Mechanisms of the lipopolysaccharide-induced inflammatory response in alveolar epithelial cell/macrophage co-culture

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Abstract. The interaction between alveolar epithelial cells (EpCs) and macrophages (MPs) serves an important role in initiating and maintaining inflammation in chronic pulmonary diseases. The aim of the present study was to investigate the molecular mechanisms of the inflammatory response in co-cultured EpCs and MPs. Briefly, a co-culture system of A549 (EpCs) and THP-1 (monocyte/MPs) cells was established in a filter-separated Transwell plate to evaluate the inflammatory response. Following lipopolysaccharide (LPS) treatment, cytokine levels were measured using ELISAs, NF-κB transcription factor activity was detected using EMSA and protein expression levels were analyzed using Western blot assays subsequently in EpCs and MPs. Co-cultured EpCs/MPs were found to secrete increased levels of interleukin (IL)-6, IL-1β, IL-8 and tumor necrosis factor (TNF)-α following LPS exposure for 6, 12, 24 and 48 h compared with either EpC or MP monocultures. Concurrently, NF-κB was revealed to be activated in MPs at 6 and 12 h, and in EpCs at 24 h. NF-κB DNA binding, Toll-like receptor 4 expression levels and the p65 phosphorylation status were also increased, which may contribute to the inflammatory response in the EpC/MP co-cultures. Notably, cytokine levels decreased following the inhibition of NF-κB expression with pyrrolidinedithiocarbamate. In conclusion, the present study successfully established an EpC/MP co-culture system using LPS, which may be a useful model for studying chronic inflammation *in vitro*.

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Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory process that predominantly affects lung parenchyma and small airways, resulting in progressive and largely irreversible airflow limitation (1). COPD-affected lungs exhibit inflammatory changes that involve immune cells, such as neutrophils and macrophages (MPs), alongside the activation of structural cells, including alveolar epithelial cells (EpCs) and fibroblasts (2,3). M§Ps are major contributors to inflammation and produce mediators that activate the inflammatory transcriptional program of other cells (4). For example, EpCs and MPs in COPD-affected lung tissues have been reported to be induced by bacteria to release inflammatory mediators, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and reactive oxygen species, in addition to secreting elastolytic enzymes, such as matrix metalloproteinases (3,5). In addition, EpCs have been observed to modulate their primary transcriptional program in response to inflammatory mediators, such as TNF-α released by MPs stimulation (6). In fact, in one study, activated EpCs were found to negatively regulate monocyte cytokine gene expression, which influenced the maturation of monocytes to MPs, and potentially further modulated cytokine release through post-translational pathways (7). EpCs have also been demonstrated to modulate MP phenotypes; co-cultured A549 cells significantly increased the expression levels of CD11b, CD14, CD54 and human leukocyte antigen-DR isotype in the human monocyte/MP cell line, THP-1 (8).

In COPD, there is an increased presence of numerous types of inflammatory mediators, such as interleukins and chemokines, which have derived from the inflammatory and structural cells of the lungs and airways (5,9). Proinflammatory cytokines are induced by numerous stimuli through the NF-κB and Janus tyrosine kinase/STAT signaling cascades. For example, the exacerbation of chronic respiratory diseases like COPD is often triggered by a bacterial or viral infection (10); lipopolysaccharide (LPS), a constituent of the outer cell membrane of Gram-negative bacteria, binds to Toll-like receptor 4 (TLR4) located in the cell membrane and activates the downstream transcriptional factor, NF-κB (11), which mediates the transcription and translation of proinflammatory

mediators, such as IL-6 and TNF-α. Thus, the present study aimed to investigate the inflammatory response, including the release of cytokines and signaling pathways, involved in LPS-induced EpCs/MPs co-culture.

Materials and methods

Cell culture. The human lung cancer A549 cell line and THP-1 cells were purchased from the American Type Culture Collection. Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 0.05 nM β-mercaptoethanol and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained at 37˚C in a 5% CO_2 atmosphere. The A549 cells were to represent EpCs, whereas THP-1 cells represented MPs. EpCs were first cultured in 24-well culture plates until they had completely adhered and subsequently the medium was removed, and the cells were washed twice with PBS to exclude serum factor effects before co-culturing. The MPs were plated in a Transwell insert and physically separated with a 0.4-μm pore polyester filter (Corning, Inc.) to avoid direct contact with the EpCs.

Cell viability assay. Following overnight culture in a 96-well plate at 37˚C, $1x10^4$ EpCs were exposed to 0.5, 1, 2 or 4 μg/ml LPS (Escherichia coli O55:B5; cat. no. L6529; Sigma-Aldrich, Merck KGaA) for 6, 12, 24 and 48 h, and EpC viabilities were quantified using MTT assays. Untreated cells were acted as control. Briefly, 100 μl MTT solution (1 mg/ml) was added to each well and following 4 h of incubation at 37˚C, the supernatants were removed and 150 μl DMSO was added/well. The absorbance/well was measured at 570 nm using ELX800 microplate reader (BioTek Instruments, Inc.).

Meanwhile, $5x10^4$ MPs were plated in a 96-well plate with 10 μl Alamar Blue™ agent (cat. no. 4020ES76; Yeasen Biotechnology Co., Ltd.) per well. At the same time, as the cells were added to the wells, 0.5, 1, 2 or 4 μg/ml LPS was added and the cells were exposed for 6, 12, 24 and 48 h. The absorbance/well was measured at 570 and 600 nm using a microplate reader. Untreated cells were used as the control.

ELISAs. From analyzing the effects of LPS on cell viability, 1 and 2 μg/ml LPS were selected as the optimum doses to use in further experiments. EpCs/MPs ($1x10^5$ of each cell type) were co-cultured in a 24-well Transwell plate and then stimulated with 1 or 2 μg/ml LPS for 6, 12, 24 and 48 h. Untreated cells were used as the control. The concentrations of IL-6, IL-1β, IL-8 and TNF-α in conditioned media were subsequently analyzed using ELISA kits (Boster Biological Technology; cat. no. IL-6, EK0410; IL-1β, EK0392; IL-8, EK0413; TNF-α, EK0525), according to the manufacturers' protocols. Subsequently, monocultures of EpCs ($1x10^5$ cells) and MPs ($1x10^5$ cells), as well as the co-culture of EpCs/MPs ($1x10^5$ of each cell type) were plated in 12-well culture plate, and following stimulation with 2 μg/ml LPS for 6, 12, 24 and 48 h, the cytokine levels were determined using the aforementioned ELISA kits.

