

LPS-induced proinflammatory cytokine expression in human airway epithelial cells and macrophages via NF- κ B, STAT3 or AP-1 activation

XUEFANG LIU^{1,2}, SUGAI YIN^{1,2}, YULONG CHEN^{1,2}, YAOSONG WU^{1,2}, WANCHUN ZHENG^{1,2}, HAORAN DONG^{1,2}, YAN BAI^{1,2}, YANQIN QIN^{1,2}, JIANSHENG LI^{1,2}, SUXIANG FENG^{1,2} and PENG ZHAO^{1,2}

¹Henan Key Laboratory of Chinese Medicine for Respiratory Disease; ²Collaborative Innovation Center for Respiratory Disease Diagnosis and Treatment and Chinese Medicine Development of Henan Province, Henan University of Chinese Medicine, Zhengzhou, Henan 450046, P.R. China

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Abstract. Lipopolysaccharide (LPS), the major outer surface membrane component of Gram-negative bacteria, is one of the main etiological factors in the pathogenesis of several lung diseases, such as chronic obstructive pulmonary disease. The respiratory epithelium and the macrophages comprise the dynamic interface between the outside environment and the host response to bacterial infection via cytokine secretion. In the present study, the mechanisms of LPS induced-inflammatory response in human lung cells and macrophages were investigated. The effects of LPS exposure on cytokine production, inflammation-related transcription factors and intracellular signaling pathway activation were assessed in human lung mucoepidermoid carcinoma H292 cells and human macrophage THP-1 cells. The results demonstrated that LPS markedly increased the expression of interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)- α , matrix metalloproteinase (MMP)-9 and tissue inhibitor of metalloproteinases-1 in H292 cells, while it increased the production of IL-6, IL-8 and TNF- α in differentiated THP-1 cells. In addition, LPS exposure activated nuclear factor (NF)- κ B and activator protein (AP)-1 signaling in H292 cells, while it activated NF- κ B and signal transducer and activator of transcription (STAT) 3 signaling in THP-1 cells. Furthermore, treatment with NF- κ B, AP-1 or STAT3 inhibitors significantly decreased the LPS-mediated expression of IL-8 and TNF- α in these cells, suggesting that these pathways might serve crucial roles in LPS-induced cytokine expression. In conclusion, LPS stimulation of H292 and THP-1 cells induced cytokine expression and NF- κ B,

mitogen-activated protein kinase and Janus kinase/STAT3 pathway activation with subsequent nuclear translocation of NF- κ B, AP-1 and STAT3, which demonstrated potential of the use of NF- κ B, AP-1 and STAT3 in therapies for conditions and diseases associated with chronic inflammation.

Introduction

Airway inflammatory diseases, such as chronic obstructive pulmonary disease (COPD) and asthma, are major causes of morbidity and mortality in patients, and place a substantial burden on healthcare systems (1). Bacterial infection, cigarette smoking and air pollutants are implicated in the onset and progression of lung inflammation (2). Lipopolysaccharides (LPS) is the major outer surface membrane component of Gram-negative bacteria and a biologically active component present in cigarette smoke (3). Thus, LPS-mediated inflammatory response is a major lung inflammation source from exposure to both gram-negative bacterial infection and cigarette smoke. During this inflammatory response, macrophages first help in endocytosis of bacterium debris, followed by generation of inflammatory cytokines and expansion of the local inflammatory response (4,5). In addition, the respiratory epithelium has an active role in the airway defense through the production of cytoprotective mucus and through coordinating local inflammation responses by producing proinflammatory cytokines. These cytokines, however, also result in bronchial maladaptations, including pulmonary dysfunction, increased mucin production and protease-antiprotease imbalance (6-8).

It has been reported that proinflammatory cytokine production is induced by many stimuli through the mitogen-activated protein kinase (MAPK), nuclear factor (NF)- κ B or Janus kinase (JAK)/signal transducer and activator of transcription (STAT3) signaling cascades. For instance, LPS-induced MAPK and JAK/STAT3 activation results in activation of the downstream transcriptional factors NF- κ B, activator protein (AP)-1, peroxisome proliferator-activated receptor (PPAR) and STAT3, which mediate the transcription and translation of proinflammatory genes (9-11). In the present study, in order to investigate the mechanisms of LPS induced-inflammatory

Correspondence to: Professor Jiansheng Li, Henan Key Laboratory of Chinese Medicine for Respiratory Disease, Henan University of Chinese Medicine, 156 Jinshui Dong Road, Zhengzhou, Henan 450046, P.R. China
E-mail: li_js8@163.com

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response in airway epithelial and macrophage cells, the effects of LPS exposure on the expression of interleukin (IL)-6, IL-8, IL-10, tumor necrosis factor (TNF)- α , matrix metalloproteinase (MMP)-9 and tissue inhibitor of metalloproteinases (TIMP)-1 were examined in human airway epithelial H292 cells and macrophage THP-1 cells. Subsequently, inflammation-related transcription factors and intracellular signaling pathways that may be involved in LPS-induced pro-inflammation cytokine production were explored.

Materials and methods

Cell culture. H292 human lung mucoepidermoid carcinoma cells and THP-1 human monocytic cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in RPMI-1640 (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) supplemented with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), penicillin (100 U/ml) and streptomycin (100 mg/ml), and incubated at 37°C with 5% CO₂. All experiments were performed with exponentially growing cells. For differentiation to a macrophage phenotype, THP-1 cells were adjusted to the desired concentrations for each experiment and incubated with 20 μ M phorbol myristate acetate (PMA) diluted in complete culture medium for 24 h. Then, the cells were washed with serum-free RPMI-1640 medium prior to each experiment.

