

Coculture with bone marrow-derived mesenchymal stem cells attenuates inflammation and apoptosis in lipopolysaccharide-stimulated alveolar epithelial cells via enhanced secretion of keratinocyte growth factor and angiopoietin-1 modulating the Toll-like receptor-4 signal pathway

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Abstract. Acute lung injury (ALI) is a common, costly and potentially lethal disease with characteristics of alveolar-capillary membrane disruption, pulmonary edema and impaired gas exchange due to increased apoptosis and pulmonary inflammation. There is no effective and specific therapy for ALI; however, mesenchymal stem cells (MSCs) have been demonstrated to be a potential option. Lipopolysaccharide (LPS) is a highly proinflammatory molecule that is used to mimic an *in vivo* inflammatory and damaged state *in vitro*. The present study investigated the effect of bone marrow-derived MSCs on an LPS-induced alveolar epithelial cell (A549 cell line) injury and its underlying mechanisms by a Transwell system. It was

identified that a high LPS concentration caused a decrease in cell viability, increases in apoptosis, inflammatory cytokine release and NF- κ B activity, disruption of the caspase-3/Bcl-2 ratio, upregulation of Toll-like receptor 4 (TLR4), myeloid differentiation factor 88 (MyD88) and toll-interleukin-1 receptor domain-containing adaptor inducing interferon (TRIF) expression, and facilitation of TLR4/MyD88 and TLR4/TRIF complex formation in A549 cells. Coculture with MSCs attenuated all of these activities induced by LPS in A549 cells. In addition, an increased level of keratinocyte growth factor (KGF) and angiopoietin-1 (ANGPT1) secretion from MSCs was observed under inflammatory stimulation. KGF and/or ANGPT1 neutralizing antibodies diminished the beneficial effect of MSC conditioned medium. These data suggest that MSCs alleviate inflammatory damage and cellular apoptosis induced by LPS in A549 cells by modulating TLR4 signals. These changes may be partly associated with an increased secretion of KGF and ANGPT1 from MSCs under inflammatory conditions. These data provide the basis for development of MSC-based therapies for ALI.

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Abbreviations: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; MSC, mesenchymal stem cell; TLR, toll-like receptor; LPS, lipopolysaccharide; LDH, lactate dehydrogenase; TNF, tumor necrosis factor; IL, interleukin; NF- κ B, nuclear factor κ -light-chain-enhancer of activated B cells; MyD88, myeloid differentiation factor 88; TRIF, toll-interleukin-1 receptor domain-containing adaptor inducing interferon; KGF, keratinocyte growth factor; ANGPT1, angiopoietin-1

Key words: mesenchymal stem cell, acute lung injury, acute respiratory distress syndrome, alveolar epithelial cell, toll-like receptor 4, lipopolysaccharide, paracrine, keratinocyte growth factor, angiopoietin-1

Introduction

Acute lung injury (ALI) and its severe form, acute respiratory distress syndrome (ARDS), are serious clinical issues with no effective treatment (1). Despite tremendous improvements in treatment modalities and understanding of the associated respiratory physiology, the mortality rate for patients with ALI/ARDS is 40% (1). The characteristic features of ALI/ARDS are refractory hypoxemia, alveolar-capillary barrier disruption, pulmonary alveoli edema and impaired gas exchange (2). Alveolar epithelial cells (AECs) are essential to maintain homeostatic pulmonary functions and are the primary injury site during ALI/ARDS (3,4). AEC damage causes fluid clearance dysfunction and decreases surfactant production (4). Numerous mechanisms are involved in diffuse

AEC damage. However, excessive inflammatory reactions and apoptosis are the two primary factors (5). Therefore, preservation and repair of AECs by controlling inflammation and apoptosis are critical in ALL/ARDS treatment (5).

Mesenchymal stem cells (MSCs) are multipotent cells that exhibit a high immunoregulatory capacity (6). Accumulating evidence indicates that MSCs are an effective modality for cell-based therapies in various ALL/ARDS models, including endotoxin-induced, live bacteria-induced and sepsis-associated models, and pancreatitis-associated lung injury (7). Studies involving MSCs have focused on the effect of MSCs on the integrity and alveolar fluid clearance function of AECs (8). At present, increasing evidence suggests that the protective effect of MSCs is largely attributed to paracrine mechanisms (9). MSCs provide beneficial paracrine effects by secreting a broad range of cytokines, chemokines and growth factors, which protects endogenous cells, inhibits apoptosis and attenuates the inflammatory response (9). However, which paracrine factors and signaling pathways are involved in the immunoregulatory effects of MSCs on inflammation and apoptosis remain unclear.

Toll-like receptor 4 (TLR4) is a sensing receptor for lipopolysaccharide (LPS) (10). TLR4 signaling serves a key role in host defense, innate immunity and inflammation, and is involved in several acute and chronic diseases (11). However, unchecked or inappropriate TLR4 activation serves as an amplifier of the inflammatory response, which causes inflammation and immunity-associated tissue damage (1). Additionally, TLR4/nuclear factor κ -B signal activation triggers apoptotic cascades (12). Control of aberrant TLR4 activation contributes to improving the prognosis of inflammation and apoptosis-associated diseases including ALI/ARDS.

We hypothesized that MSCs may secrete several soluble factors to attenuate LPS-induced inflammation and apoptosis through the inhibition of TLR4 signals. Therefore, the present study was performed to investigate the effects of bone marrow-derived MSCs (BM-MSCs) on the inflammatory reaction and apoptosis of LPS-stimulated AECs in the A549 cell line, and to explore the involved mechanisms using a coculture system.

Materials and methods

Cell lines and culture. The human AEC line A549 and human BM-MSCs were purchased from Procell Life Science & Technology Co., Ltd (Wuhan, China) and Cyagen Biosciences Inc. (Guangzhou, China), respectively. The cells were maintained in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotics (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere with 5% CO₂. Characterization of surface markers [endoglin (CD105⁺), CD44 antigen (CD44⁺), hematopoietic progenitor cell antigen CD34 (CD34⁻) and receptor-type tyrosine-protein phosphatase C (CD45⁻)] and verification of the differentiation potential of MSCs (osteogenesis, adipogenesis and chondrogenesis) was performed by the supplier (Cyagen Biosciences Inc. Guangzhou, China).

Cell Counting Kit-8 (CCK-8) assay of A549 cells. A549 cells were seeded in 96-well plates (Corning Incorporated, Corning, NY, USA) at a density of 2x10³ cells/well and treated with 0, 10, 20, 40, 80, 100, 150 and 200 µg/ml LPS (*Escherichia coli* 0127: B8; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 6 h. The cell viability assay was performed using a CCK-8 kit (Beyotime Institute of Biotechnology, Haimen, China), following the manufacturer's protocol. CCK-8 solution (10 µl) was added to each well, followed by incubation for 2 h at 37°C. The optical density at 450 nm was then measured with a microplate reader (BioTek Instruments, Inc., Vermont, USA).

Coculture system and experimental design. MSCs and A549 cells were cocultured using an indirect Transwell system (Corning Incorporated). For the Cell Counting Kit-8 (CCK-8) assay, 96-well plates were used (Corning Incorporated). Briefly, 2x10³ A549 cells/well were seeded in 96-well plates. Then, 0, 1x10³, 1x10⁴ or 1x10⁵ MSCs/well were plated in the upper chamber of Transwell plates and cocultured with A549 cells overnight. LPS (100 µg/ml) was added to the lower chamber for 6 h. A cell viability assay was then performed using a CCK-8 kit (Beyotime Institute of Biotechnology). For the other experiments, 6-well plates were used (Corning Incorporated). Briefly, A549 cells (2x10³/well) were seeded in the bottom chamber of the Transwell plate, and MSCs (1x10⁵/well) were seeded in the upper chamber. The coculture groups were A549 + LPS, A549/MSC + LPS, A549 alone and A549/MSC + PBS. Following overnight coculture at 37°C in a humidified chamber with 5% CO₂, 100 µg/ml LPS was added for 6 h. Then, A549 cells were collected for apoptosis analysis by using flow cytometry, and total/nucleus protein of A549 cell was extracted for protein expression, NF- κ B and coimmunoprecipitation analyses as described subsequently. Furthermore, the culture supernatants were collected for ELISA assays as described subsequently.

