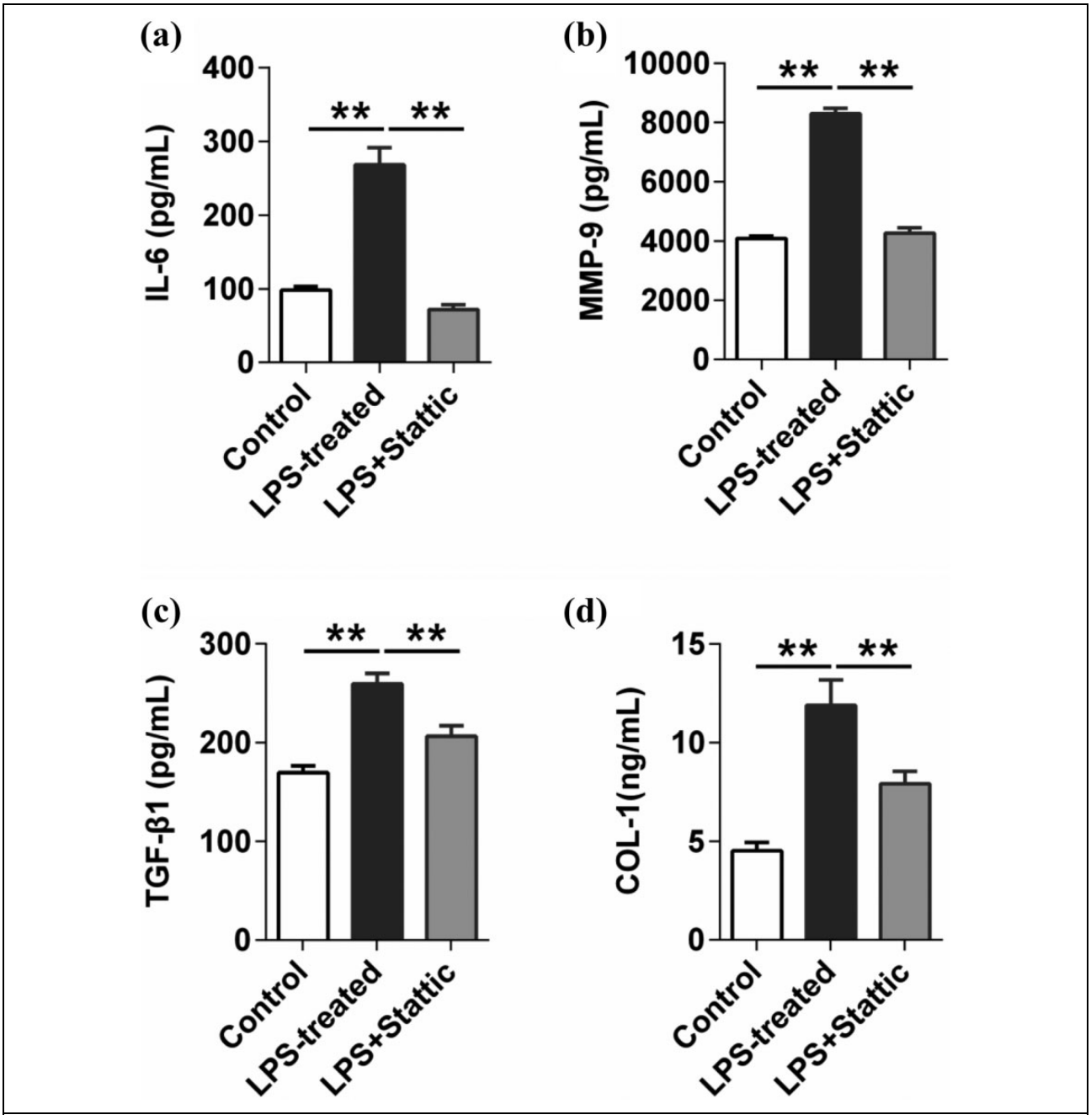


**Figure 6.** Stattic treatment reduced inflammatory markers secretions in the coculture. Coculture A549/THP-1 cells were pretreated with Stattic for 2 h following LPS for 24 h, supernatants were applied to ELISA to determine the cytokines level. Stattic treatment could reduce the level of (a) IL-6, (b) MMP-9, (c) TGF-$\beta$1, and (d) COL-1. The values are presented as mean $\pm$ standard deviation of three independent experiments ($n = 3$), $^{**}p < 0.01$ versus LPS-treated group. LPS: lipopolysaccharide; IL-6: interleukin-6; MMP-9: matrix metalloproteinase-9.

between JAK2/STAT3 with EMT in A549 cells, we pretreated the coculture with Stattic, followed by LPS exposure. The data showed that inhibition of STAT3 resulted in its decreased DNA binding and downregulation of cytokines. These results indicate that LPS induces EMT of alveolar epithelial cells cocultured with macrophages possibly through the JAK2/STAT3 signaling pathway. Collectively, we speculate that LPS induced the secretion of IL-6 in the current A549/THP-1 coculture system. Then, the secreted IL-6 acts on A549 cells and THP-1 macrophages to increase the production of TGF-$\beta$1 via

activating the JAK2/STAT3 pathway contributing to the EMT-related changes.

In conclusion, we believe that we have successfully established a coculture system in which EMT of A549 cells is orchestrated in the presence of THP-1 macrophages using LPS. In addition, LPS induces EMT of A549 cells possibly via the JAK/STAT signaling pathway in this coculture. This system is an efficient model for simulation of alveolar epithelial cell and macrophage interactions which occur in vivo and can serve as a useful tool for in vitro studies of pulmonary diseases involving EMT.

## Author contributions

JL and PZ designed the outline of the study. YQ, CL, and XM performed experiments, conceived the study, and drafted and revised the manuscript. YC, SF, XL, HD, and WZ were involved in performing experiments, acquisition of data, and statistical analysis. All authors read and approved the final version of manuscript.

## Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The research was supported by National Natural Science Fund of China (Grant Nos 81130062 and 81403367). We are grateful for the funding support from the Collaborative Innovation Center for Respiratory Disease Diagnosis and Treatment and Chinese Medicine Development of Henan Province.

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