Detection of NF-κB DNA-binding activity of EpCs and MPs using an electrophoretic mobility shift assay (EMSA). Following the induction with 2 μg/ml LPS for 6, 12, 24 and 48 h, EpCs and MPs were centrifuged for 5 min with the speed of 500 x g at 4˚C to extract nuclear proteins using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (cat. no. 78833; Thermo Fisher Scientific, Inc.). Nuclear extract protein was quantified using a bicinchoninic acid (BCA) assay (Boster Biological Technology). The activity of NF-κB was detected using an EMSA. The following NF-κB oligonucleotide probes were obtained from Beyotime Institute of Biotechnology (cat. no. 1302131410): Forward, 5'-AGTTGAGGGGACTTTCCC AGGC-3' and reverse, 3'-TCAACTCCCCTGAAAGGGTCC G-5'. Binding reactions were performed at room temperature for 20 min in 10 μl binding buffer (cat. no. GS006; Beyotime Institute of Biotechnology), containing 10 μg nuclear extracts, nuclease-free water, EMSA gel-shift buffer and oligonucleotide probes. The biotin-labeled DNA oligo probe without any protein extract was regarded as the negative control. DNA-protein complexes were separated from free DNA probes at 120 V on 5% polyacrylamide gels with 0.5X Tris Boric acid EDTA buffer. Following electrophoresis, the gels were transferred to a nylon membrane and detected using a chemiluminescent substrate (cat. no. 1419701; EMD Millipore). The signal intensity was quantified using an image analyzer (Bio-Rad Laboratories, Inc.).

Western blotting. The expression levels of upstream proteins of the NF-κB signaling pathway were detected using western blotting following 24 h of EpC/MP co-culture treated with 2 μg/ml LPS. Total protein was obtained by RIPA lysis buffer (cat. no. R0010; Beijing Solarbio Science and Technology Co., Ltd.), and quantified using a BCA assay. Then 20 μg protein/lane was separated via 8% SDS-PAGE. The separated proteins were subsequently transferred to polyvinylidene fluoride membranes (EMD Millipore) and blocked with 5% skimmed milk powder diluted in Tris-buffered saline -0.05% Tween 20 (TBST) buffer at room temperature for 1 h. The membranes were incubated with the following primary antibodies at 4˚C overnight: Anti-TLR4 (1:500; cat. no. 19811-1-AP; ProteinTech Group, Inc.), anti-phosphorylated (p)-p65 (1:2,000; cat. no. ab76302; Abcam), anti-p65 (1:2,000; cat. no. ab32536; Abcam), anti-p-ERK (1:2,000; cat. no. ab223500; Abcam), anti-ERK (1:1,500; cat. no. ab201015; Abcam) and anti-GAPDH (1:2,000; cat. no. ab128915; Abcam). Following the primary antibody incubation, the membranes were washed five times with TBST buffer and incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:15,000; cat. no SA00001-2; ProteinTech Group, Inc.) at room temperature for 2 h. Subsequently, the membranes were washed five times with TBST. Protein bands were visualized with an enhanced chemiluminescence reagent (EMD Millipore) and a Gel Doc XR+ system (Bio-Rad Laboratories, Inc.). Expression levels were quantified using Image Lab software version 5.1 (Bio-Rad Laboratories, Inc.).

Investigating the effect of NF-κB inhibition on the cytokine levels. To confirm whether the levels of proinflammatory cytokines were regulated by the NF-κB signaling pathway, co-cultured EpCs/MPs were pretreated with 100 μM pyrrolidinedithiocarbamate (PDTC; Beyotime Institute of Biotechnology), a selective NF-κB inhibitor (12), at 37˚C for 2 h prior to stimulation with 2 μg/ml LPS for a further 24 h. The levels of IL-6, IL-1β, IL-8 and TNF-α in the co-culture supernatant were measured using the aforementioned commercial ELISA kits.

