

Original Research

Perfluorocarbon inhibits lipopolysaccharide-induced macrophage inflammatory protein-2 expression and activation of ATF-2 and c-Jun in A549 pulmonary epithelial cells

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Abstract: The signaling pathway that mediates the anti-inflammatory effects of perfluorocarbon (PFC) in alveolar epithelial cells treated with lipopolysaccharide (LPS) remains unclear. To evaluate the role of macrophage-inflammatory protein-2 (MIP-2), four A549 treatment groups were utilized: (1) untreated control, (2) 10 µg/mL of LPS, (3) 10 µg/mL of LPS+30% PFC and (4) 30% PFC. MIP-2 mRNA expression was determined by qPCR and ELISA. Mitogen-activated protein kinase (MAPK) activation was determined by Western blot analysis, and MIP-2 expression was determined by qPCR following treatment with MAPK inhibitors. PFC suppressed LPS-induced MIP-2 mRNA levels ($P \leq 0.035$) and MIP-2 secretion ($P \leq 0.046$). LPS induced ATF-2 and c-Jun phosphorylation, which was suppressed by PFC. Finally, inhibitors of ERK, JNK, and p38 suppressed LPS-induced MIP-2 mRNA expression. Thus, PFC inhibits LPS-induced MIP-2 expression and ATF-2 and c-Jun phosphorylation. To fully explore the therapeutic potential of PFC for acute lung injury (ALI), *in vivo* analyses are required to confirm these effects.

Key words: Acute lung injury, lipopolysaccharide, macrophage inflammatory protein 2, mitogen-activated protein kinase, perfluorocarbon.

Introduction

Acute lung injury (ALI) is a critical pulmonary complication with a high incidence and mortality rate (1-3). Neutrophil-mediated tissue injury is a common mechanism underlying the development of organ dysfunction during ALI (4). In response to LPS, neutrophils accumulate in the alveolar space, secreting proinflammatory cytokines and chemokines (5,6), which culminates in destruction of the alveolar-capillary membrane with severe consequences for pulmonary gas exchange (7,8). Although great advances in understanding the pathophysiology of ALI have been achieved, currently available therapies have not reduced the mortality or increased the quality of life for survivors.

Perfluorocarbon (PFC) is a stable, inert compound that is highly soluble and has low surface tension (9). In addition to being used as an intrapulmonary agent in partial liquid ventilation (10), as contrast agents for enhancement of ultrasound images, and as blood substitutes for an intravascular oxygen carrier (11,12), some reports have demonstrated the efficacy of PFC liquids as a delivery vehicle for pulmonary administration of antibiotics and gene vectors (12). Furthermore, histologic analysis has revealed reduced inflammatory responses in lungs treated with PFC (13). Given these properties, PFC has been investigated as part of the treatment for ALI or acute respiratory distress syndrome (ARDS) since 1962. Improved oxygenation and respiratory function in response to liquid ventilation with PFC have been reported in various clinical (14,15) and animal (16-19) models of ALI. In addition, PFC can modulate the *in vitro* cellular responses associated with inflammation, such as suppression of macrophage and neutrophil infiltration and activation (20,21). In A549 cells stimulated with tumor necrosis factor-alpha (TNF- α), perflubron blocked interleukin-8 (IL-8) release (22). Furthermore, PFC decreased respiratory syncytial virus (RSV)-induced lung inflammation in mice by a mechanism that

involves the inhibition of NF- κ B-dependent production of inducible chemokines in the lung (23). Similarly, in RAW 264.7 cells treated with lipopolysaccharide (LPS), a major component of the cell wall of Gram-negative bacteria and an important trigger of ALI (24,25), PFC reduced nitric oxide (NO) levels by suppressing NF- κ B activation (26). PFC also reduced *Chlamydia pneumoniae*-mediated TNF- α and macrophage-inflammatory protein-2 (MIP-2) release, decreased NF- κ B activation and suppressed Toll-like receptor 4 (TLR4) expression (27). Furthermore, our lab has shown that PFC inhibited the LPS-induced inflammatory response in A549 alveolar epithelial cells (28) as well as pulmonary vascular endothelial cells (PMVECs) (29) from LPS-induced inflammatory injury by blocking the TLR-4 and NF- κ B signaling pathway.