Cell viability assay. For the cell viability assay, H292 (2x10⁴ cells/well) and THP-1 cells (1x10⁴ cells/well, pretreated with 20 μ M PMA) were plated in sextuplicate 96-well plates for 24 h. The cells were then stimulated with the indicated concentrations of LPS for 6, 12, 24 and 48 h. Normal complete RPMI-1640 media without LPS was used as a negative control. Subsequently, MTT solution was added for 4 h, following which the supernatants were removed and 150 μ l dimethyl sulfoxide was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm.

Cytokine analysis. H292 (2x10⁴ cells/well) and THP-1 cells (1x10⁴ cells/well, pretreated with 20 μ M PMA) were seeded in triplicate into 96-well tissue culture plates for 24 h. The cells were then stimulated with 1, 2 or 2.5 μ g/ml of LPS for 6, 12, 24 and 48 h. The levels of human cytokines were measured in the collected supernatants with human IL-6 (cat. no. EK0410), IL-8 (cat. no. EK0413), IL-10 (cat. no. EK0416), TNF- α (cat. no. EK0525), MMP-9 (cat. no. EK0465) and TIMP-1 (cat. no. EK0520) ELISA kits (Boster Biological Technology, Ltd., Wuhan, China), according to the manufacturer's protocols. For the inhibitor experiments, H292 and THP-1 cells were pretreated with pyrrolidine dithiocarbamate (PDTTC), SP00125 or static (all Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 4 h, followed by exposure to LPS for 48 h, prior to measuring IL-8 and TNF- α levels in the cell culture supernatants with ELISA kits, as aforementioned.

Electrophoretic mobility shift assay (EMSA). Nuclear protein was extracted from LPS-stimulated H292 and THP-1 cells using a total nuclear protein extraction kit (Beyotime Institute of Biotechnology, Shanghai, China), according to the

manufacturer's protocol. Protein concentration was measured with the bicinchoninic acid (BCA) method.

H292 and differentiated THP-1 cells were treated with LPS for 24 h. The DNA binding activities of AP-1, PPAR, NF- κ B and STAT3 in the nuclear extracts were assessed by EMSA using the EMSA kit (Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer's protocol. The sequences of the oligonucleotides used were as follows: AP1, forward 5'-CGCTTGATGACTCAGCCGGAA-3' and reverse 3'-GCGAACTACTGAGTCGGCCTT-5'; AP-1 mutated probe, forward 5'-CGCTTGATGACTTGGCCGGAA-3' and reverse 3'-GCGAACTACTGAACCGGCCTT-5'; PPAR, forward 5'-CAAAACTAGGTCAAAGGTCA-3' and reverse 3'-GTT TTGATCCAGTTTCCAGT-5'; PPAR mutated probe, forward 5'-CAAAACTAGCACAAAGCACA-3', 3'-GTTTTGATC GTGTTTCGTGT-5' and reverse STAT3, forward 5'-GAT CCTTCTGGGAATTCCTAGATC-3' and reverse 3'-CTA GGAAGACCCCTTAAGGATCTAG-5'; STAT3 mutated probe, forward 5'-GATCCTTCTGGGCCGTCTAGATC-3' and reverse 3'-CTAGGAAGACCCGGCAGGATCTAG-5'; NF- κ B, forward 5'-AGTTGAGGGGACTTTCCAGG C-3' and reverse 3'-TCAACTCCCCTGAAAGGGTCCG-5'; NF- κ B mutated probe, forward 5'-AGTTGAGGCGACTTT CCCAGGC-3' and reverse 3'-TCAACTCCGCTGAAAGGG TCCG-5'. Briefly, 10 μ g nuclear extract was added to 20 fmol biotin-labeled oligonucleotides, 1X binding buffer, 2.5% Glycerol, 5 mM MgCl₂, 0.1 mM EDTA and 0.05% NP-40 at 24°C for 40 min. The reactions were analyzed by electrophoresis on 5% polyacrylamide gels in 0.5X TBE buffer at 180 V for 35 min, and were transferred to positively-charged nylon membranes and cross-linked in a Stratagene cross-linker (Agilent Technologies, Inc., Santa Clara, CA, USA). The signals were visualized using chemiluminescent detection (Beyotime Institute of Biotechnology) on X-ray film.

Western blotting assay. H292 cells and differentiated THP-1 cells were stimulated with 1 μ g/ml LPS for 24 h. Cells were washed with ice-cold PBS and then lysed with radio-immunoprecipitation assay lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.). Protein concentration was determined using a BCA protein assay. Samples containing 30 μ g of protein were mixed with SDS sample buffer and boiled. Protein was separated by 10% SDS-polyacrylamide gel electrophoresis prior to being transferred electrophoretically into polyvinylidene fluoride membrane. The membranes were blocked for 1.5 h at room temperature with 5% nonfat milk and then incubated with primary antibodies at 4°C overnight followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at 22°C. The bands were visualized by film exposure following incubation with enhanced chemiluminescence reagent (Beijing Solarbio Science & Technology Co., Ltd.). Antibodies used were as follows: Anti-TLR4 (Proteintech Group, Inc., Chicago, IL, USA; cat. no. 19811-1-AP; 1:500), anti-inhibitor of κ B α (I κ B α ; Proteintech Group, Inc.; cat. no. 10268-1-AP; 1:500), anti-p65 (Proteintech Group, Inc.; cat. no. 10745-1-AP; 1:500), anti-p38 (Proteintech Group, Inc.; cat. no. 14064-1-AP; 1:500), anti-Jun proto-oncogene AP-1 transcription factor subunit (JUN; Proteintech Group, Inc.; cat. no. 10024-2-AP; 1:500), anti-JAK1 (Proteintech Group, Inc.; cat. no. 66466-1-Ig; 1:500),