A549 cell apoptosis evaluation by flow cytometry. Following LPS stimulation as aforementioned, A549 cells were collected, washed with ice-cold PBS and stained with final concentration of 0.2 µg/ml Annexin V-fluorescein isothiocyanate (FITC) and final concentration of 2 µg/ml propidium iodide (PI; Beyotime Institute of Biotechnology) at 4°C in the dark, following the manufacturer's protocol. Annexin V-FITC and PI fluorescence emissions were detected in FL1 and FL2 channels using a flow cytometer (BD Biosciences, San Jose, CA, USA). The apoptotic ratio was determined by FlowJo 7.6.4 software (Tree Star, Inc., Ashland, OR, USA).

Western blot analysis of caspase-3, B-cell lymphoma 2 (Bcl-2), myeloid differentiation factor 88 (MyD88), TLR4 and toll-interleukin-1 receptor domain-containing adaptor inducing interferon (TRIF) in A549 cells. Following LPS stimulation as aforementioned, A549 cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer containing a protease inhibitor cocktail (Beyotime Institute of Biotechnology). Protein concentrations were measured using a bicinchoninic acid (BCA) assay (Beyotime Institute of Biotechnology). Protein samples were analyzed by western blot analysis, as described previously (10). Equal amounts of protein (30 µg) were separated by 10% SDS-PAGE (Beyotime Institute of Biotechnology) and transferred onto a polyvinylidene

fluoride filter membrane (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and blocked with 5% dry non-fat milk (Beyotime Institute of Biotechnology) at 37°C for 2 h. The membrane was incubated with anti-cleaved Caspase-3 (1:1,000, cat. no. AC033), anti-phosphorylated (phosphor)-Bcl-2 (1:1,000, cat. no. AB116) (both Beyotime Institute of Biotechnology), anti-MyD88 (1:1,000, cat. no. sc-136970), anti-TRIF (1:500, cat. no. sc-514384), anti-TLR4 (1:500, cat. no. sc-293072) and anti- β -actin (1:1,000, cat. no. sc-58673) (all Santa Cruz Biotechnology, Inc.) primary antibodies overnight at 4°C, and then with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:1,000, cat. no. A0216; Beyotime Institute of Biotechnology) at 37°C for 2 h. The membrane was washed, and the signals were detected by enhanced chemiluminescence (Beyotime Institute of Biotechnology). Protein expression levels were normalized by Quantity One (version 4.62; Bio-Rad Laboratories Inc., Hercules, CA, USA).

ELISA analysis of cytokine levels and NF- κ B activity in A549 cells. Following LPS stimulation as aforementioned, culture supernatants were collected and centrifuged at 1,000 x g for 20 min at 4°C. Subsequently, tumor necrosis factor- α (TNF- α) (cat. no. CSB-E04740h), interleukin-8 (IL-8) (cat. no. CSB-E04641h), and IL-1 β (cat. no. CSB-E08053h) levels were measured by ELISA kits (Cusabio Technology, LLC., Wuhan, China), following the manufacturer's protocols. In addition, nuclear extracts of A549 cells were prepared using a Nuclear Extract kit (Active Motif, Carlsbad, CA, USA), following the manufacturer's instructions. Protein concentrations of nuclear extracts were quantified as aforementioned using a BCA assay. DNA-binding activities of NF- κ B p65 were assessed by performing an ELISA using the TransAM™ NF- κ B Transcription Factor Assay kit (cat. no. 40596; Active Motif), following the manufacturer's protocol.

Coimmunoprecipitation in A549 cells. A549 cells were pooled and lysed with RIPA lysis buffer containing the protease inhibitor cocktail following LPS stimulation as aforementioned. Cell lysates were centrifuged at 14,000 x g for 15 min at 4°C. Then, protein concentrations were quantified using a BCA kit as aforementioned. The supernatant was incubated overnight with anti-TLR4 (1:100; cat. no. sc-293072; Santa Cruz Biotechnology, Inc.) or normal goat IgG (1:100; cat. no. A7028; Beyotime Institute of Biotechnology) for 3 h at 4°C, and then with 20 μ l protein A/G-agarose (Beyotime Institute of Biotechnology) at 4°C while rocking overnight. The pellets obtained following centrifugation at 4°C in 14,000 x g for 5 sec were washed five times with washing buffer (50 mM Tris, pH 7.5, 7 mM MgCl₂, 2 mM EDTA, and 1 mM PMSF) and resolved by 1X SDS-PAGE after boiling for 10 min. The immunocomplexes were analyzed by western blot analysis using anti-MyD88, anti-TRIF or anti-TLR4 (Santa Cruz Biotechnology) antibodies as described previously.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) of keratinocyte growth factor (KGF) and angiopoietin-1 (ANGPT1) mRNAs in MSCs. Following coculture with A549 cells and LPS treatment for 6 h, total RNA from each group (MSC, MSC + A549 + PBS, MSC + LPS

and MSC + A549 + LPS) was prepared and reverse transcribed into cDNA, and then RT-qPCR was performed as described previously (1), using β -actin as an internal standard. The primer sequences were as follows: KGF (sense), 5'-GAACAAGGAAGGAAA ACTCTATGCAA-3'; KGF (antisense), 5'-AAGTGGGCTGTTTTTGTCTTTCT-3'; ANGPT1 (sense), 5'-TGGCTGCAAAA ACTTGAGAAT TAC-3'; ANGPT1 (antisense) 5'-TTCTGGTCTGCTCTG CAGTCTG-3'. The relative expression of each target gene was calculated by the 2^{- $\Delta\Delta$ Cq} method (13).

ELISAs of KGF and ANGPT1 levels. Following LPS (100 μ g/ml) stimulation as aforementioned for 6 h, the culture supernatants of each group from co-culture system were collected as described previously. Then, KGF (Human KGF ELISA Kit; cat. no. CSB-E08939h) and ANGPT1 (Human ANGPT1 ELISA Kit; cat. no. CSB-EL001706HU) levels were measured by ELISA kits (Cusabio Technology, LLC.), following the manufacturer's protocols.

Preparation of MSC conditioned medium (MSC-CM). MSC-CM was prepared as follows: MSCs (1x10⁵/well) were seeded and cocultured with A549 cells (2x10³/well) in 6-well plates overnight as aforementioned. LPS (100 μ g/ml) was added into the plate for 6 h. The conditioned medium was harvested, filtrated with a 0.22 μ m filter under sterile conditions and stored at -20°C until use.

KGF/ANGPT1 neutralization experiment. Various concentrations (0, 0.5, 1.0, 2.0 and 4.0 μ g/ml) of KGF (cat. no. AF-251-SP; R&D Systems, Inc. Minneapolis, MN, USA) and ANGPT1 (cat. no. A0604; Merck KGaA) neutralizing antibodies were added to MSC-CM to neutralize KGF and/or ANGPT1 activities in MSC-CM, respectively as described previously (14-16). Following incubation at 37°C for 1 h, the concentration of KGF (Human KGF ELISA Kit; cat. no. CSB-E08939h) and ANGPT1 (Human ANGPT1 ELISA Kit; cat. no. CSB-EL001706HU) in MSC-CM was detected by ELISA kits as aforementioned. CM treated with unspecific IgG (2 μ g/ml) antibodies (cat. no. sc-52000; Santa Cruz Biotechnology, Inc.) served as respective negative controls. Following preparation of various kinds of CM (MSC-CM, MSC-CM + IgG, MSC-CM + KGF-Ab, MSC-CM + ANGPT1-Ab and MSC-CM + KGF/ANGPT1-Ab), A549 cells were seeded and cultured in appropriate plates overnight as aforementioned. Then, LPS (100 μ g/ml) or LPS combined with various kinds of CM was added to the plates for 6 h to stimulate A549 cells. The A549 cell viability assay was performed and the levels of TNF- α , IL-8 and IL-1 β in cell culture supernatants were calculated by ELISA assays as aforementioned.

Statistical analysis. Statistical analyses were performed using SPSS software version 16.0 (SPSS Inc, Chicago, IL, USA). Data are presented as means \pm standard error of the mean and were compared by one-way analysis of variance. If the variance was homogeneous, the Least Significant Difference test was adopted for group comparisons. If the variance was not homogeneous, Dunnett's T3 test was used for group comparisons. P<0.05 was considered to indicate a statistically significant difference.