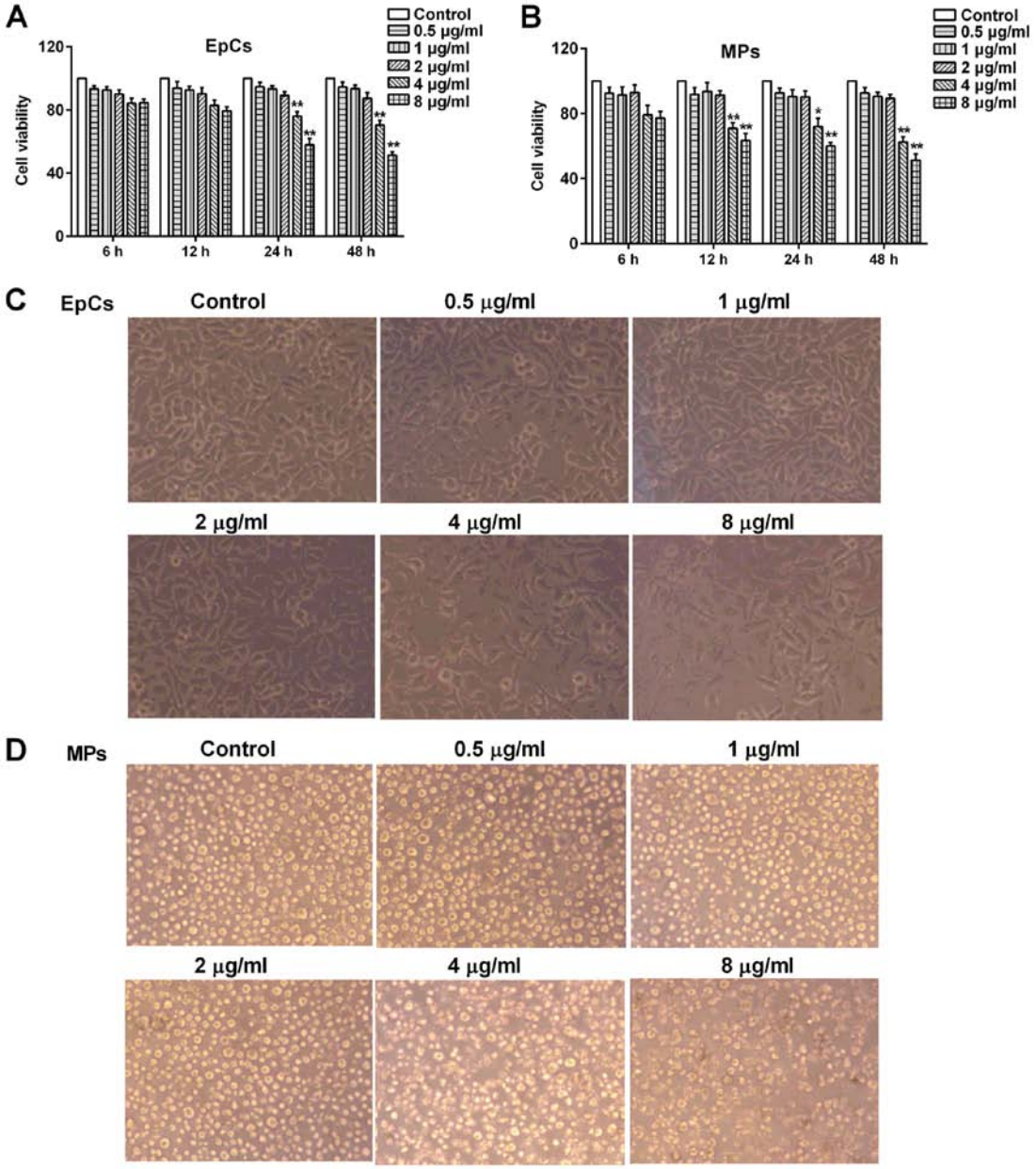

Figure 1. Viability and proliferation of EpCs and MPs in response to LPS stimulation. Following the stimulation with different concentrations of LPS, (A) EpCs viability was analyzed using an MTT assay and (B) MPs viability was analyzed using an alamarBlue assay. (C) EpCs and (D) MPs morphology were visualized using light microscopy (100X) following different concentrations of LPS treatment for 48 h. Results are presented as the mean ± SD of three independent experimental repeats. *P<0.05, **P<0.01 vs. control group. LPS, lipopolysaccharide; EpC, epithelial cell; MP, macrophage.

Statistical analysis. Statistical analysis was performed using SPSS 21.0 software (IBM Corp.) and data are presented as the mean ± SD. Statistical differences between groups were determined using one-way ANOVAs, followed by a Tukey's post hoc test for multiple comparisons in data with >2 groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of LPS stimulation on cell proliferation and viability. Cytotoxicity assays, which reflect both the level of cell damage and alterations in cellular proliferation, were used to investigate the cytotoxic effects of LPS-treated EpCs and MPs. Following the treatment with LPS at various concentrations (0.5, 1, 2, 4 and 8 μg/ml) for 6, 12, 24 and 48 h, the viability of EpCs were analyzed using an MTT assay, whereas MP viability was determined using an alamarBlue assay. LPS was found to be non-toxic to EpCs or MPs at concentrations between 0.5-2 μg/ml (Fig. 1A and B); however, 4 and 8 μg/ml LPS significantly decreased the cell viability of both EpCs and MPs at 12, 24 and 48 h. In addition, 0.5-4 μg/ml LPS did not affect the cell morphology of EpCs (Fig. 1C) or MPs (Fig. 1D), thus 1 and 2 μg/ml LPS were selected as doses of LPS to use in subsequent experiments.

Effect of LPS on cytokine levels in EpC and MP monocultures, and EpC/MP co-cultures. Co-cultured EpCs/MPs were stimulated with 1 or 2 μg/ml LPS for different durations and the levels of secreted cytokines were analyzed using ELISAs.