In addition to NF- κ B signaling, the inflammatory reaction in ALI and ARDS was closely associated with mitogen-activated protein kinase (MAPK) signaling (30-32). MAPKs, including p38 MAPK, extracellular regulated kinase (ERK), and c-Jun NH2-terminal kinase (JNK), are activated by many proinflammatory stimuli and play an important role in the pathogenesis and development of inflammation (33). However, whether MAPK activation mediates the anti-inflammatory effect of PFC remains to be determined. Thus, the present study was undertaken to examine the hypothesis that PFC modulates LPS-induced MAPK signaling and MIP-2 expression and secretion. Because of the limitations of

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primary cells, many laboratories use LPS-treated A549 cells to investigate pathogen-associated ALI *in vitro* (34,35) as the inflammatory response induced by LPS is an important trigger of ALI (24,25,36,37).

Materials and Methods

Cell culture

The human lung adenocarcinoma cell line, A549 cells, was purchased from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (both from Solarbio Science and Technology, Beijing, China) and 100 U/mL of penicillin/streptomycin in a humidified 5% CO₂ 37°C incubator. For experiments, 2×10⁶ cells/well were plated in 6-well plates (Corning, Ithaca, NY, USA) and cultured for 24 h to form an 80–90% confluent monolayer. Prior to all experiments, the cells were starved overnight in serum-free DMEM medium.

Experimental protocol

PFC was purchased from Huajieshi Medical Treatment Facility Company Ltd (Shanghai, China). A 30% (v/v) solution of PFC was prepared by diluting it in DMEM at a ratio of 3:7 (v/v). In preliminary experiments, A549 cells were treated with 0.1, 1, 10 and 100 µg/mL of LPS (*Escherichia coli* serotype 055:B5; Sigma Aldrich, St. Louis, MO, USA); 10 µg/mL of LPS was the optimal for inducing MIP-2 mRNA and protein expression (data not shown). For all of the subsequent experiments, A549 cells were separated into the following treatment groups: (1) untreated control group, (2) 10 µg/mL of LPS (LPS group), (3) 10 µg/mL of LPS + 30% (v/v) PFC (L+P group) and (4) 30% (v/v) PFC (PFC group). Because PFC is not miscible with the medium, cells treated with PFC were constantly shaken.

To examine the effects of MAPK inhibitors on the production of MIP-2, A549 cells were treated with 1 µM U0126 (an ERK1/2 inhibitor), SB203580 (a p38 inhibitor), or SP600125 (a JNK inhibitor) alone or in combination. The cells were then co-treated with 10 µg/mL of LPS for 1, 6, or 12 h after which the cells were harvested and stored at -80°C pending for further measurements.

Quantitative polymerase chain reaction (qPCR) analysis

The cells were harvested at 1, 2, 4, 6, 8 and 12 h after the stimulation with or without LPS in the presence or absence of 30% PFC. Total RNA was isolated using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA purity and concentration were assayed with a Gene Quant Pro spectrophotometer (Amersham Biosciences, Piscataway, NJ, USA). The mRNAs were then reverse transcribed directly into cDNA using a RT-PCR kit (Takara, Shiga, Japan), following the manufacturer's instructions. mRNA was quantified by real-time PCR using the SYBR®Premix Ex Taq™ kit (Takara) on a Bio-Rad iQ5 Real-time Detection System (Bio-Rad, Hercules, USA). The PCR amplification conditions were as follows: initial denaturation at

95°C for 30 s followed by 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 20 s. The sense and antisense PCR primers for MIP-2 were 5'-CAG-TGCTTGACAGACCCTG-3' and 5'-AGCTTTCTGCC-CATTCTTG-3', respectively. The sense and antisense PCR primers for GAPDH were 5'-CAAAGACCTG-TACGCCAACACAGT-3' and 5'-ACTCCTGCT-TGCTGATCCACATCT-3', respectively. Relative MIP mRNA quantities were determined using the 2^{-ΔΔCt} method with data normalized to the *GAPDH* gene.