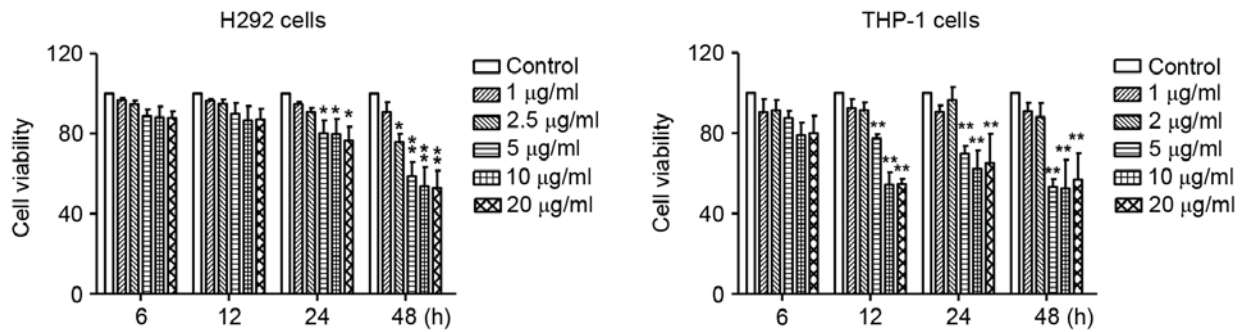


Figure 1. Effects of LPS stimulation on cell viability of H292 and THP-1 cells. Cells were stimulated with the indicated concentrations of LPS for 6, 12, 24 or 48 h, then cell viability was evaluated by MTT assay. Untreated cells were used as control. Results were expressed as mean \pm standard deviation of three separate experiments. *P<0.05 and **P<0.01 vs. control of each time point. LPS, lipopolysaccharide.

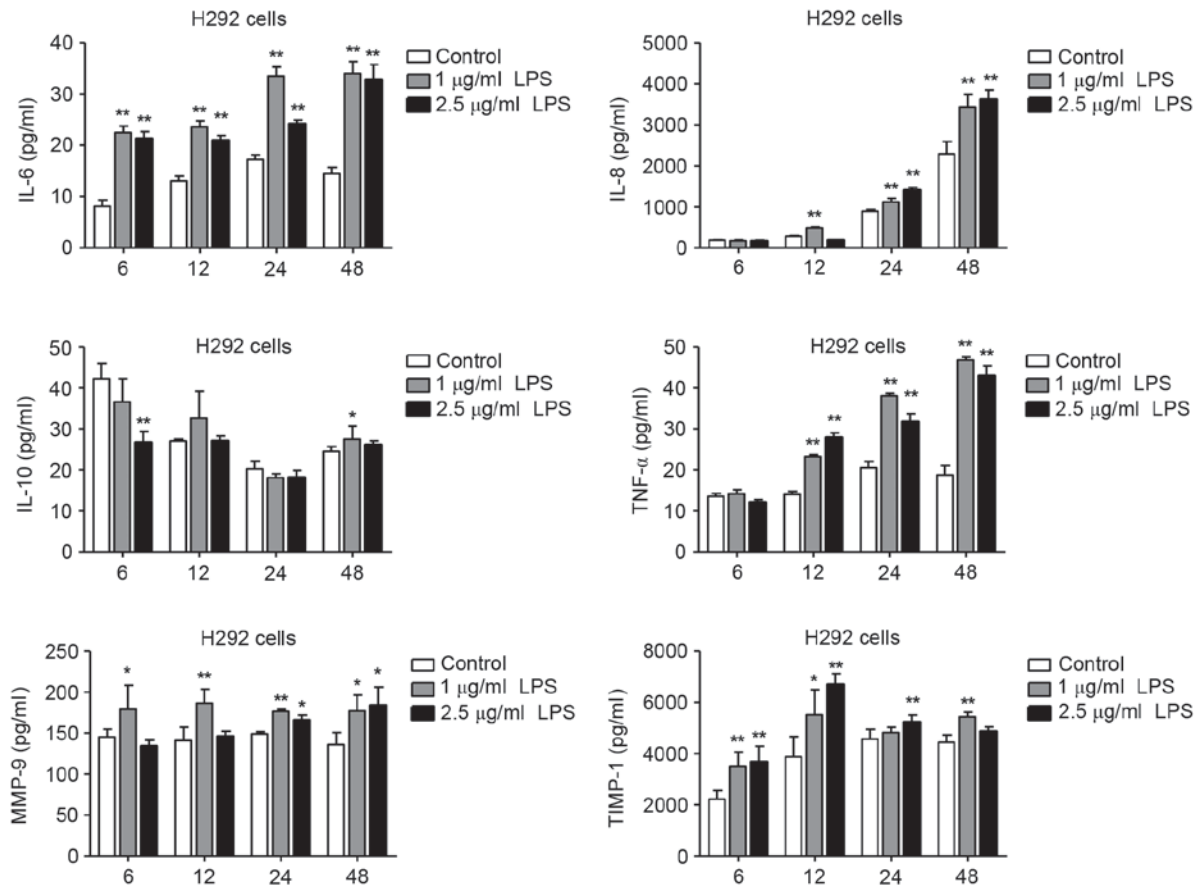


Figure 2. Effects of LPS stimulation on cytokine production of H292 cells. H292 cells were treated with 1 and 2.5 µg/ml LPS for 6, 12, 24, 48 h, and protein production was assessed by ELISA. Results were expressed as mean \pm standard deviation of three separate experiments. *P<0.05 and **P<0.01 vs. control of each time point. LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases.

anti-JAK2 (Proteintech Group, Inc.; cat. no. 17670-1-AP; 1:500), anti- β -actin rabbit polyclonal antibody (Proteintech Group, Inc.; cat. no. 20536-1-AP; 1:4,000), anti-p-STAT3 (Bioworld Technology, Inc., St Louis Park, MN, USA; cat. no. BS4181; 1:800), anti-p-p65 (Bioworld Technology, Inc.; cat. no. BS5088, 1:600), anti-p-p38 (Bioworld Technology, Inc.; cat. no. 4522; 1:500), HRP-conjugated Affinipure goat anti-mouse IgG(H+L) (Proteintech Group, Inc.; cat. no. SA00001-1; 1:5,000) and HRP-conjugated Affinipure Goat Anti-Rabbit IgG(H+L) (Proteintech Group, Inc.; cat. no. SA00001-2; 1:5,000).