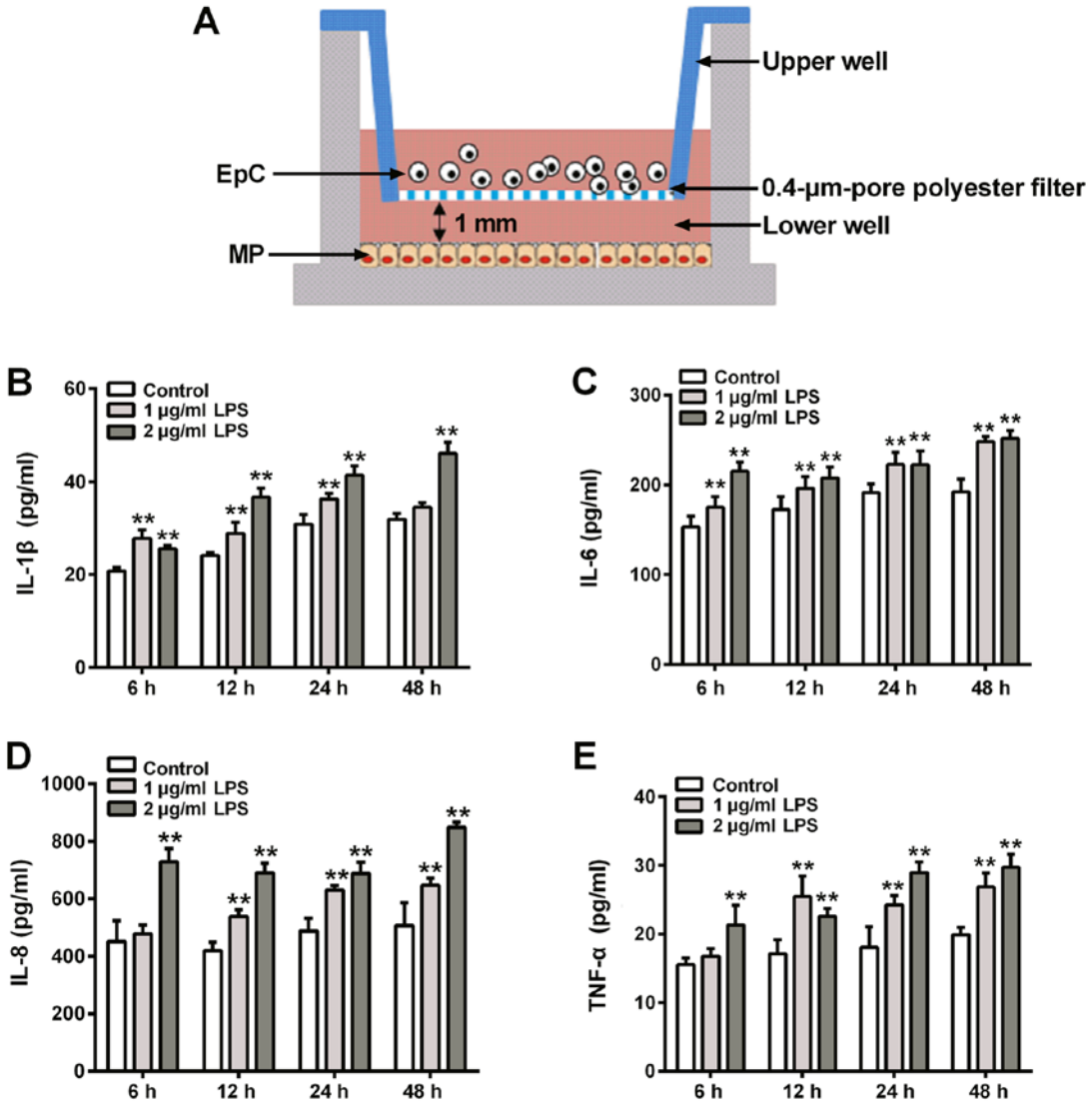

Figure 2. Effect of different concentrations of LPS on the levels of cytokine secretion in EpC/MP co-culture. (A) EpCs and MPs were co-cultured in Transwell plates and stimulated with 1 or 2 μg/μl LPS for 6, 12, 24 and 48 h. The cytokine levels of (B) IL-1β, (C) IL-6, (D) IL-8 and (E) TNF-α were analyzed using ELISAs. Results are presented as the mean ± SD of three independent experimental repeats. **P<0.01 vs. control group. LPS, lipopolysaccharide; EpC, epithelial cell; MP, macrophage; IL, interleukin; TNF, tumor necrosis factor.

The co-culture system is demonstrated in Fig. 2A. The data presented in Fig. 2B-E showed that 2 μg/ml induced greater IL-1β, IL-6, IL-8 and TNF-α expression levels at 6, 12, 24 and 48 h. Therefore, 2 μg/ml LPS constituted the LPS dose used in subsequent experiments. Subsequently, EpCs and MPs cultured both together, and separately, were exposed to 2 μg/ml LPS for 6, 12, 24 and 48 h before determining the cytokine concentrations in the supernatants. The cytokine levels of IL-1β, IL-6, IL-8 and TNF-α were not significantly increased in EpCs following LPS stimulation compared with the control group, except for IL-1β levels at 48 h (Fig. 3); however, the incubation of MPs with LPS resulted in significantly increased levels of IL-6, IL-1β, IL-8 and TNF-α at 48 h, IL-6, IL-1β, IL-8 at 24 h, IL-1β, IL-8 at 12 h, IL-1β and TNF-α at 6 h, compared with the control group (Fig. 3). Upon the co-culturing of the cells, LPS stimulation significantly increased the levels of IL-6, IL-1β, IL-8 and TNF-α at all time points compared with the control cells (Fig. 3). Collectively, these data suggested that LPS may not promote inflammation in EpCs, but may be able to in MPs from 12 h. The LPS-induced inflammatory response was amplified in the EpC/MP co-cultures, which indicated that this co-culture system may be suitable to use as an inflammatory model to research chronic inflammatory diseases.

Effect on NF-κB DNA-binding. It is well established that TLR4 recognizes bacterial LPS and triggers an inflammatory response, mainly through the TLR4 receptor (13). TLR4 activation induces the activity of the NF-κB nuclear transcription factor, which ultimately results in the release of proinflammatory cytokines (14). Thus, NF-κB DNA-binding was detected in the present study and it was revealed that LPS significantly induced NF-κB activation in MPs at 6 and 12 h compared with the control (Fig. 4A-C), whereas NF-κB DNA-binding was significantly increased in EpCs at 24 h compared with the control (Fig. 4D-F).

Expression levels of upstream proteins of the NF-κB signaling pathway. As a stimulus, LPS binds to TLR4 in the cell membrane to activate downstream factors, such as increasing