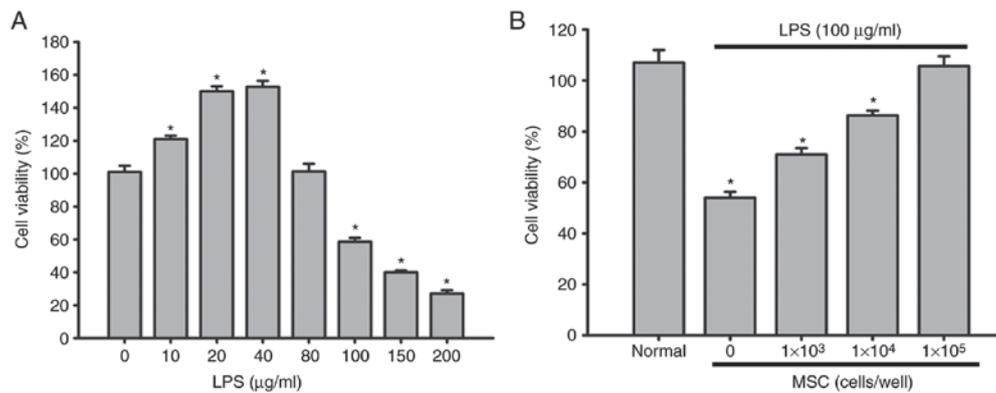


Figure 1. Coculture with MSCs attenuates the inhibition of A549 cell viability induced by LPS. (A) Effect of LPS on A549 cell viability. A549 cells were treated with LPS (0–200 µg/ml) for 6 h. Cell viability was determined by CCK-8 assay. Data are expressed as means ± SEM of three independent experiments. * $P < 0.05$ vs. LPS (0 µg/ml). (B) Coculture with MSCs increases A549 cell viability. A549 cells cocultured with various numbers of MSCs were treated with LPS (100 µg/ml) for 6 h. A549 cell viability was determined by CCK-8 assay. Data are expressed as means ± SEM of three independent experiments. * $P < 0.05$ vs. normal A549 cells. LPS, lipopolysaccharide; MSC, mesenchymal stem cell; Normal, normal and untreated A549 cells. CCK-8, Cell Counting Kit-8; SEM, standard error of the mean.

Results

Effect of LPS on the viability of A549 cells. As demonstrated in Fig. 1A, treatment with 10–40 µg/ml LPS for 6 h promoted A549 cell proliferation in a dose-dependent manner ($P < 0.05$). However, when the LPS concentration was ≥ 80 µg/ml, the survival rate of A549 cells was significantly decreased ($P < 0.05$). Specifically, 150 µg/ml LPS exhibited obvious cytotoxicity in A549 cells, decreasing cell viability to ~40% (Fig. 1A). Therefore, 100 µg/ml LPS was used for subsequent experiments, which has also been applied in previous studies (17,18).

MSCs increase the proliferation of LPS-damaged A549 cells. The CCK-8 assay demonstrated that A549 cell proliferation was suppressed following exposure to 100 µg/ml LPS ($P < 0.05$). However, following coculture with MSCs, the viability of A549 cells was significantly increased in an MSC cell number-dependent manner ($P < 0.05$; Fig. 1B).

MSCs inhibit LPS-induced apoptosis of A549 cells. Levels of apoptosis were assessed by flow cytometry of Annexin-FITC/PI-stained cells. The number of apoptotic cells was increased by treatment with LPS compared with the control ($P < 0.05$). However, coculture with MSCs weakened the promoting effect of LPS on apoptosis ($P < 0.05$). Following pretreatment with MSCs, the apoptotic rate of A549 cells was decreased under noninflammatory conditions ($P < 0.05$; Fig. 2).

MSCs modulate caspase-3 and Bcl-2 expression levels in A549 cells. The expression levels of caspase-3 and Bcl-2, which are important effector molecules during apoptosis (19), were analyzed. Cleaved caspase-3 expression was upregulated, whereas phospho-Bcl-2 expression was downregulated following LPS treatment ($P < 0.05$). However, coculture with MSCs significantly reversed these changes in protein expression ($P < 0.05$). MSCs also markedly modulated caspase-3 and Bcl-2 expression in A549 cells under non-inflammatory conditions ($P < 0.05$; Fig. 3).

MSCs attenuate the production of LPS-induced inflammatory factors in A549 cells. ELISAs demonstrated that the concentrations of TNF- α , IL-8 and IL-1 β were low in the culture supernatant of untreated A549 cells. Following LPS stimulation, there was a marked increase in the levels of TNF- α , IL-8 and IL-1 β ($P < 0.05$; Fig. 4). However, the increases induced by LPS were inhibited following coculture with MSCs ($P < 0.05$; Fig. 4). Notably, coculture with MSCs inhibited the release of TNF- α , IL-8 and IL-1 β in the absence of LPS stimulation compared with the untreated A549 cells ($P < 0.05$; Fig. 4).

MSCs attenuate the LPS-induced NF- κ B activation in A549 cells. NF- κ B is a critical transcription factor required for the maximal expression of a number of cytokines (10). As presented in Fig. 5, all LPS-stimulated A549 cells exhibited a marked increase in NF- κ B DNA binding in comparison with the A549 cells not treated with LPS. The increases in NF- κ B activity induced by LPS were decreased following coculture with MSCs ($P < 0.05$; Fig. 5). Concomitantly, coculture with MSCs inhibited the NF- κ B activation in the absence of LPS stimulation compared with the untreated A549 cells ($P < 0.05$; Fig. 5).

MSCs decrease the expression of MyD88, TLR4 and TRIF in A549 cells. In response to TLR4 activation, the adaptors MyD88, TLR4 and TRIF are overexpressed (11). Therefore, the expression of MyD88, TLR4 and TRIF were measured by western blot analysis. LPS significantly upregulated the expression of MyD88, TLR4 and TRIF in A549 cells ($P < 0.05$; Fig. 6). However, coculture with MSCs decreased MyD88, TLR4, and TRIF expression in LPS-stimulated A549 cells ($P < 0.05$; Fig. 6). Under non-inflammatory conditions, coculture with MSCs downregulated MyD88, TLR4 and TRIF expression in A549 cells ($P < 0.05$; Fig. 6).

MSCs suppress TLR4/MyD88 and TLR4/TRIF complex formation in A549 cells. TLR4/MyD88 and TLR4/TRIF complex formation is a prerequisite for TLR signal transduction (1). Therefore, the effects of MSCs on these complexes