Statistical analysis. Data were presented as mean \pm standard error. Results were analyzed by one-way analysis of variance followed by Tukey's range test using the SPSS 17.0 software package (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results and Discussion

Effect of LPS exposure on cell viability. First, the cytotoxic effects of LPS were determined by MTT assay in human lung mucoepidermoid carcinoma H292 cells and monocyte

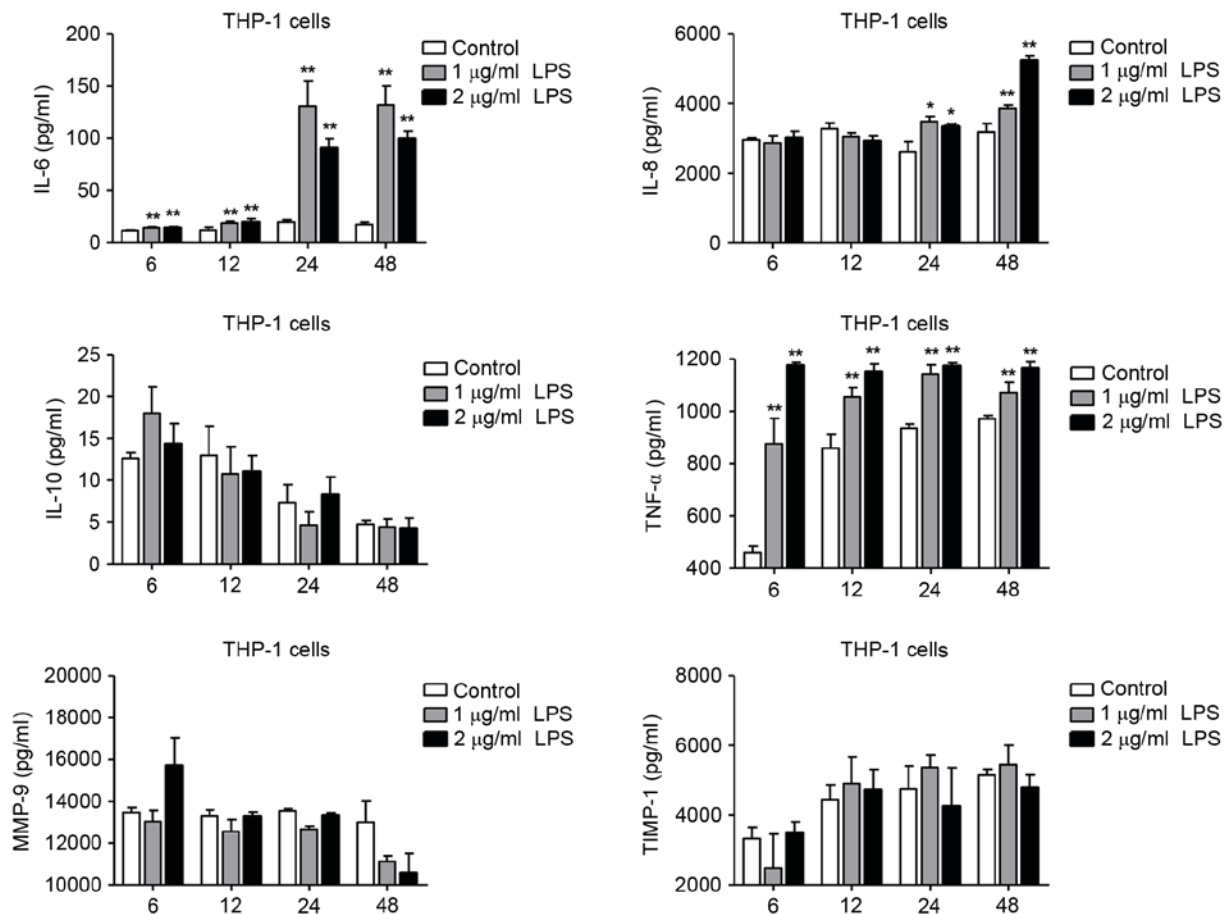


Figure 3. Effects of LPS stimulation on cytokine production of THP-1 cells. THP-1 cells were treated with 1 and 2 $\mu\text{g/ml}$ LPS for 6, 12, 24, 48 h, and protein production was assessed by ELISA. Results were expressed as mean \pm standard deviation of three separate experiments. * $P < 0.05$ and ** $P < 0.01$ vs. control of each time point. LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases.

THP-1 cells treated with various concentrations of LPS for 6, 12, 24 and 48 h. As illustrated in Fig. 1, compared with the untreated controls, no significant change was observed in the viability of H292 cells treated with 1 and 2.5 $\mu\text{g/ml}$ LPS, and the viability of THP-1 cells treated with 1 and 2 $\mu\text{g/ml}$ LPS, indicating that LPS was not cytotoxic at these concentrations. Higher concentration (5–20 $\mu\text{g/ml}$) of LPS were significantly cytotoxic to both H292 and THP-1 cells (Fig. 1). Therefore, the concentrations of 1–2.5 and 1–2 $\mu\text{g/ml}$ were selected for H292 and THP-1 cells, respectively, in all subsequent experiments.