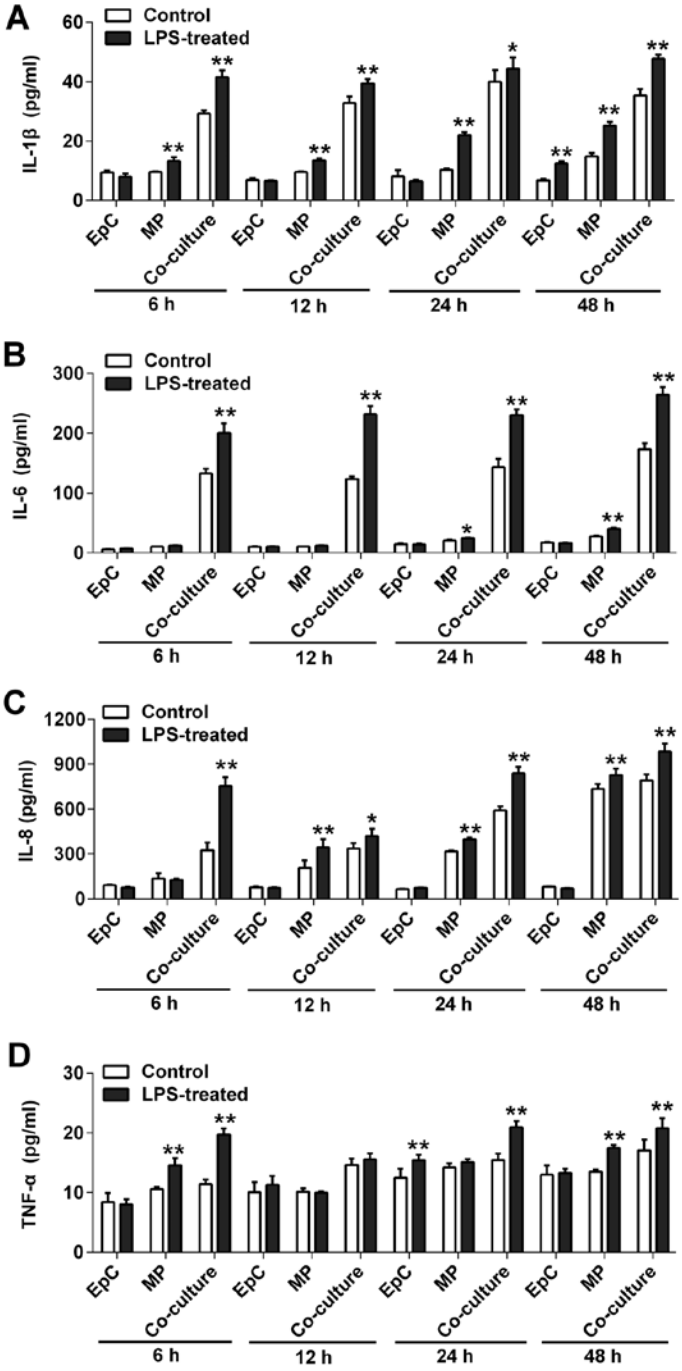

Figure 3. Effect of LPS on cytokine secretion in EpC and MP monoculture, and EpC/MP co-culture. The levels of (A) IL-1β, (B) IL-6, (C) IL-8 and (D) TNF-α were analyzed using ELISAs in EpC and MP monocultures, in addition to EpC/MP co-culture, following 2 μg/μl LPS stimulation for 48 h. Results are presented as the mean ± SD of three independent experimental repeats. *P<0.05, **P<0.01 vs. control group. LPS, lipopolysaccharide; EpC, epithelial cell; MP, macrophage; IL, interleukin; TNF, tumor necrosis factor.

p65 expression levels and subsequently, its phosphorylation levels (15). TLR4 expression levels and the p65 phosphorylation status were significantly increased in LPS-induced co-cultured EpCs/MPs compared with the control cells (Fig. 5). In addition to the activation of NF-κB, LPS also activates a series of major intracellular signaling transduction pathways, such as ERK, which serves crucial roles in inflammation (16,17). Therefore, the expression levels and phosphorylation status of ERK1/2 in EpCs and MPs were analyzed. Notably, it was identified that LPS had no effect on the expression levels of p-ERK1/2 or ERK1/2 in the EpC/MP co-culture (Fig. 5). Therefore, although the evidence is lacking, it was hypothesized that the interaction between co-cultured EpCs and MPs may affect the expression levels of ERK1/2.

Effects of the NF-κB inhibitor on the levels of cytokines. The above findings indicated that LPS may induce cytokine release by activating the nuclear transcription factor, NF-κB. To confirm its role in LPS-induced cytokine secretion, the levels of IL-1β, IL-6, IL-8 and TNF-α were analyzed following the incubation of co-cultured EpCs and MPs with the NF-κB inhibitor, PDTC. It was revealed