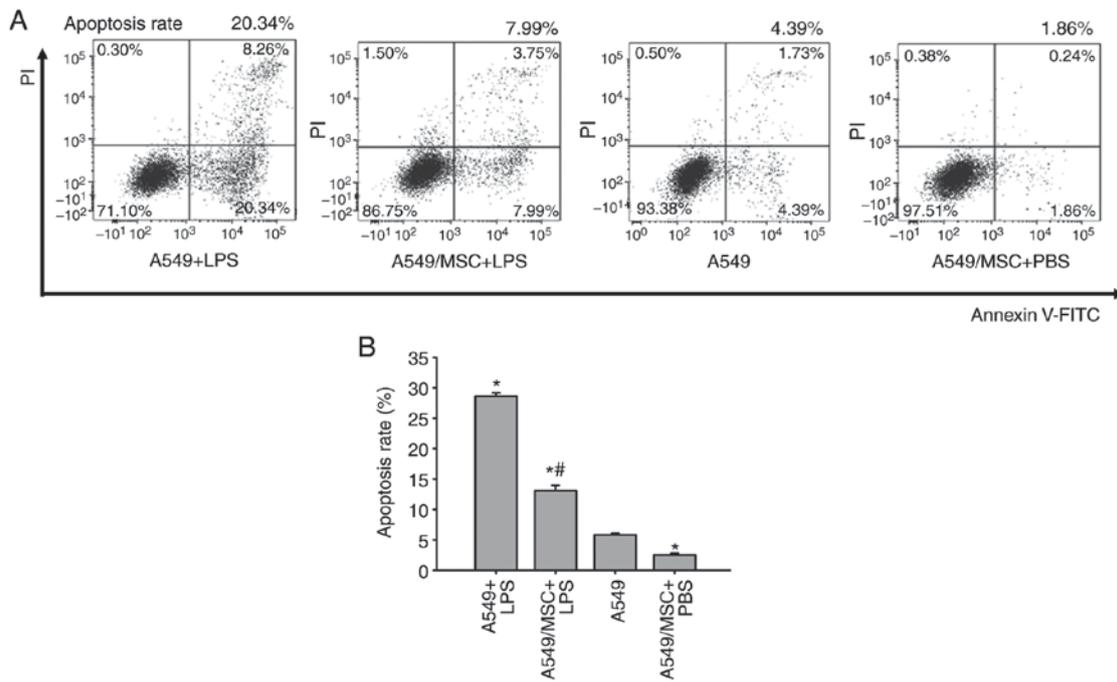


Figure 2. Coculture with MSCs inhibits A549 cell apoptosis induced by LPS. (A) A549 cells with or without coculture with MSCs were treated with LPS (100 $\mu\text{g/ml}$) or PBS for 6 h, and apoptosis of A549 was analyzed by flow cytometry. Representative examples of flow cytometry analysis for A549 cell apoptosis rate in each group are presented. (B) The cellular apoptosis rate analyses of all groups. Data are expressed as means \pm standard error of the mean of three independent experiments. * $P < 0.05$ vs. normal A549 cells, # $P < 0.05$ vs. A549 + LPS group. LPS, lipopolysaccharide; MSC, mesenchymal stem cell; FITC, fluorescein isothiocyanate; PI, propidium iodide; A549 + LPS, LPS-stimulated A549 cells; A549/MSC + LPS, A549 cells cocultured with MSCs following LPS-stimulation; A549, untreated A549 cells; A549/MSC + PBS, A549 cells cocultured with MSCs following PBS-treatment.

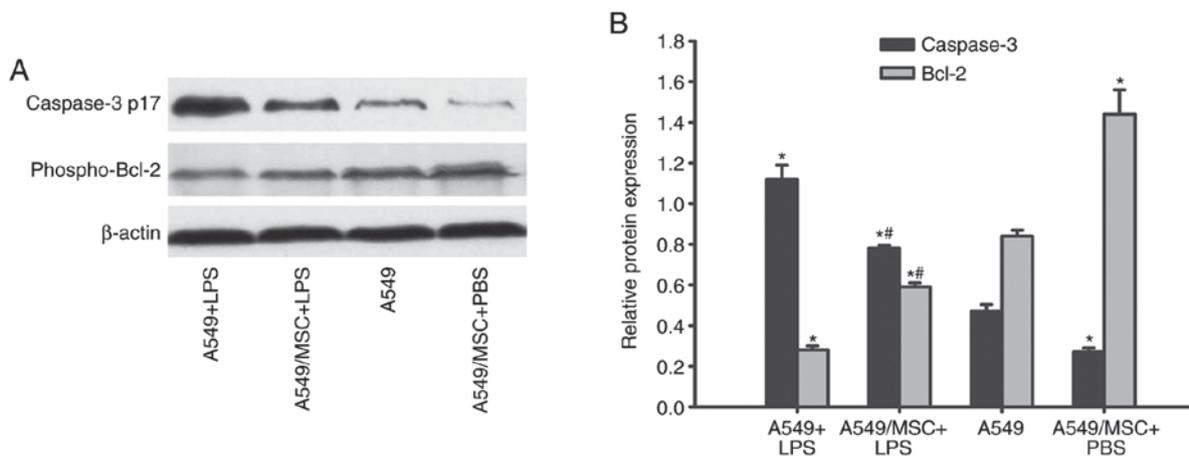


Figure 3. Coculture with MSCs modulates protein expression of caspase-3 and Bcl-2 in A549 cells. A549 cells with or without coculture with MSCs were treated with LPS (100 $\mu\text{g/ml}$) or PBS for 6 h. The cleaved-caspase-3 and phosphor-Bcl-2 protein levels in A549 cells were determined by western blot analysis. (A) Representative examples of cleaved-caspase-3 and phosphor-Bcl-2 protein bands. (B) Densitometric analyses of MyD88 and TRIF bands. Data are expressed as means \pm standard error of the mean of three independent experiments. * $P < 0.05$ vs. normal A549 cells, # $P < 0.05$ vs. A549 + LPS group. LPS, lipopolysaccharide; MSC, mesenchymal stem cell; phosphor, phosphorylated; Bcl-2, B-cell lymphoma 2; caspase-3 p17, cleaved caspase fragment; A549 + LPS, LPS-stimulated A549 cells; A549/MSC + LPS, A549 cells cocultured with MSCs following LPS-stimulation; A549, untreated A549 cells; A549/MSC + PBS, A549 cells cocultured with MSCs following PBS-treatment.

were examined by coimmunoprecipitation. As demonstrated in Fig. 7, enhanced formation of these complexes was observed following LPS stimulation ($P < 0.05$). However, the interactions of TLR4/MyD88 and TLR4/TRIF were attenuated by coculture with MSCs with or without LPS stimulation ($P < 0.05$; Fig. 7). MyD88 and TRIF expression was not detected after coprecipitation with normal non-specific IgG (Fig. 7).

KGF and ANGPT1 expression in MSCs. KGF and ANGPT1 are MSC-derived paracrine factors that target the alveolar epithelium (8). Therefore, the expression levels of KGF and ANGPT1 in MSCs prior to and following treatments were compared by RT-qPCR and ELISAs. KGF and ANGPT1 mRNA and protein expression of MSCs cocultured with A549 cells was similar to that of normal MSCs prior to LPS stimulation ($P > 0.05$; Fig. 8). Following LPS stimulation, KGF

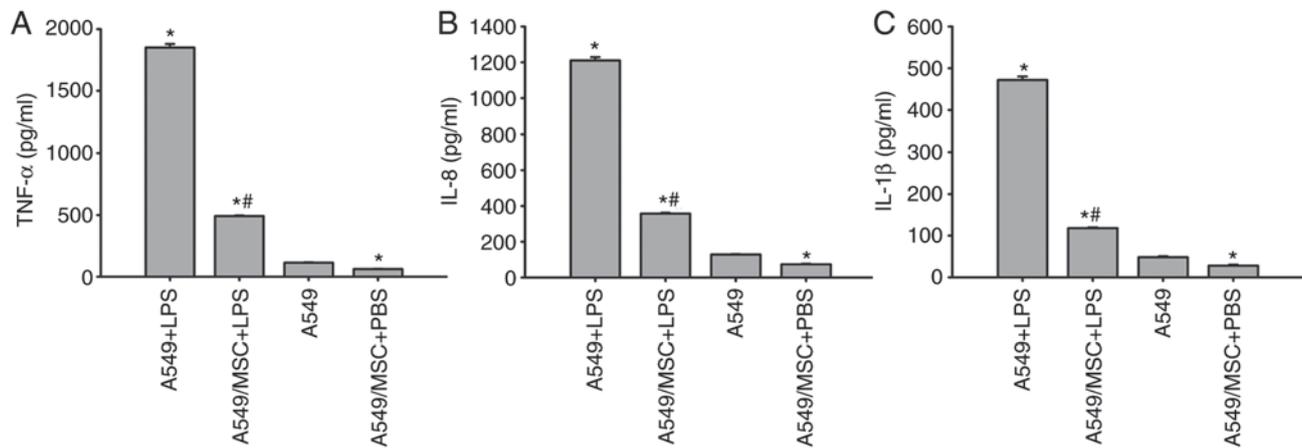


Figure 4. Coculture with MSCs inhibits the production of (A) TNF- α (A), (B) IL-8 and (C) IL-1 β derived from A549 cells induced by LPS. A549 cells with or without coculture with MSCs were treated with LPS (100 μ g/ml) or PBS for 6 h. Coculture supernatants were collected, and the levels of TNF- α , IL-8 and IL-1 β were then measured by ELISAs. Data are expressed as means \pm standard error of the mean of three independent experiments. * P <0.05 vs. normal A549 cells, # P <0.05 vs. A549 + LPS group. LPS, lipopolysaccharide; MSC, mesenchymal stem cell; TNF- α , tumor necrosis factor α ; IL, interleukin; A549 + LPS, LPS-stimulated A549 cells; A549/MSC + LPS, A549 cells cocultured with MSCs following LPS-stimulation; A549, untreated A549 cells; A549/MSC + PBS, A549 cells cocultured with MSCs following PBS-treatment.