Enzyme linked immunosorbent assay (ELISA) detection of MIP-2 secretion

MIP-2 levels in the cell culture supernatants were determined using an ELISA kit (Lianshuo Biological Technology Company, Shanghai, China), according to the instructions recommended by the manufacturer. The optical density of each well was determined at 450 nm.

Western blot analysis

After the cells were lysed with cell lysis buffer containing 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.6, 1 mM EDTA, pH 8.0, 1 µg/mL of aprotinin, 100 µg/mL of phenylmethylsulfonyl fluoride (PMSF), 30 µg of protein was separated by electrophoresis in a 8% sodium dodecyl sulfate polyacrylamide gel (SDS PAGE) and transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk/PBS and then incubated with the following primary antibodies at 4°C overnight: phospho-ERK 1/2 (Thr202/Thr204), ERK 1/2, phospho-JNK (Thr183/Thr185), JNK, phospho-p38 (Thr180/Thr182), p38, phospho-ATF-2, phospho-c-Jun, and β-actin (all from Cell Signaling Technology, Beverly, MA, USA). The membranes were then incubated with horseradish peroxidase-linked anti-rabbit IgG (1:1000-1:20000, Cell Signaling Technology) at room temperature for 1 h. The specific protein bands were visualized using enhanced chemiluminescence (ECL) (Amersham Life Sciences, Amersham, UK). The bands were quantified by scanning densitometry using image analyzing software (Multi Gauge Ver 3.2; Fujifilm, Tokyo, Japan).

Statistical analysis

All data were obtained in triplicate and represent mean ± SD. One-way ANOVA analysis was used to compare the group means among multiple time points (1, 2, 4, 6, 8, and 12 h). The Bonferroni post-hoc test was performed when a significant treatment effect was found in ANOVA. All statistical assessments were two-sided and evaluated at the 0.05 level of significance. Statistical analyses were performed with the SPSS software for Windows, version 18.0 (SPSS Inc, Chicago, IL, USA).

Results

Effect of PFC on LPS-induced MIP-2 mRNA expression and secretion in A549 cells

We first assessed MIP-2 expression in response to LPS and PFC after 1, 4, 6, 8, and 12 h. As shown in Figure 1, LPS significantly increased MIP-2 mRNA expression at 1, 4, and 6 h ($P=0.006$, $P=0.006$, and $P=0.029$, respectively). Furthermore, PFC significantly

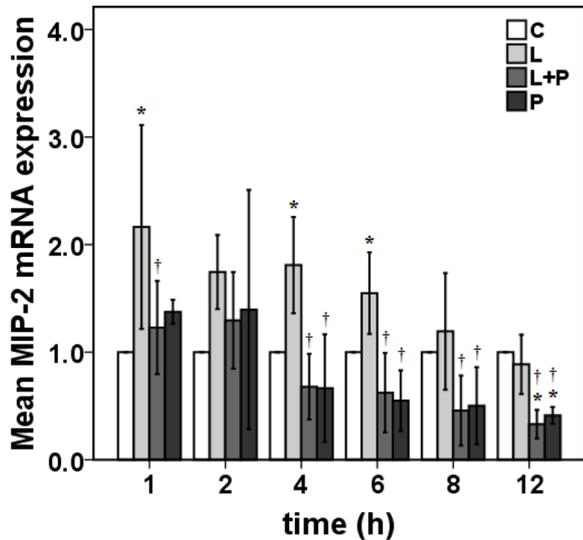


Figure 1. Effects of LPS and PFC on MIP-2 mRNA expression. MIP-2 mRNA expression was determined by RT-PCR analysis at 1, 2, 4, 6, 8, and 12 h. The data were normalized to GAPDH and presented as mean \pm standard deviation (N = 3). C, control; L, lipopolysaccharide; L+P, lipopolysaccharide+perfluorocarbon; and P, perfluorocarbon. *significantly different compared with the C group at the same time point. †significantly different compared with the L group at the same time point.

suppressed LPS-induced MIP-2 mRNA levels at 1, 4, 6, 8, and 12 h (all $P < 0.035$). However, the PFC group had MIP-2 mRNA levels comparable with the control group at 1, 2, 4, 6, and 8 h (all $P > 0.05$).