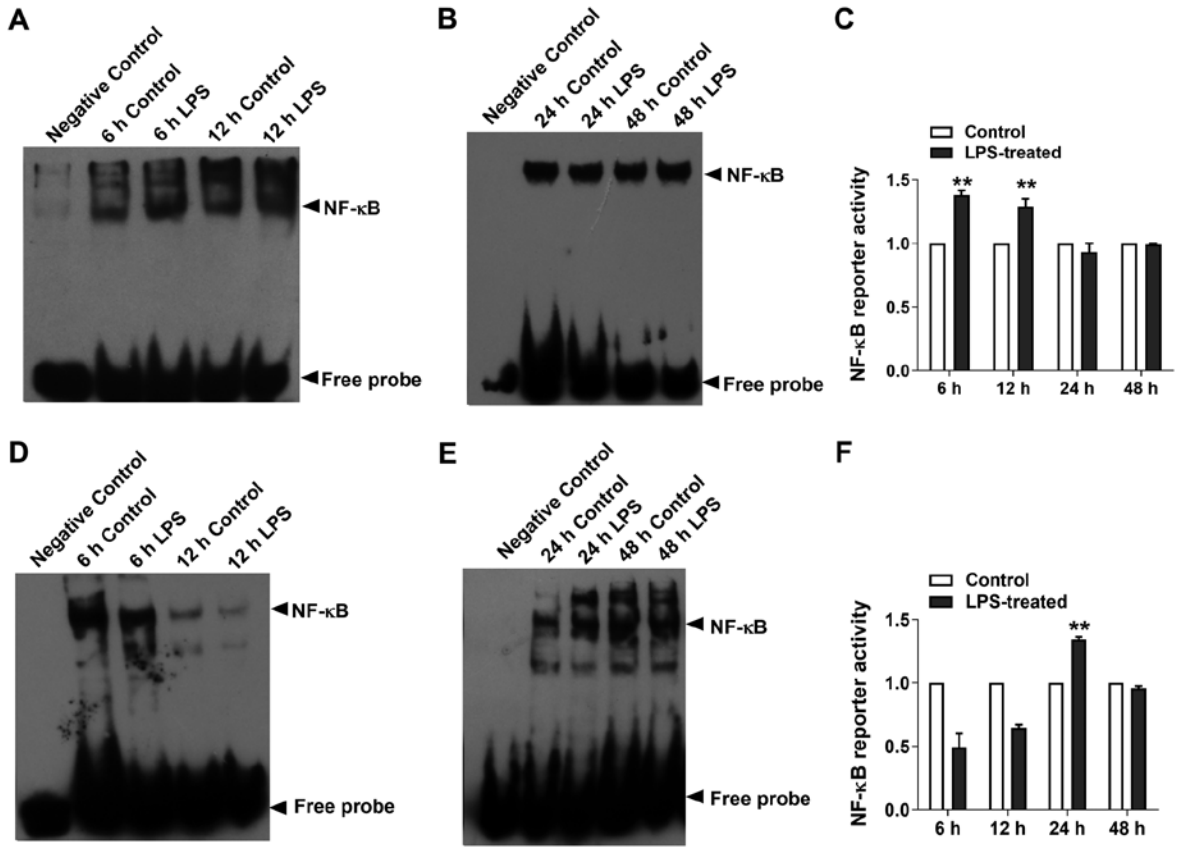

Figure 4. Effect of LPS on the DNA binding of NF-κB in EpCs/MPs co-culture. Co-cultured EpCs and MPs were stimulated with LPS for 6, 12, 24 and 48 h. Nuclear extract proteins from the cells were isolated and an electrophoretic mobility shift assay was performed to determine the NF-κB DNA binding of (A-C) MPs and (D-F) EpCs. The biotin-labeled DNA oligo probe without any protein extract was regarded as the negative control. Three independent experimental repeats were performed. Results are presented as the mean ± SD. **P<0.01 vs. control group. LPS, lipopolysaccharide; EpC, epithelial cell; MP, macrophage.

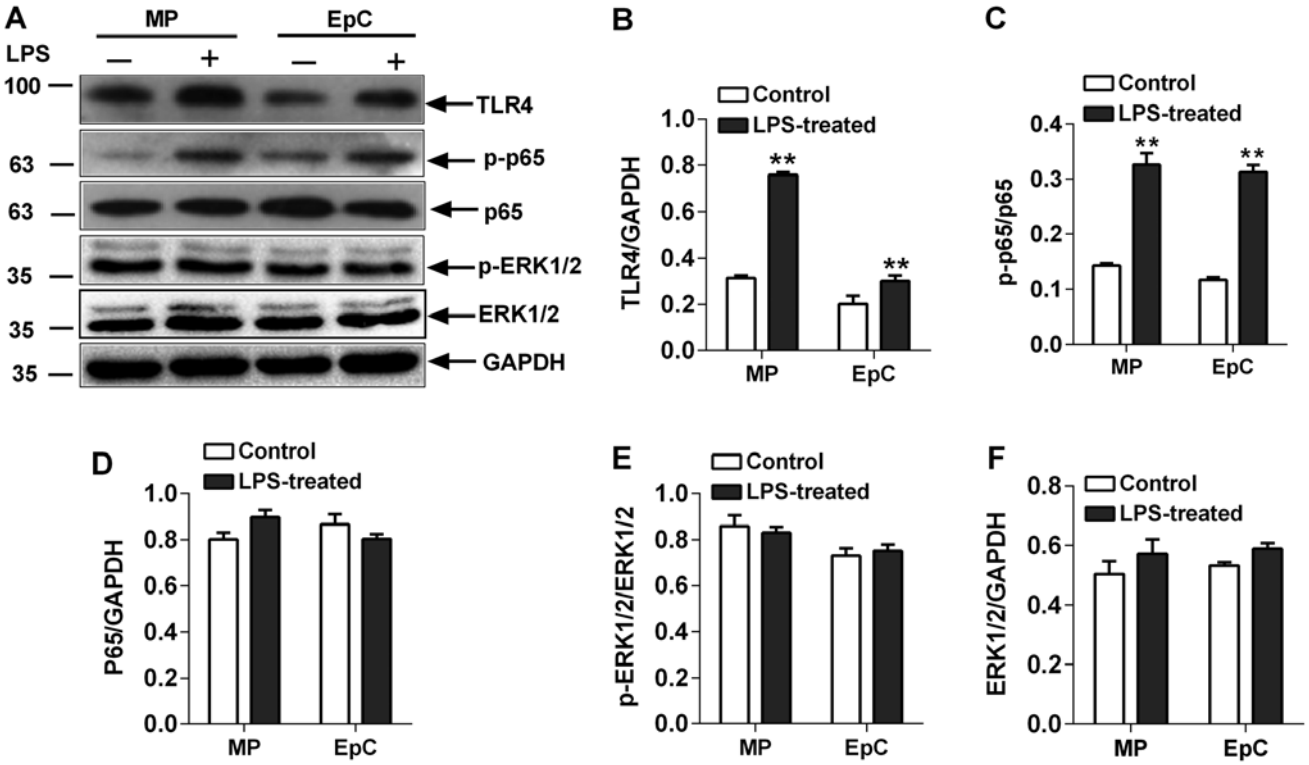

Figure 5. Effect of LPS on expression of proteins of NF-κB pathway in EpC/MP co-culture. EpCs and MPs were co-cultured for 24 h in the presence or absence of LPS. Western blotting was used to analyze the expression levels of TLR4 (A and B), p-p65 (A and C), p65 (A and D), p-ERK1/2 (A and E) and ERK1/2 (A and F). Data are presented as the mean ± SD of three independent experimental repeats. **P<0.01 vs. control group. LPS, lipopolysaccharide; EpC, epithelial cell; MP, macrophage; TLR, Toll-like receptor; p-, phosphorylated.