Levels of IL-6, -8, -10, TNF- α , MMP-9 and TIMP-1 in LPS-exposed H292 and THP-1 cells. LPS is a potent proinflammatory activator of monocytes and macrophages *in vivo* and *in vitro* (3,12). In the present study, H292 and differentiated THP-1 cells were exposed to different concentrations of LPS for 6, 12, 24 and 48 h, then the expression levels of IL-6, -8, -10, TNF- α , MMP-9 and TIMP-1 were detected by ELISA. Compared with untreated H292 cells, treatment with 1 and 2.5 $\mu\text{g/ml}$ LPS resulted in increased levels of IL-6, -8, TNF- α , MMP-9 and TIMP-1 (Fig. 2). IL-10 levels in the LPS-treated H292 cells, however, were not significantly altered (Fig. 2). In differentiated THP-1 cells, stimulation with 1 and 2 $\mu\text{g/ml}$ LPS resulted in an increase of IL-6, -8 and TNF- α levels compared with control (Fig. 3). However, the expression levels of IL-10, MMP-9 and TIMP-1 were not significantly altered in THP-1

cells following LPS stimulation (Fig. 3). These results demonstrated that LPS could induce IL-6, -8, and TNF- α expression in both the H292 and THP-1 cells. In addition, LPS treatment significantly increased the expression levels of MMP-9 and TIMP-1 in H292 cells, but not in differentiated THP-1 cells. Taken together, these data suggest that LPS stimulation had the different effects on cytokine expression profiles in H292 and differentiated THP-1 cells, and this may be related to differences in the physiological characteristics of these two cell types. H292 cells have characteristics similar to those of alveolar and bronchiolar epithelial cells and are widely used in alveolar and bronchiolar epithelial models, and the gene expression profile of H292 cells is similar to that of primary nasal epithelial cells from healthy human controls (13). THP-1 cells, a human leukemia-derived monocytic cell line, is the most commonly used cellular model for investigating human macrophage function (12).

Effect of LPS treatment on NF- κB , AP-1, PPAR and STAT3 DNA-binding activity. The transcription factors NF- κB , AP-1, PPAR and STAT3 are the main signal transduction molecules activated in response to LPS-induced inflammatory response (12,14). For example, NF- κB translocates to the nucleus and promotes the transcription of proinflammatory genes, including the biologically active cytokine TNF- α and proinflammatory interleukins in activated macrophages (14).