MIP-2 protein secretion by A549 cells into the cell culture supernatant was next determined by ELISA. As shown in Figure 2, LPS significantly increased MIP-2 protein secretion at 1 and 2 h ($P = 0.016$ and $P = 0.008$, respectively), which was suppressed by PFC at both time points ($P = 0.046$ and $P = 0.003$, respectively). However, PFC did not significantly affect the basal MIP-2 protein secretion at all time points analyzed (all $P > 0.05$).

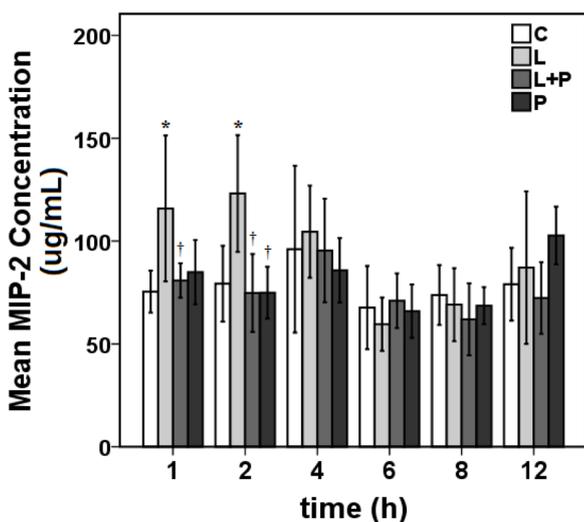


Figure 2. Effects of LPS and PFC on MIP-2 secretion by A549 cells. MIP-2 protein secretion was determined by ELISA at 1, 2, 4, 6, 8, and 12 h. The data were presented as mean \pm standard deviation (N = 3). C, control; L, lipopolysaccharide; L+P, lipopolysaccharide+perfluorocarbon; and P, perfluorocarbon. *significantly different compared with the C group at the same time point. †significantly different compared with the L group at the same time point.

These data suggested that PFC inhibited LPS-upregulated MIP-2 expression and secretion without altering basal MIP-2 levels.

Effect of PFC on LPS-induced MAPK activation in A549 cells

The effects of LPS and PFC on ERK, JNK, P38, phospho-ATF-2, and phospho-c-Jun protein expression (Figure 3), as well as ERK, JNK, and P38 phosphorylation (Figure 4) were determined by Western blot analysis. With the exception of the 12 h-timepoint, there were no significant differences in ERK protein expression among the four groups, except for the ERK/actin level of L+P group was significantly lower compared with the L group at 12 h (Figure 3A). JNK and P38 protein expression dramatically declined at 6 h and thereafter. There were no significant differences in JNK and P38 protein expression among the four groups at all time points analyzed (Figure 3B, 3C). As shown in Figure 3D, LPS significantly increased phospho-ATF-2 protein expression at 2, 4, 6, and 8 h (all $P < 0.008$), which was decreased by PFC 1, 2, 6, and 8 h (all $P < 0.001$). Howe-