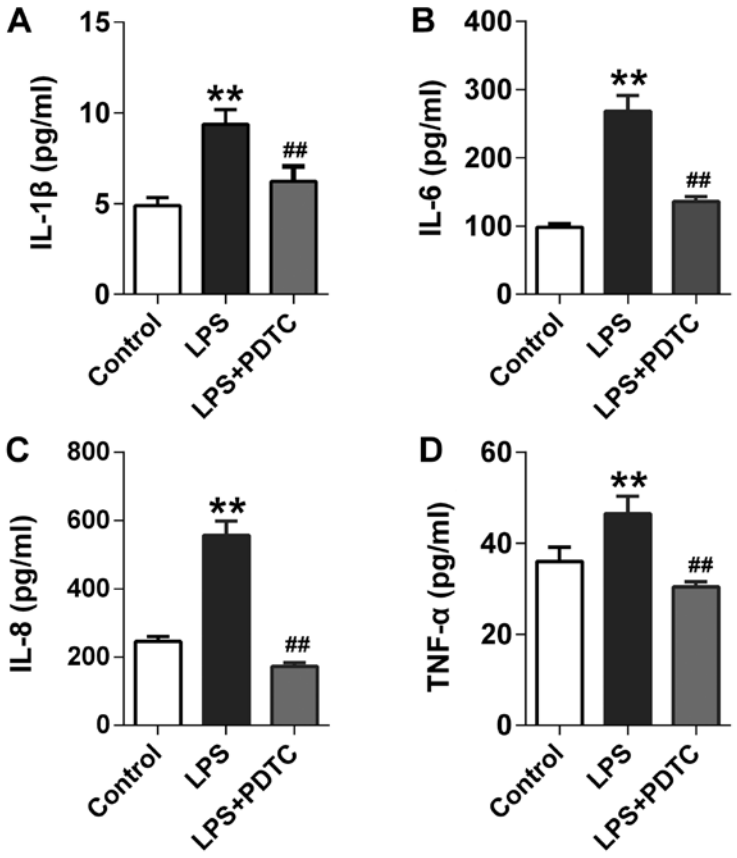

Figure 6. PDTC pretreatment reduces LPS-induced proinflammatory cytokine secretion. Epithelial cell/macrophage co-cultures were treated with 100 μM PDTC for 2 h and then 2 μg/ml LPS for 24 h before ELISAs were performed to determine the levels of (A) IL-1β (B) IL-6, (C) IL-8 and (D) TNF-α. Data are presented as the mean ± SD of three independent experimental repeats. **P<0.01 vs. control group. $^{\#\#}$P<0.01 vs. LPS group. LPS, lipopolysaccharide; PDTC, pyrrolidinedithiocarbamate; IL, interleukin; TNF, tumor necrosis factor.

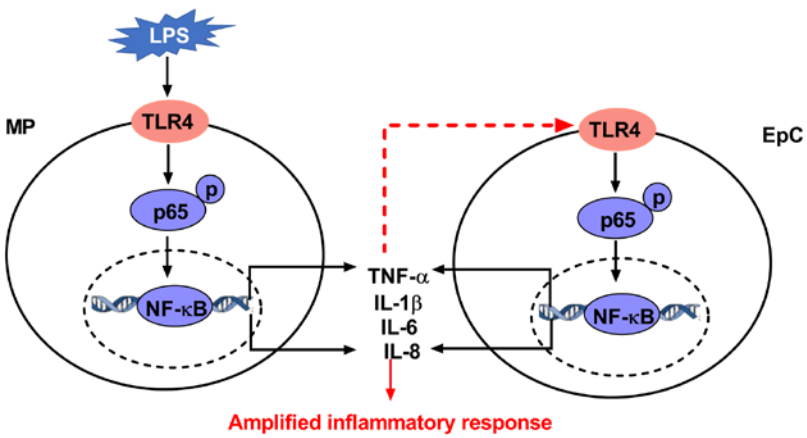

Figure 7. Schematic diagram of the hypothesized working model of LPS-induced inflammation in EpC/MP co-culture. LPS initially binds to TLR4 located on the MP membrane, which causes downstream factors, such as the phosphorylation of p65, to induce the activity of NF-κB. Activation of the NF-κB of transcription factor initiates the transcription of cytokine genes, such as TNF-α. These cytokines in turn activate NF-κB EpCs (shown as red dotted lines), which contributes to the amplified inflammatory response. The highlights in red indicate the novel findings from the present study. Red dotted lines, hypothesized process; red solid lines, findings from the present work. LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; EpC, epithelial cell; MP, macrophage; TLR, Toll-like receptor.

that PDTC significantly decreased the LPS-induced release of IL-1β, IL-6, IL-8 and TNF-α (Fig. 6A-D). These findings suggested that LPS may induce cytokine release in EpCs/MPs co-culture through the activation of NF-κB. Collectively, the results indicated that LPS may initially bind to TLR4, located on the MPs membrane, and promote downstream factors, like p-p65, to contribute to NF-κB activity. Activation of the NF-κB transcription factor may subsequently initiate the transcription of cytokine genes, such as TNF-α and IL-6, with the secreted cytokines then activating NF-κB through TLR4 in EpCs to amplify the inflammatory response (Fig. 7).

Discussion

Chronic systemic inflammation in COPD has been associated with the constant production of cytokines by MPs, neutrophils and lung structural cells, which is correlated with disease progression and an exacerbated frequency of occurrence (2,18). The air-blood barrier is mainly composed of alveolar EpCs and MPs, with EpCs serving as diffusional barriers and MPs providing an immunological barrier (19). In a previous study, monocultured EpCs were treated with conditioned medium from monocytes to investigate the inflammatory response (20). In the present study, a new co-culture model of EpCs and MPs was established to investigate the inflammatory response in EpCs and MPs cultured together, and separately, in the presence and absence of LPS stimulation. The study revealed that LPS could not promote inflammation in A549 cells (EpCs), but could in THP-1 cells (MPs), whereas an amplified inflammatory response was observed in co-cultured EpCs/MPs. Contradictory to the findings of the present study, a previous study reported that the expression levels of IL-1β, IL-6 and TNF-α were increased in A549 cells treated with 10 μg/ml LPS (21). In addition, in another study, 50 μg LPS significantly increased the levels of proinflammatory cytokines, including IL-1β, IL-2, TNF-α and transforming growth factor-β in animal lung tissue lysate, in addition to increasing the expression levels of NF-κB and histone deacetylase 3 in A549 cells (22). These paradoxical findings may be due to the different concentrations and incubation times of LPS.