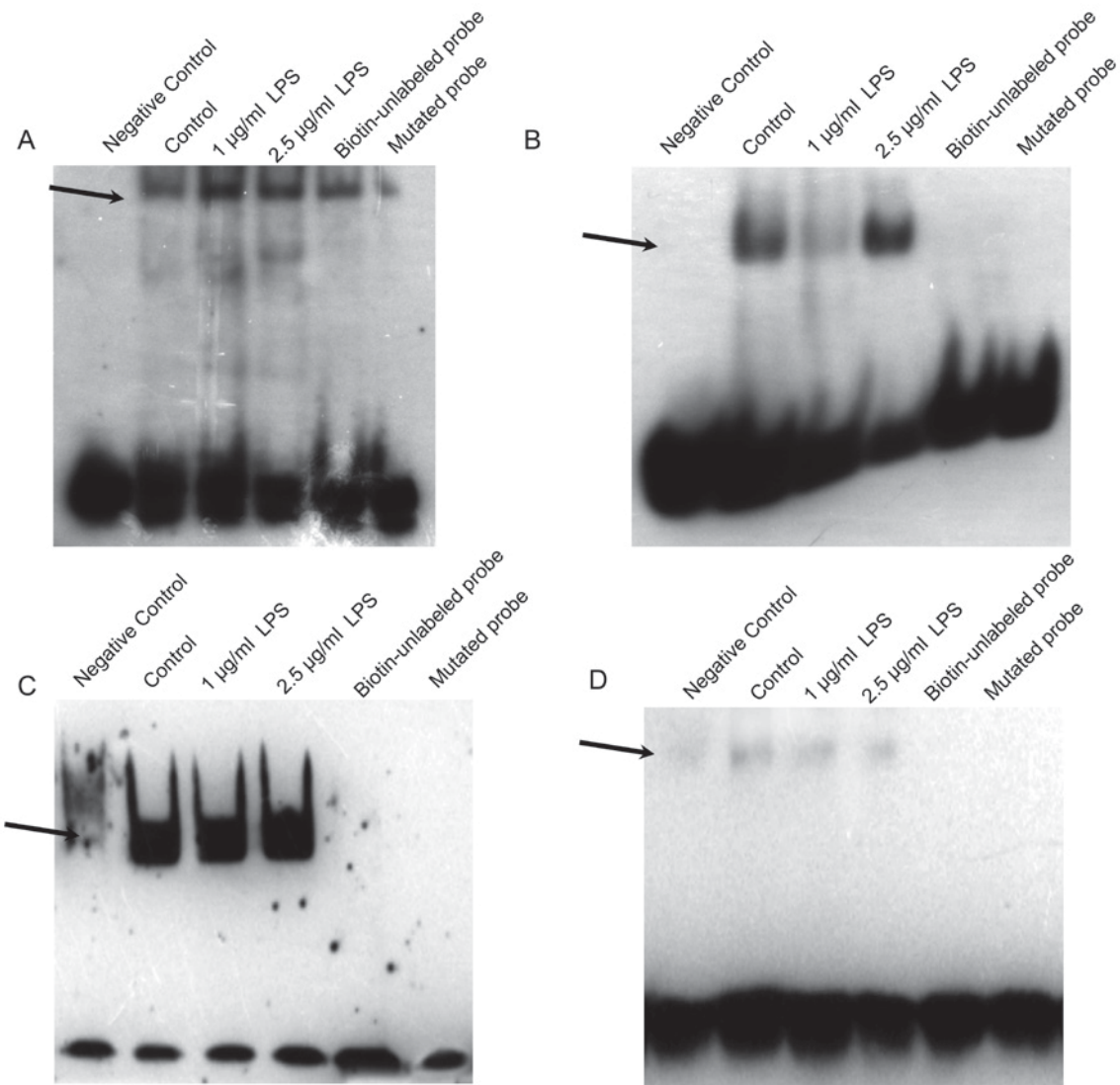


Figure 4. Effects of LPS stimulation on NF- κ B, AP-1, PPAR and STAT3 DNA-binding activity in H292 cells. Nuclear extracts were prepared from H292 cells and EMSA was performed by incubation of extracts with the (A) NF- κ B, (B) AP-1, (C) PPAR or (D) STAT3 DNA-binding consensus sequences. Negative control, the biotin-labelled DNA oligo probe run without any protein extract; biotin-unlabeled protein, the biotin-unlabelled DNA oligo probe run with protein extract; mutated probe, the biotin-labelled DNA oligo probe with binding site mutation, run with protein extract. A total of three separate experiments were performed. Representative gel images are shown. LPS, lipopolysaccharide; NF- κ B, nuclear factor- κ B; AP, activator protein; PPAR, peroxisome proliferator-activated receptor; STAT, signal transducer and activator of transcription.

Thus, the activation of NF- κ B, AP-1, PPAR and STAT3 was investigated in H292 and THP-1 cells stimulated with LPS by EMSA. As illustrated in Fig. 4, treatment of H292 cells with 1 or 2.5 μ g/ml LPS for 24 h markedly increased the NF- κ B and AP-1 binding activities, but had no effect on the PPAR and STAT3 binding activities. Treatment of differentiated THP-1 cells with 1 and 2 μ g/ml LPS for 24 h markedly increased the NF- κ B and STAT3 binding activities, slightly increased the AP-1 binding activity, but had no effect on PPAR (Fig. 5). These data indicated that LPS-induced cytokine expression in H292 cells likely resulted from the activation of the NF- κ B and AP-1 transcription factors. Similarly, LPS-mediated cytokine expression in THP-1 cells was associated with the activation of the NF- κ B, AP-1 and STAT3 transcription factors.

Effect of LPS exposure on the NF- κ B, MAPK and STAT3 signaling pathways. The activation of NF- κ B signaling

increases the I κ B phosphorylation or degradation, and subsequent NF- κ B transactivation, translocation and promoter binding (15). In addition, NF- κ B activation in the airways of allergen-challenged mice is attenuated by Toll-like receptor (TLR) 2 or TLR4 gene deletion, suggesting that TLR2 or TLR4 contribute to NF- κ B signaling (16,17). Similarly, the AP-1 and STAT3 promoter-binding activity are regulated by MAPK and JAK/STAT signaling (18,19). Therefore, it was hypothesized that LPS may induce NF- κ B, MAPK and JAK/STAT pathway activation, leading to NF- κ B, AP-1 and STAT3 nuclear translocation and cytokine expression in the H292 and THP-1 cells investigated in the present study.

In order to further examine the effects of LPS on pathway activation in H292 and THP-1 cells, cells were treated with 1 μ g/ml LPS and the activation of NF- κ B and MAPK signaling pathways in H292 cells, and the activation of NF- κ B and JAK/STAT3 signaling pathways in THP-1 cells, were