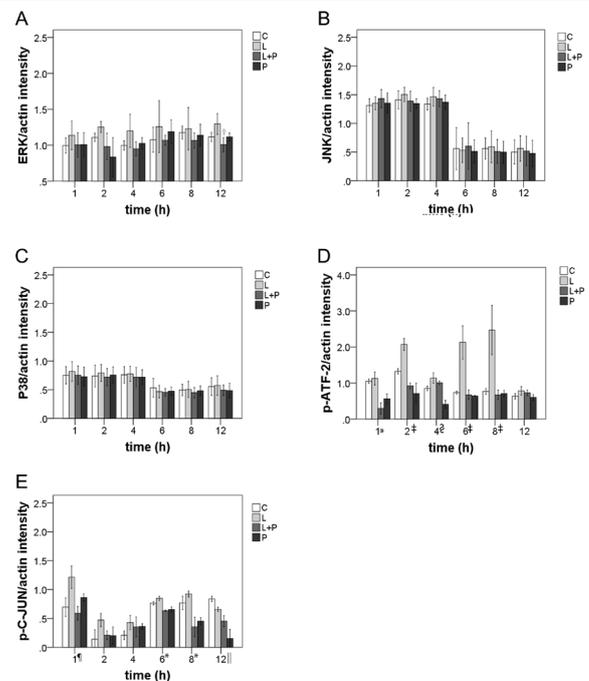


Figure 3. PFC suppresses LPS-induced ATF-2 and c-Jun activation. The expression of (A) ERK, (B) JNK, (C) P38, and (D) phospho-ATF-2, and (E) phospho-c-Jun was determined by Western blot analysis. The data were normalized to β -actin levels and presented as mean \pm standard deviation (N = 3). C, control; L, lipopolysaccharide; L+P, lipopolysaccharide+perfluorocarbon; and P, perfluorocarbon. *Indicates significantly lower levels were observed in L+P and P groups compared with the C and L group at the same time point. †The ERK/actin level of L+P group was significantly lower compared with the L group at the time point of 12h. ‡ indicates the level in L group was significantly higher than the other three groups. § indicates the level in P group was significantly lower than the other three groups. ¶ indicates a significantly higher level was observed in the L group compared to C group; and a significantly lower level was observed in the L+P group compared to L group. || indicates a significantly lower level was observed in the L+P group compared to C group; and a significantly lower level was observed in the P group compared to C, L, L+P groups.

ver, PFC significantly decreased ATF-2 protein expression compared to the corresponding control group at 1, 2, and 4 h (all $P < 0.001$). Increased the phospho-c-Jun protein expression was also observed with LPS at 1 h (all $P < 0.001$), which was suppressed by PFC at 1, 6, 8 h (all $P < 0.0010$; Figure 3E).

The differences in ERK and JNK phosphorylation levels between the four treatment groups were only observed at the 4 h-timepoint (Figure 4A and 4B). PFC significantly decreased ERK phosphorylation at 4 h as compared with the other treatment groups (all $P < 0.001$; Figure 4A). In addition, the level of phosphorylated JNK in cells treated with LPS and PFC was significantly lower than in control cells or those treated with LPS alone ($P = 0.009$ and $P = 0.004$, respectively; Figure 4B). Furthermore, significant differences in P38 phosphorylation levels between the four treatment groups were only observed at the 6 h-timepoint, at which time LPS significantly increased P38 phosphorylation levels compared to the control group ($P < 0.001$). The LPS-induced P38 phosphorylation was significantly inhibited ($P < 0.001$) in cells treated with PFC to levels similar to the control group. However, PFC alone did not alter P38 phosphorylation as compared to the control group. Representative gel images of Western blot were shown in Figure 5.

Effects of MAPK inhibitors on LPS-induced MIP-2 mRNA expression in A549 cells

To determine if the effects of LPS on MIP-2 expression were mediated by MAPK signaling, A549 cells were next treated with LPS in the presence or absence of inhibitors of ERK (U0126, U), JNK (SP600125, SP), or p38 (SB203580, SB) signaling (Figure 6). At