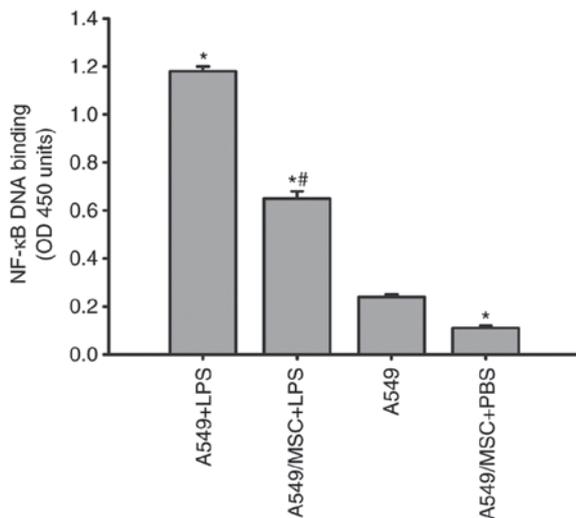


Figure 5. Coculture with MSCs inhibits NF- κ B DNA binding activity in A549 cells induced by LPS. A549 cells with or without coculture with MSCs were treated with LPS (100 μ g/ml) or PBS for 6 h. Nuclear extracts of A549 cells were collected, and the activity of NF- κ B was then measured by ELISA. Data are expressed as means \pm standard error of the mean of three independent experiments. * P <0.05 vs. normal A549 cells, # P <0.05 vs. A549 + LPS group. LPS, lipopolysaccharide; MSC, mesenchymal stem cell; NF- κ B, nuclear factor κ -light-chain-enhancer of activated B cells; OD, optical density; A549 + LPS, LPS-stimulated A549 cells; A549/MSC + LPS, A549 cells cocultured with MSCs following LPS-stimulation; A549, untreated A549 cells; A549/MSC + PBS, A549 cells cocultured with MSCs following PBS-treatment.

and ANGPT1 mRNA and protein levels were upregulated in non-cocultured MSCs, which were increased in the MSC/A549 coculture system (P <0.05; Fig. 8).

Effect of KGF and ANGPT1 neutralizing antibody on KGF and ANGPT1 levels in MSC-CM. KGF and ANGPT1 concentration levels were additionally examined by ELISAs following the addition of anti-KGF (0.5, 1.0, 2.0 and 4.0 μ g/ml) and anti-ANGPT1 (0.5, 1.0, 2.0 and 4.0 μ g/ml) antibodies to MSC-CM. The

results indicated that the levels of KGF and ANGPT1 were \sim 1,360 pg/ml and 416 ng/ml, respectively, in MSC-CM after 6 h of LPS stimulation. However, KGF in MSC-CM was significantly neutralized with \geq 2 μ g/ml anti-KGF antibody (P <0.05), while \geq 1 μ g/ml anti-ANGPT1 antibody significantly neutralized ANGPT1 in MSC-CM (P <0.05; Fig. 9).

Effect of KGF and ANGPT1 neutralizing antibody on the viability of A549 cells. To analyze the specific effect of KGF and ANGPT1 on the viability of A549 cells, the effect of MSC-CM on cell viability in the presence of neutralizing antibodies against KGF and ANGPT1 was evaluated. The results demonstrated that MSC-CM reversed the inhibitory effect of LPS on cell viability of A549 cells (P <0.05; Fig. 10). However, the beneficial effect of MSC-CM on cell viability was abrogated by the administration of KGF or ANGPT1 neutralizing antibody (P <0.05; Fig. 10). Compared with monotherapy, the combination of anti-KGF and anti-ANGPT1 antibodies additionally impaired the protective effect of MSC-CM on cell viability of A549 cells, but the difference did not reach significance (P >0.05; Fig. 10). Unspecific IgG antibodies had no effect on the cytoprotective effect of MSC-CM (P >0.05; Fig. 10).

Effect of KGF and ANGPT1 neutralizing antibodies on the release of TNF- α , IL-8 and IL-1 β in A549 cells. As indicated in Fig. 11, treatment with MSC-CM significantly inhibited the production of TNF- α , IL-8 and IL-1 β induced by LPS in A549 cells (P <0.05; Fig. 11). In addition, unspecific IgG antibodies had no effect on the inhibitory effect of MSC-CM on the production of inflammatory cytokines (P >0.05; Fig. 11). However, the anti-KGF and anti-ANGPT1 antibodies diminished this inhibitory effect of MSC-CM on the production of TNF- α , IL-8 and IL-1 β induced by LPS in A549 cells (P <0.05 vs. MSC-CM + LPS and MSC-CM + IgG + LPS; Fig. 11). Concurrently, treatment with anti-KGF antibodies combined with anti-ANGPT1 antibodies additionally reversed the beneficial effect of MSC-CM and promoted the release of A549 cell-derived inflammatory cytokines under LPS simulation (P <0.05; Fig. 11).

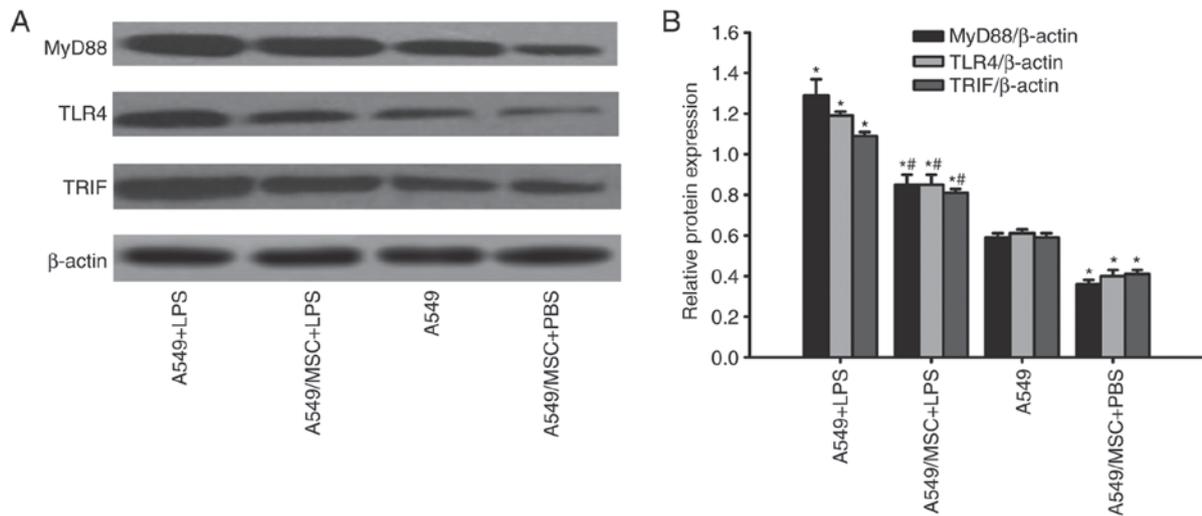


Figure 6. Coculture with MSCs suppresses TLR4, MyD88 and TRIF protein expression in A549 cells. A549 cells with or without coculture with MSCs were treated with LPS (100 μ g/ml) or PBS for 6 h. TLR4, MyD88 and TRIF protein expression in A549 cells was detected by western blot analysis. (A) Representative examples of TLR4, MyD88, and TRIF bands. (B) Densitometric analyses of TLR4, MyD88 and TRIF bands. Data are expressed as means \pm standard error of the mean of three independent experiments. * P <0.05 vs. normal A549 cells, # P <0.05 vs. A549 + LPS group. LPS, lipopolysaccharide; MSC, mesenchymal stem cell; TLR4, Toll-like receptor-4; MyD88, myeloid differentiation factor 88; TRIF, toll-interleukin-1 receptor domain-containing adaptor inducing interferon; A549 + LPS, LPS-stimulated A549 cells; A549/MSC + LPS, A549 cells cocultured with MSCs following LPS-stimulation; A549, untreated A549 cells; A549/MSC + PBS, A549 cells cocultured with MSCs following PBS-treatment.