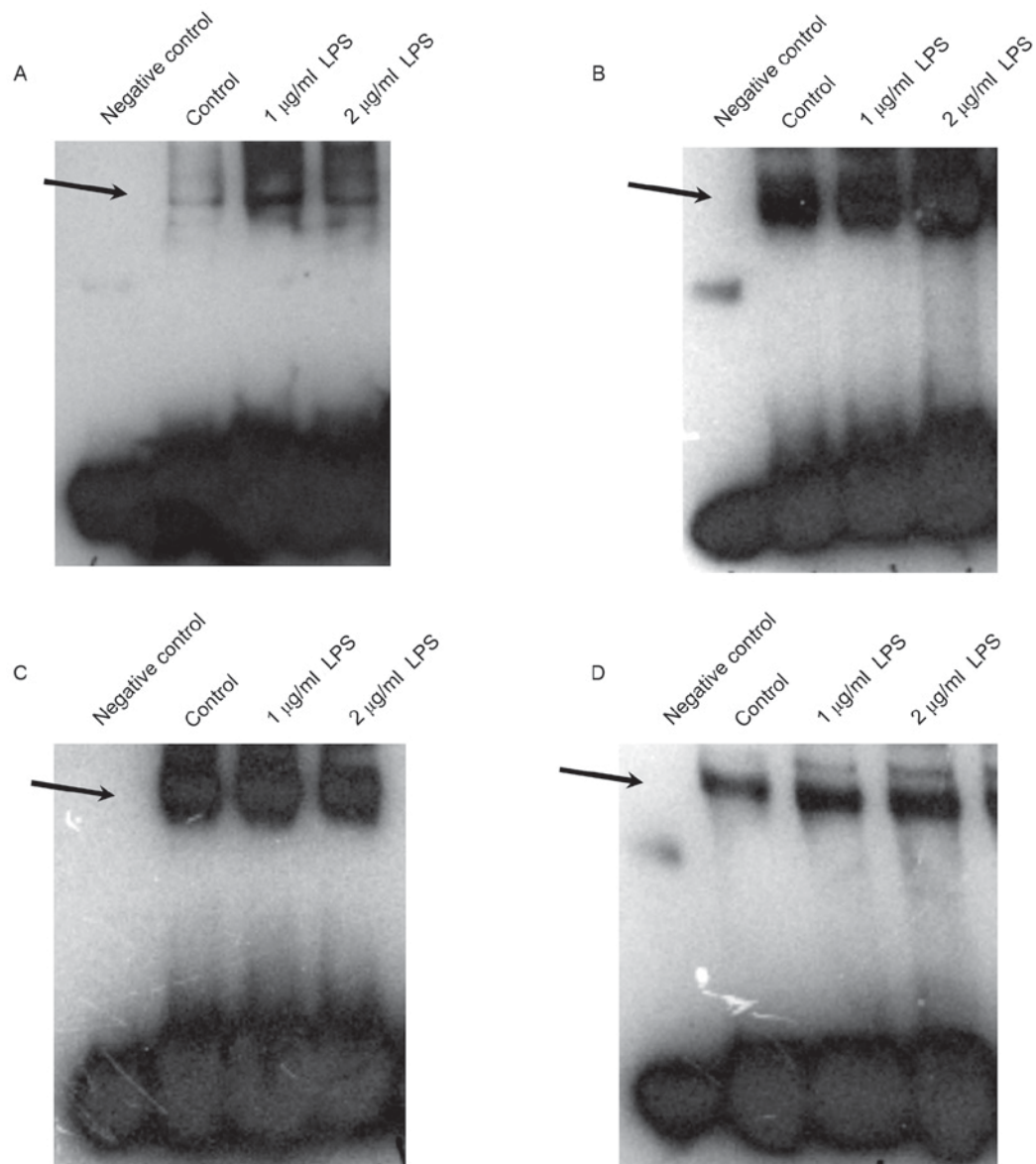


Figure 5. Effects of LPS stimulation on NF- κ B, AP-1, PPAR and STAT3 DNA-binding activity in THP-1 cells. Nuclear extracts were prepared from the THP-1 cells and EMSA was performed by incubation of extracts with the (A) NF- κ B, (B) AP-1, (C) PPAR and (D) STAT3 DNA-binding consensus sequences. Negative control, the biotin-labelled DNA oligo probe run without any protein extract. A total of three separate experiments were performed. Representative gel images are shown. LPS, lipopolysaccharide; NF- κ B, nuclear factor- κ B; AP, activator protein; PPAR, peroxisome proliferator-activated receptor; STAT, signal transducer and activator of transcription.

tested by western blotting. As the results demonstrated, LPS treatment for 24 h resulted in an obvious increase in p65 and p38 phosphorylation, as well as a marked increase in TLR4, I κ B α , p65, p38 and JUN protein expression levels in H292 cells (Fig. 6A). In the differentiated THP-1 cells, LPS treatment for 24 h resulted in a marked increase of STAT3 and p65 phosphorylation, as well as an increase in TLR4, I κ B α and Janus kinase (JAK) 1 protein expression levels, while JAK2 expression was not altered (Fig. 6B). These results indicated that NF- κ B, MAPK and their downstream signals were activated in H292 cells, while NF- κ B and STAT3 downstream signals were activated in THP-1 cells following LPS exposure.

LPS has been reported to bind to the TLR4 receptor, and to induce activation of the NF- κ B pathway which then results in the release of proinflammatory cytokines (20). In the present study, LPS treatment was demonstrated to increase TLR4

expression in both H292 and THP-1 cells, and to activate NF- κ B and MAPK signaling in H292 cells and NF- κ B and STAT3 signaling in THP-1 cells. These findings suggested that LPS may bind to TLR4 receptor and activate different pathways in the human airway H292 and the human macrophage THP-1 cells.

Treatment with NF- κ B, STAT3 and AP-1 inhibitors reverses the LPS-induced cytokine expression. The role of the NF- κ B, AP-1 or STAT3 pathway activation in the LPS-mediated cytokine expression in H292 and differentiated THP-1 cells was further explored. H292 and THP-1 cells were pretreated with PDTC (a NF- κ B inhibitor; 100 μ M), SP00125 (an AP-1 inhibitor; 100 μ M) or stattic (a STAT3 inhibitor; 100 μ M) for 4 h, followed by exposure to LPS for 48 h. The results demonstrated that treatment with PDTC or SP00125 significantly