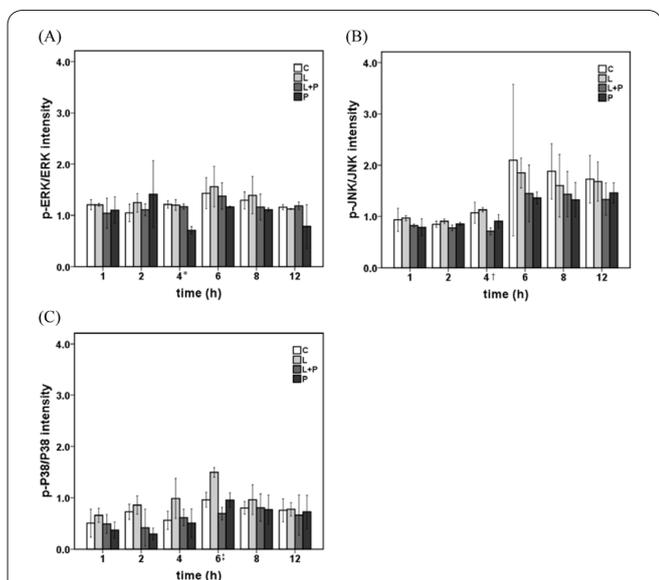


Figure 4. Effects of LPS and PFC on MAPK activation. Phosphorylated (A) ERK, (B) JNK, and (C) P38 levels were determined by Western blot analysis. The data were normalized to total protein levels of the corresponding protein and presented as mean \pm standard deviation ($N = 3$). C, control; L, lipopolysaccharide; L+P, lipopolysaccharide+perfluorocarbon; and P, perfluorocarbon. *The phosphorylated ERK level of the P group was significantly higher than the other three groups at 4 h. †The phosphorylated JNK level of the L+P group was significantly different from the C and L groups at 4 h. ‡The phosphorylated P38 levels of the L group was significantly higher than the other three groups at 6 h.

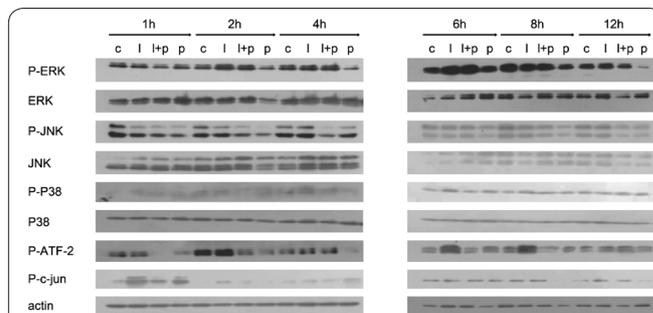


Figure 5. The representative images of Western blot. C, control; L, lipopolysaccharide; L+P, lipopolysaccharide+perfluorocarbon; and P, perfluorocarbon.

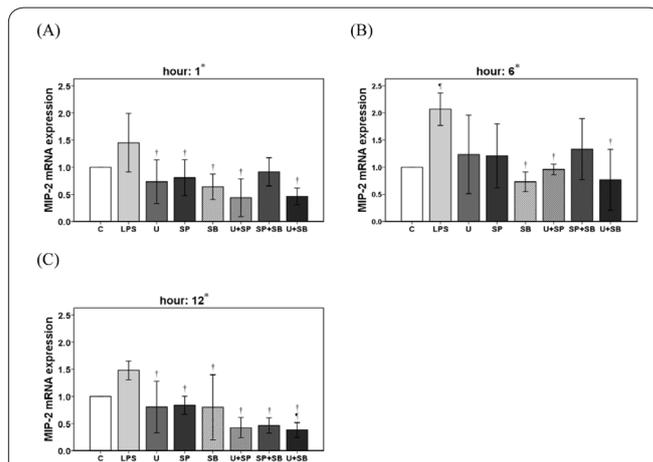


Figure 6. The effects of MAPK inhibitors on MIP-2 mRNA expression. A549 cells were treated with the indicated inhibitors for (A) 1, (B) 6, and (C) 12 h after which MIP-2 mRNA expression was determined by RT-PCR analysis. Relative mRNA quantities were determined using the $2^{-\Delta\Delta Ct}$ method with data normalized to the *GAPDH* gene, and presented as mean \pm standard deviation ($N = 3$). C, control; L, lipopolysaccharide; MAPK, mitogen-activated protein kinases; U, U0126; SP, SP600125; and SB, SB203580. †significantly different compared with the C group at the same time point. ‡significantly different compared with the L group at the same time point.