For the co-culture experiments, the time course for the release of cytokines was established, and a variable increase in the levels secreted was observed following LPS treatment. As these cytokines are regulated by the NF-κB signaling pathway, the activity of the NF-κB nuclear transcription factor was also analyzed (23-25). The initiation of the inflammatory response requires various inflammatory mediators that are regulated by inducible transcription factors, including IL-6 and TNF-α which bind to the promoter regions of their respective target genes (11,26,27). The EpCs/MPs co-culture released increased levels of cytokines compared with either EpCs or MPs monoculture following exposure to LPS. Furthermore, only IL-1β and IL-8 levels were increased at 12 h in monocultured MPs; although MPs released more cytokines following the stimulation with LPS for 24 and 48 h, the levels remained much lower compared with in the co-cultured cells. However, increased levels of the proinflammatory cytokine, TNF-α, were observed following 6, 24 and 48 h of LPS exposure, and increased levels of IL-1β, IL-6 and IL-8 were observed across all time intervals from 6 to 48 h, which indicated that these cytokines may be important factors for maintaining persistent inflammation. Some co-culture models of chronic inflammatory have been studied for more than 7 days (19,28); in the current study, EpCs and MPs viability following LPS treatment was monitored for 7 days; however, the A549 (EpCs) cells began to die after 72 h (the OD value was quite low), whereas THP-1 cells (MPs) grew well from 0 to 7 days (data not shown). In a previous study, the inflammatory status of airway epithelial cells and MPs was determined at 24 and 48 h (29), thus in the present study, the inflammatory effects were observed until up to 48 h of co-culture. Therefore, in the present study, the increased inflammatory status was investigated following the co-culture of cells.

The results of the present study indicated that co-cultured EpCs and MPs may amplify the inflammatory response, and that the interactions between these two cell types might be responsible. Consistent with these findings, a previous study also found that co-cultured A549 cells and monocytes released increased amounts of IL-6 and IL-8 in response to endotoxin (30). Additionally, expression levels of the epithelial marker, E-cadherin, were discovered in the co-culture system previously (31).

NF-κB also regulates cytokine secretions, such as TNF-α, IL-1β, IL-6 and IL-8 in MPs and EpCs, which mediate their expression levels by directly binding to motifs in the promoter region of their target genes (32,33). In the current study, the investigations into NF-κB DNA-binding activity revealed that NF-κB was initially activated in MPs upon being co-cultured with EpCs for 6 h. Thus, NF-κB was activated first in MPs, suggesting that MPs may orchestrate the inflammatory response, which is consistent with the fact that they are activated during the early stages of chronic inflammation (34). In the present study, from the findings it was hypothesized that LPS initially binds to TLR4 located on the membrane of THP-1 cells, promoting downstream factors, such as NF-κB inhibitor α and p-p65, to initiate NF-κB activity; activated NF-κB may subsequently initiate the transcription of cytokine genes, such as TNF-α and IL-6, which in turn may activate NF-κB in EpCs. Taking this into consideration, NF-κB DNA binding in co-cultured EpCs/MPs was analyzed for 24 and 48 h and the EMSA data demonstrated that NF-κB DNA binding increased in EpCs from 24 h. Based on the current study, LPS could not induce inflammation in EpCs; however, inflammation has been observed in TNF-α-induced EpCs (unpublished data). TNF-α levels were increased in the co-culture system and the activity of NF-kB was also increased in MPs, but earlier than that in EpCs. Therefore, it was hypothesized that MP-induced TNF-α expression levels increased the activity of NF-kB in EpCs. Consistent with the present study, an isolated report observed a significant increase in TNF-α, IL-1β and IL-6 expression levels in LPS-induced THP-1 cells, which was related to NF-κB activation (29).

Transcription factor activation is a multistep process induced by various upstream signal transduction pathways (35). TLR4 is a pattern-recognizing receptor that identifies LPS, which is associated with Gram-negative bacteria and is highly expressed in MPs (36,37). Activation of the NF-κB and ERK1/2/activator protein 1/STAT3 signaling pathways has been reported in LPS-induced THP-1 cells (38). The current study further analyzed the upstream factors of NF-κB in co-cultured EpCs/MPs; LPS increased TLR4 expression levels in both EpCs and MPs and the expression levels of phosphorylated p65 were significantly increased. It has previously been reported that LPS activates a series of signaling transduction pathways, including ERK, which serves crucial roles in inflammation (25). In the present study, the expression levels and phosphorylation status of ERK1/2 were analyzed in EpCs and MPs; however, the current results revealed that LPS had no effect on the expression levels or phosphorylation status of ERK1/2 in EpC/MP co-culture, which may be because the phosphorylation of ERK1/2 occurred at an earlier stage (39). Although there is no direct evidence to date, it was

hypothesized that the interaction between co-cultured EpCs and MPs may affect ERK1/2 expression. However, one limitation of the present study was that as gene expression occurs earlier than protein expression, only protein expression levels of factors involved in the NF-κB signaling pathway were investigated. Nonetheless, the most important and novel observation of the present study was that in the co-culture system, the interaction between EpCs and MPs led to an amplified inflammatory response to LPS.

Considering these findings, the effects of LPS-induced inflammation on cytokine levels were investigated using the transcription factor inhibitor, PDTC. The results demonstrated that PDTC significantly decreased IL-6, IL-1β, IL-8 and TNF-α levels, but to varying degrees. Consistent with the findings in the present study, Liu *et al* (29) reported that PDTC markedly attenuated the expression levels of IL-1β, IL-6 and TNF-α through inhibiting the activation of NF-κB in MPs. However, the effects of PDTC on protein expression and the activation of nuclear transcription factors should be further investigated in future studies.

In conclusion, the findings of the present study suggested that LPS may promote an inflammatory response in MPs, but not in EpCs. However, LPS may be able to amplify the inflammatory response in co-cultured EpCs/MPs through activating the nuclear transcription factor, NF-κB, and the expression levels of proteins in the NF-κB signaling pathway. Previously, this research has focused on investigating the anti-inflammatory mechanisms of traditional Chinese herbs in treating COPD. Traditional Chinese herbs have multiple targets and multiple levels of action, thus from the findings of the present study, it can be suggested that cell co-cultures may be more useful models for investigating their mechanisms of action compared with monocultures. The present study was the first time that the present research group had used this combination of cells as a co-culture model, although a previously established co-culture system using RAW 264.7/NIH3T3 cells has been established (unpublished data). Future studies should aim to determine the stability of this inflammatory model to help with future *in vitro* studies of the chronic inflammatory response.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JL and YC designed the study; YQ, XM and CL conceived and performed the experiments, and drafted and revised the manuscript; and PZ, SF, XL, HD and WZ all performed the experiments, acquired the data and performed the statistical analysis. All authors read and approved the final manuscript.

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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