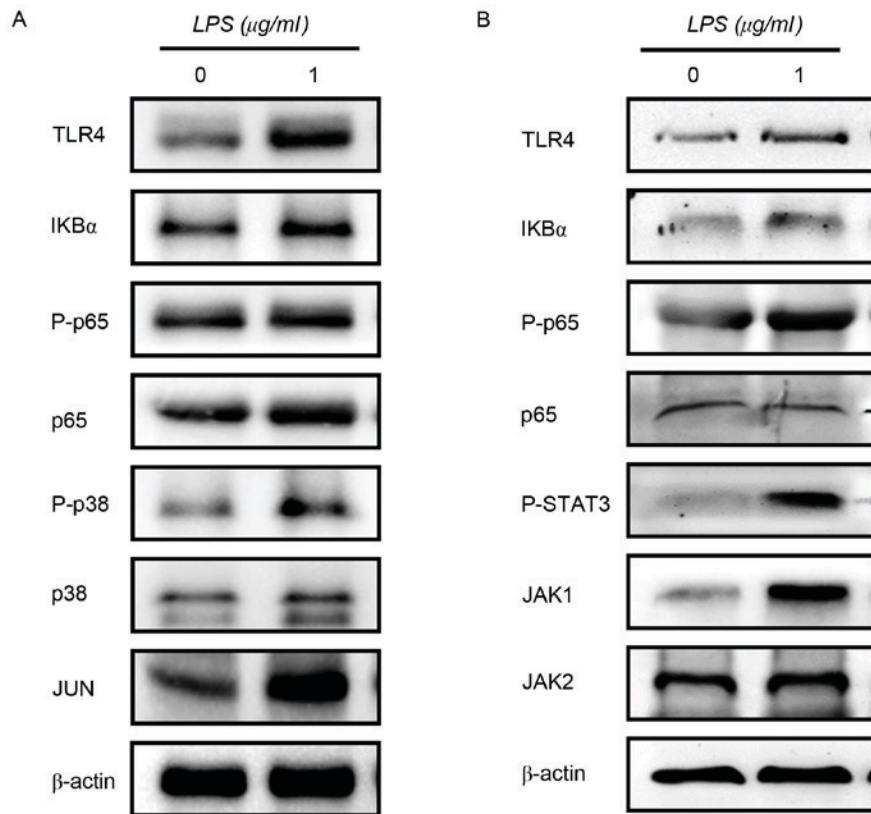


Figure 6. Effect of LPS stimulation on the NF- κ B, MAPK and STAT3 signaling pathways. (A) H292 and (B) THP-1 cells were treated with 1 μ g/ml LPS for 24 h and protein expression levels of the indicated signaling molecules were examined by western blotting. Representative images are shown from three independent repeats. LPS, lipopolysaccharide; NF- κ B, nuclear factor- κ B; MAPK, mitogen-activated protein kinase; STAT, signal transducer and activator of transcription; TLR, Toll-like receptor; I κ B, inhibitor of κ B; P-, phosphorylated; p65, RELA proto-oncogene NF- κ B subunit; p38, mitogen-activated protein kinase 14; JUN, Jun proto-oncogene AP-1 transcription factor subunit; JAK, Janus kinase.

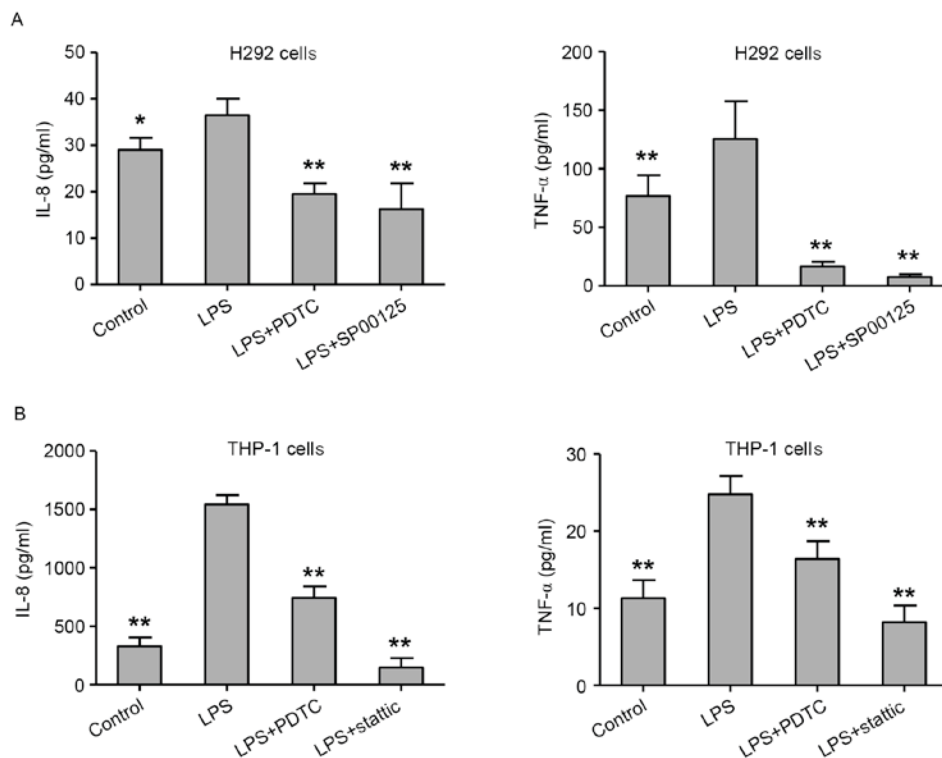


Figure 7. Role of NF- κ B, AP-1 and STAT3 signaling in LPS-mediated cytokine production. (A) H292 and (B) THP-1 cells were pretreated with or without PDTC, SP00125 or stattic for 4 h. Then, cells were exposed to 1 μ g/ml LPS for 48 h, and expression levels of IL-8 or TNF- α were analyzed by ELISA. Results were expressed as mean \pm standard deviation of three separate experiments. * P <0.05 and ** P <0.01 vs. LPS alone. LPS, lipopolysaccharide; NF- κ B, nuclear factor- κ B; AP, activator protein; STAT, signal transducer and activator of transcription; PDTC, pyrrolidine dithiocarbamate.

inhibited the LPS-induced expression of IL-8 and TNF- α in H292 cells, compared with cells treated with LPS alone (Fig. 7A). Similarly, treatment with PDTC or stattic significantly decreased the LPS-induced expression of IL-8 and TNF- α in THP-1 cells, compared with cells treated with LPS alone (Fig. 7B). These results suggest that NF- κ B, AP-1 or STAT3 signaling might serve critical roles in LPS-mediated cytokine expression in lung epithelial cells and macrophages.

In conclusion, LPS stimulation increased the production of IL-6, IL-8, TNF- α , MMP-9 and TIMP-1 in H292 cells, and it increased the expression of IL-6, IL-8, and TNF- α in THP-1 cells. In addition, LPS stimulation increased the DNA-binding activation of NF- κ B and AP-1 in H292 cells, and the DNA-binding activation of NF- κ B and STAT3 in THP-1 cells. NF- κ B, MAPK and STAT3 downstream signaling was also activated by LPS stimulation. Furthermore, NF- κ B, AP-1 or STAT3 inhibitors significantly reversed the LPS-mediated IL-8 and TNF- α induction in H292 and THP-1 cells. The present study suggests that these signals might serve vital roles in LPS-induced cytokine expression in human airway cells and macrophages.

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Competing interests

The authors declare that they have no competing interests.

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