Discussion

In the present study, it was demonstrated that high concentrations of LPS (>80 μ g/ml) were cytotoxic in AECs, suppressed AEC proliferation, induced apoptosis and provoked severe inflammatory reactions through activation of TLR4 signaling in AECs. Coculture with BM-MSCs reversed all of these detrimental effects of LPS and inhibited TLR4 signal transduction in AECs. The protective effect of MSCs may be partly associated with their paracrine secretion of KGF and ANGPT1. To the best of our knowledge, the present study is the first to demonstrate that coculture with MSCs protects AECs from LPS-induced injury via enhanced secretion of KGF and ANGPT1 and consequent inhibition of TLR4 signaling.

TLR4 is an innate immune receptor that is expressed in monocytes/macrophages, neutrophils, and dendritic, epithelial and endothelial cells (20). LPS causes conformational changes of TLR4. Toll/interleukin-1 receptor-like (TIR) domains of TLR4 recruit TIR domain-containing adaptor proteins MyD88 in the MyD88-dependent pathway or TRIF in the MyD88-independent pathway (11). MyD88-dependent and -independent pathways serve key roles in the activation of NF- κ B (21). In the present study, it was identified that coculture with MSCs downregulated TLR4, MyD88 and TRIF expression and hampered TLR4/MyD88 and TLR4/TRIF complex formation. Inhibition of the LPS-induced 'cytokine storm' and NF- κ B activation in A549 cells by coculture with MSCs may be attributed to decreases in the expression these adaptors and their complex formations. These data suggested that coculture with MSCs attenuated the LPS-induced inflammation by inhibiting TLR4 signal activation.

Excessive apoptosis of AECs is a primary factor in ALI progression (22). It has been demonstrated that TLR4 signal activation cross talks with caspase activation (12). Caspase activation has been implicated as the final common pathway

to the induction of apoptosis (19). In the present study, it was identified that 100 μ g/ml LPS upregulated the pro-apoptotic protein caspase-3 and downregulated anti-apoptotic protein Bcl-2. Coculture with MSCs reversed the modulation of these apoptosis-associated proteins. The proliferative effect of MSCs on A549 cells was associated with a suppression of the decrease in Bcl-2 and increase in caspase-3 levels. Considering the cross talk between TLR4 activation and apoptotic cascades, and the results of the present study, the inhibitory effect of MSCs on LPS-induced apoptosis and caspase activation may be attributed to the inhibition of TLR4 signaling. Additional investigation is required to reveal the detailed association between TLR4 signals and apoptosis in LPS-stimulated AECs. Notably, the data from the present study demonstrated that high doses of LPS induced A549 cell apoptosis, whereas low doses promoted A549 cell proliferation, which is in concordance with previous studies (23,24). Low doses of LPS promote cell proliferation by activating multiple signaling pathways including the phosphatidylinositol 3-kinase/protein kinase B pathway (24). Different doses of LPS may have distinct modulatory effects on various cell types (25).

Excessive inflammation and pneumocyte apoptosis are the primary pathologies of ALI (26,27). MSCs are potent tools for improving these lesions (28). MSCs may exert preventive or inhibitory effects on the inflammatory response via TLR3-regulated mitogen-activated protein kinase and TLR2/4-NF- κ B signaling pathway in LPS-induced lung injury (26,29). MSCs have been suggested to possess additional functions for the treatment of ALI/ARDS, including homing to inflammatory sites, differentiating into pneumocytes, secreting multiple soluble factors that may repair injured pneumocytes and performing immunomodulatory effects (8,30,31). However, current data suggest that the therapeutic effects of MSCs are largely mediated through paracrine factors (8). Among MSC-derived factors, KGF

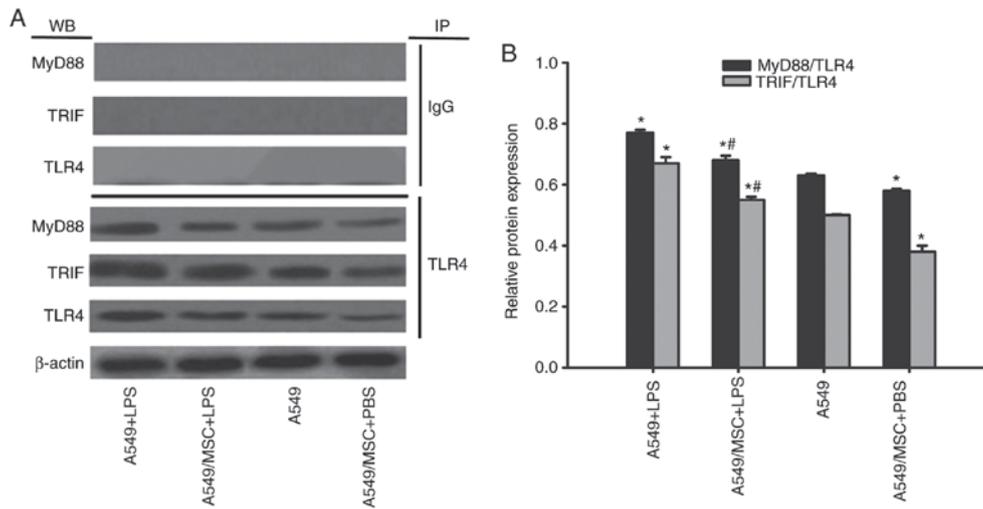


Figure 7. Coculture with MSCs suppresses TLR4/MyD88 and TLR4/TRIF complex formation in A549 cells. A549 cells with or without coculture with MSCs were treated with LPS (100 $\mu\text{g}/\text{ml}$) or PBS for 6 h. The interaction of TLR with MyD88 or TRIF was evaluated by coimmunoprecipitation. (A) Representative coimmunoprecipitated blots of MyD88 and TRIF. (B) Densitometric analyses of MyD88 and TRIF bands. Data are expressed as means \pm standard error of the mean of three independent experiments. * $P < 0.05$ vs. normal A549 cells, # $P < 0.05$ vs. A549 + LPS group. LPS, lipopolysaccharide; MSC, mesenchymal stem cell; TLR4, Toll-like receptor-4; MyD88, myeloid differentiation factor 88; TRIF, toll-interleukin-1 receptor domain-containing adaptor inducing interferon; WB, western blot analysis; IP, immunoprecipitation; A549 + LPS, LPS-stimulated A549 cells; A549/MSC + LPS, A549 cells cocultured with MSCs following LPS-stimulation; A549, untreated A549 cells; A549/MSC + PBS, A549 cells cocultured with MSCs following PBS-treatment.

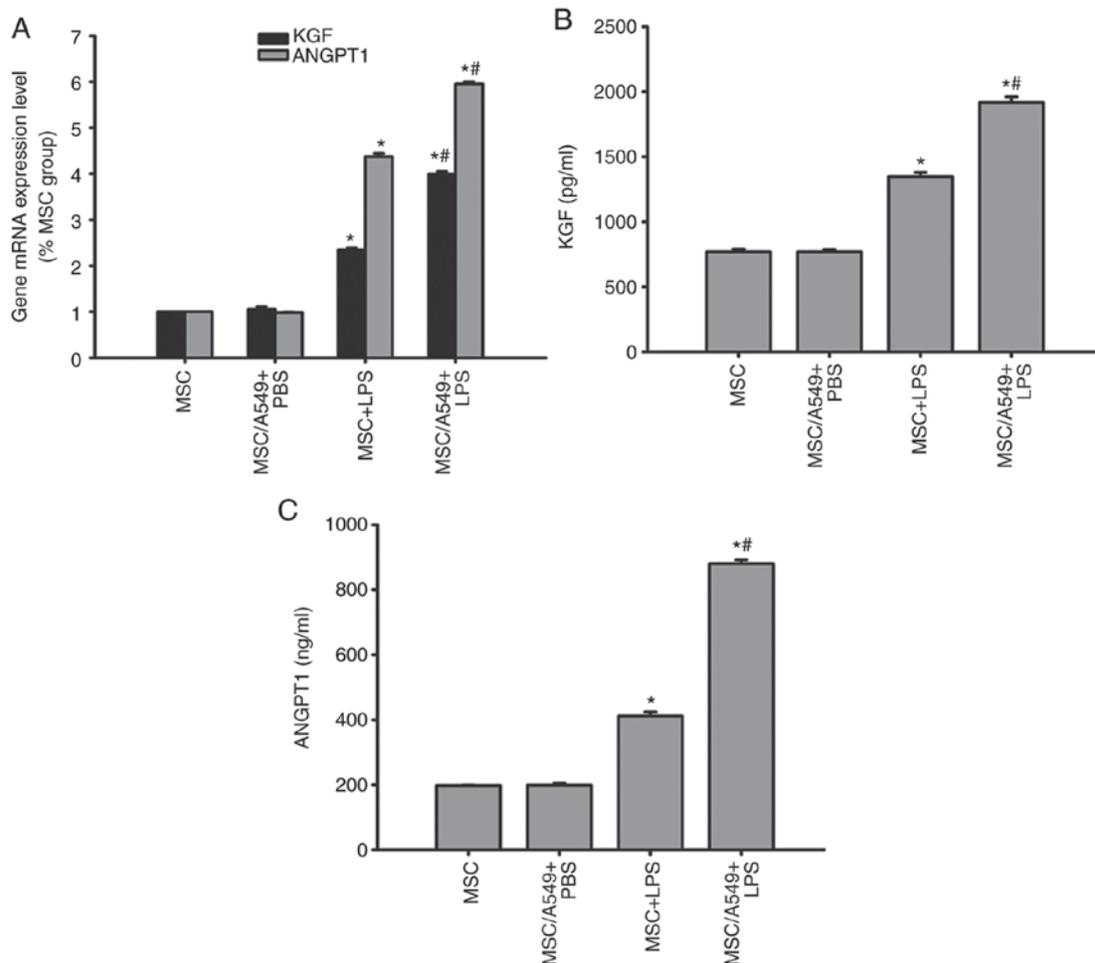


Figure 8. Inflammatory stimulation enhances the expression of paracrine factors. MSCs with or without coculture with A549 cells were treated with LPS (100 $\mu\text{g}/\text{ml}$) or PBS for 6 h. (A) KGF and ANGPT1 mRNA levels in MSCs were detected by reverse transcription quantitative polymerase chain reaction. The concentrations of (B) KGF and (C) ANGPT1 in culture supernatants were measured by ELISAs. Data are expressed as means \pm standard error of the mean of three independent experiments. * $P < 0.05$ vs. normal MSCs, # $P < 0.05$ vs. MSC + LPS group. LPS, lipopolysaccharide; MSC, mesenchymal stem cell; KGF, keratinocyte growth factor; ANGPT1, angiopoietin-1; MSC + LPS, LPS-stimulated MSCs; MSC/A549 + LPS, MSCs cocultured with A549 following LPS-stimulation; MSC/A549 + PBS, MSCs cocultured with A549 cells following PBS-treatment.

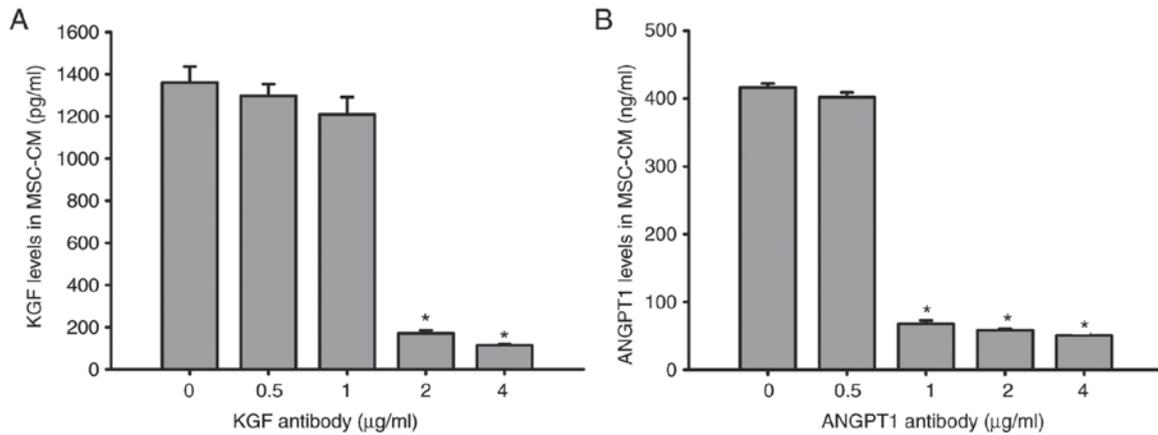


Figure 9. Inhibitory effect of anti-KGF and anti-ANGPT1 antibody on (A) KGF and (B) ANGPT1 in MSC-CM. MSC-CM was prepared and collected, and then was treated with KGF and ANGPT1 neutralizing antibodies. The concentrations of KGF and ANGPT1 in MSC-CM were measured by ELISAs. Data are expressed as means \pm standard error of the mean of three independent experiments. * $P < 0.05$ vs. 0 $\mu\text{g/ml}$. KGF, keratinocyte growth factor; ANGPT1, angiopoietin-1; MSC, mesenchymal stem cell; MSC-CM, MSC conditioned medium.

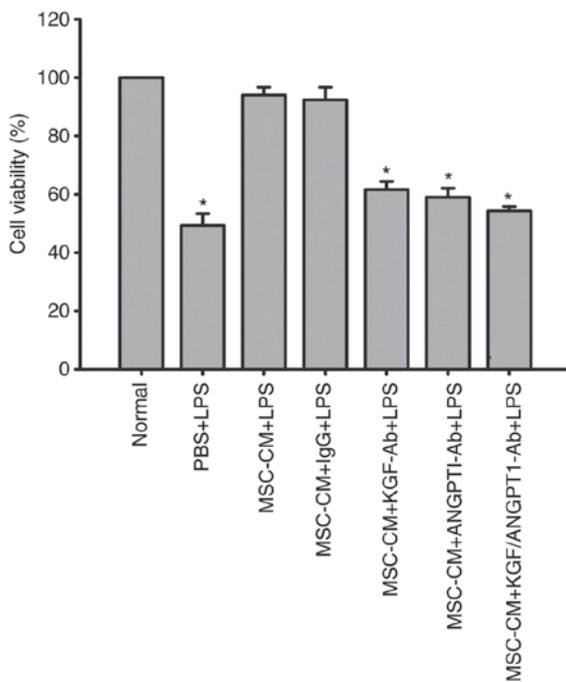


Figure 10. Coculture with MSCs improves A549 cell viability by secreting paracrine KGF and ANGPT1. A549 cells were treated with LPS (100 $\mu\text{g/ml}$) or LPS combined with various types of CM for 6 h. Then, A549 cell viability was determined by CCK-8 assays. Data are expressed as means \pm standard error of the mean of three independent experiments. * $P < 0.05$ vs. normal A549 cells. KGF-Ab, keratinocyte growth factor-neutralizing antibody; ANGPT1-Ab, angiopoietin-1-neutralizing antibody; LPS, lipopolysaccharide; IgG, Immunoglobulin G; MSC, mesenchymal stem cell; MSC-CM, MSC conditioned medium; Normal, normal and untreated A549 cells; PBS + LPS, A549 cells treated with PBS and LPS; MSC-CM + LPS, A549 cells treated with MSC-CM and LPS; MSC-CM + IgG + LPS, A549 cells treated with IgG-pretreated MSC-CM and LPS; MSC-CM + KGF-Ab + LPS, A549 cells treated with KGF-Ab-pretreated MSC-CM and LPS; MSC-CM + ANGPT1-Ab + LPS, A549 cells treated with ANGPT1-Ab-pretreated MSC-CM and LPS; MSC-CM + KGF/ANGPT1-Ab + LPS, A549 cells treated with KGF/ANGPT1-Ab-pretreated MSC-CM and LPS.

The present study revealed that the mRNA and protein expression levels of KGF and ANGPT1 were markedly upregulated following LPS stimulation. In the MSC/A549 coculture system, KGF and ANGPT1 expression levels were additionally increased. The additional increase in KGF and ANGPT1 expression may correlate with proinflammatory cytokines that also have a stimulatory effect on KGF and ANGPT1 expression (33). In coculture systems, LPS may exert its synergistic effects with proinflammatory cytokines, including TNF- α , IL-8 and IL-1 β , released by A549 cells to additionally promote paracrine factor secretion. Otherwise, MSCs attenuated inflammation and inhibited apoptosis in A549 cells without LPS stimulation. It appeared that MSCs also secreted a certain amount of KGF and ANGPT1 to protect AECs under non-inflammatory conditions. Notably, KGF and ANGPT1 neutralizing antibodies exhibited inhibitory effects on KGF and ANGPT1 in MSC-CM and impaired the protective and anti-inflammatory effect of MSC-CM. Therefore, we hypothesized that the beneficial effect of MSCs on inflammation and cell viability in AECs following LPS-stimulation was partly dependent on enhanced KGF and ANGPT1 expression. This result additionally verified the association between enhanced KGF/ANGPT1 expression and the beneficial effect of MSCs on A549 cells. In future studies, cytokine array analysis would be useful to determine whether other paracrine factors are involved in the epithelial protective effect of MSCs in addition to KGF and ANGPT1.

Primary human type II AECs are difficult to isolate and cultivate (34). Although the A549 cell line is cancerous, it is an AEC line with type II cell characteristics, which has been widely used as a suitable surrogate for a primary human type II AEC line to study ALI/ARDS (2,35). Therefore, A549 cells were selected for the experiments in the present study. The fact that beneficial effects of MSCs and TLR4 signal inhibition were observed in A549 cells indicate that the data are valuable to reveal the protective mechanisms of MSCs *in vitro*, despite the subtle differences between A549 cells and primary human AECs. Additionally, a 2-dimensional culture system was adopted in the present study, which lacked

and ANGPT1 with anti-inflammatory, anti-permeability and epithelial proliferative effects are targeted to AECs (8,32).

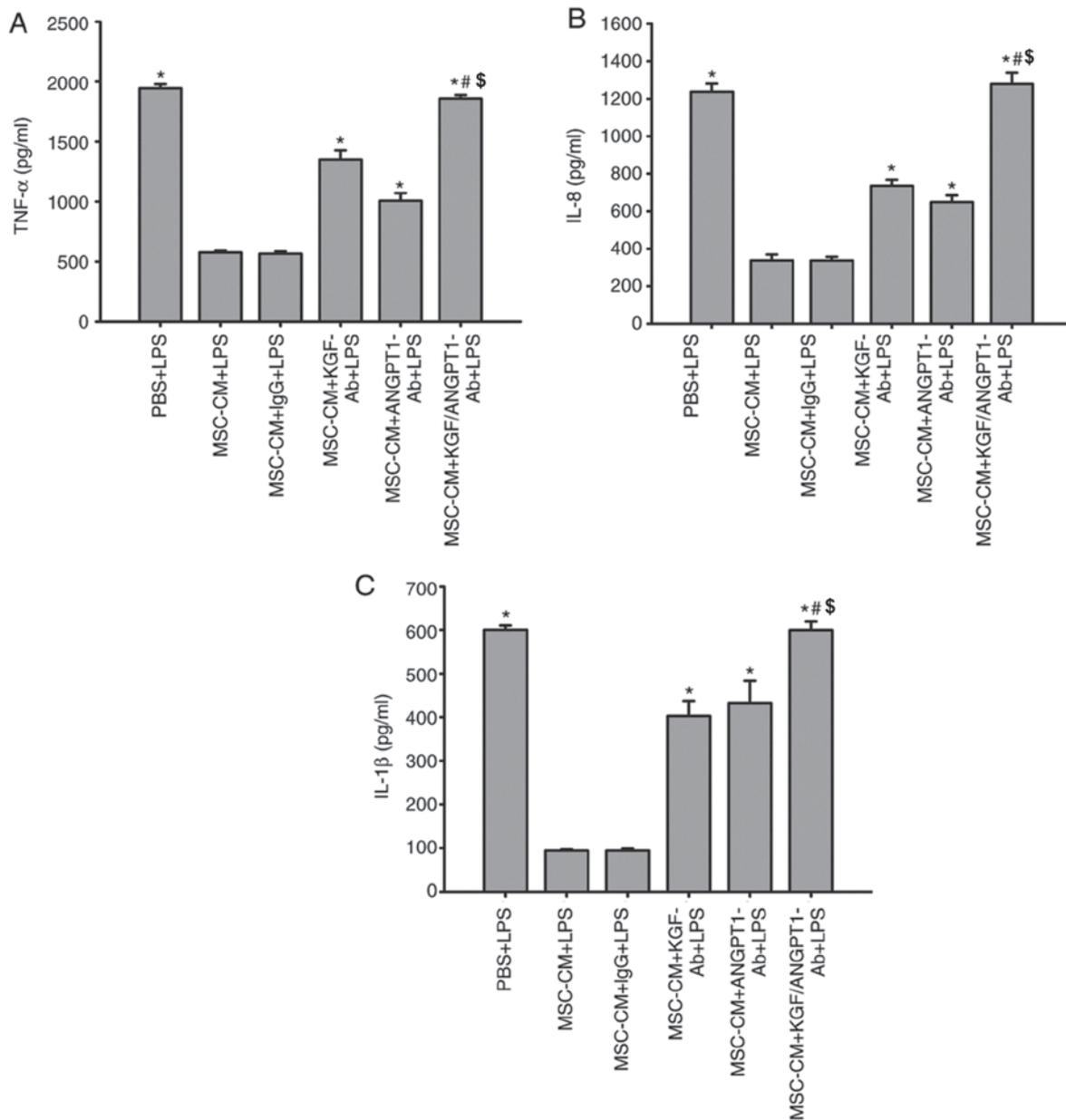


Figure 11. Coculture with MSCs inhibits the production of (A) TNF- α , (B) IL-8 and (C) IL-1 β derived from A549 cells by secreting paracrine KGF and ANGPT1. A549 cells were treated with LPS (100 μ g/ml) or LPS combined with various types of CM for 6 h. The levels of TNF- α , IL-8 and IL-1 β were then measured by ELISAs. Data are expressed as means \pm standard error of the mean of three independent experiments. *P<0.05 vs. MSC-CM + LPS, [#]P<0.05 vs. MSC-CM + KGF-Ab + LPS and [§]P<0.05 vs. MSC-CM + ANGPT1-Ab + LPS. KGF-Ab, keratinocyte growth factor-neutralizing antibody; ANGPT1-Ab, angiopoietin-1-neutralizing antibody; LPS, lipopolysaccharide; IgG, Immunoglobulin G; MSC, mesenchymal stem cell; MSC-CM, MSC conditioned medium; TNF- α , tumor necrosis factor α ; IL, interleukin; PBS + LPS, A549 cells treated with PBS and LPS; MSC-CM + LPS, A549 cells treated with MSC-CM and LPS; MSC-CM + IgG + LPS, A549 cells treated with IgG-pretreated MSC-CM and LPS; MSC-CM + KGF-Ab + LPS, A549 cells treated with KGF-Ab-pretreated MSC-CM and LPS; MSC-CM + ANGPT1-Ab + LPS, A549 cells treated with ANGPT1-Ab-pretreated MSC-CM and LPS; MSC-CM + KGF/ANGPT1-Ab + LPS, A549 cells treated with KGF/ANGPT1-Ab-pretreated MSC-CM and LPS.

an extracellular matrix and inflammatory cells. A 3-dimensional cell culture system, which is closer to the *in vivo* cell microenvironment, will be used to corroborate the results in future studies.

In conclusion, the present study demonstrated that coculture with BM-MSCs attenuates LPS-induced inflammation and apoptosis in AECs via the TLR4 signaling pathway. The modulation of TLR4 signals involves downregulation of adaptor proteins TLR4, MyD88 and TRIF and suppression of TLR4/MyD88 and TLR4/TRIF complex formation. These

beneficial effects of MSCs may be ascribed to enhanced secretion of KGF and ANGPT1 under inflammatory conditions. These data provide a novel insight into MSC-based therapeutic strategies for treating ALI. Future studies will focus on the aforementioned issues and detailed mechanisms of MSC-mediated inhibition of TLR4 signals.

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Availability of data and material

All data generated or analyzed during this study are included in this article.

Authors' contributions

JM designed the present study. XC performed the analysis of NF- κ B DNA binding activity, collected data, made figures and wrote manuscript. LT performed the cell culture, cell viability, western blot analysis and reverse transcription quantitative polymerase chain reaction assay. WW performed the ELISAs, and coimmunoprecipitation and flow cytometric assays. ZH made substantial contributions to the analysis of data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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