1 and 12 h, U, SP, SB, U+SP, and U+SB significantly inhibited LPS-induced MIP-2 mRNA expression (all $P < 0.05$, Figure 6A and 6C). At 6 h, only SB, U+SP, and U+SB significantly inhibited LPS-induced MIP-2 mRNA expression (all $P < 0.05$, Figure 6B).

Discussion

The effects of PFC on the expression of the chemokine, MIP-2, as well as MAPK signaling were examined in the present study. PFC significantly suppressed LPS-induced MIP-2 mRNA levels and secretion. Moreover, LPS-induced ATF-2 and c-Jun phosphorylation was suppressed by PFC; however, no significant changes in ERK, JNK, or P38 phosphorylation were detected. Finally, inhibitors of ERK, JNK, and p38 suppressed LPS-induced MIP-2 mRNA expression in A549 cells.

The mechanism by which PFC may suppress the inflammatory response in ALI was examined using LPS-treated A549 cells as an *in vitro* model of pathogen-associated ALI that has been employed in previous studies (24,25,34-37,38). In the present study, LPS induced MIP-2 expression and secretion as well as the expres-

sion of JNK and activation of ATF-2 and c-Jun. This is consistent with *in vivo* models of ALI induced by LPS (38,39).

In both acute and chronic diseases, LPS-induced inflammation activates multiple intracellular signaling cascades, including the P38, ERK, and JNK signal transduction pathways (40), which plays a significant role in the recruitment of leukocytes to the sites of inflammation via regulating the synthesis and release of proinflammatory mediators by activated macrophages (41). In addition, MAPKs may play a pivotal role in the development of ALI (42,43); LPS-induced p38 signaling is required for subsequent cytokine release (44). Furthermore, p38 and JNK contributed to sepsis-induced organ injury (45,46), and ERK signaling plays an important role in LPS-mediated pulmonary inflammation (47). In the present study, LPS induced ATF-2 and c-Jun phosphorylation, which was suppressed by PFC. However, LPS did not influence the levels of phosphorylated MAPKs as seen *in vivo* (40), which may be due to differences in the model utilized. Because activated MAPKs can phosphorylate and activate other kinases or transcription factors (48), further studies will analyze the potential downstream mediators that are induced by LPS in A549 cells and examine the effect of PFC on their activity. In addition, studies will be undertaken to examine the impact of PFC on ATF-2 and c-Jun expression as PFC can inhibit both TLR-4 and NF- κ B (28), which may in turn modulate ATF-2 and c-Jun expression.

MIP-2, a rodent homologue of human IL-8 belonging to the CXC family of cytokines, induces neutrophil chemotaxis (49-51) in inflammatory reactions (52), including that observed in ALI (53). In the present study, LPS upregulated MIP-2 expression and secretion, which is similar to that reported for *in vivo* ALI models (39,40) as well as in primary cultures of rat alveolar epithelial cells (54). In addition, PFC suppressed LPS-induced MIP-2 expression and secretion by A549 cells. This is consistent with previous reports in which PFC inhibited the release of other inflammatory mediators, including IL-1b, IL-6, IL-8 and TNF- α , by macrophages (55,56). It is also consistent with a study by Nakata et al. (57) in which PFC inhibited IL-8 secretion by AECs induced by LPS. Further studies will also examine whether ATF-2 and/or c-Jun activation mediates the effect of PFC on LPS-induced MIP-2 expression, especially given that MIP-2 promoter activation was induced by c-Jun in RAW264.7 cells treated with LPS (58).

To determine if MAPK signaling mediates the effects of LPS on MIP-2 expression, we treated the cells with various MAPK inhibitors. In the acute phase, inhibitors of ERK, JNK, and p38 suppressed LPS-induced MIP-2 mRNA expression in A549 cells. Further studies will assess if the effects of PFC on LPS-induced MIP-2 expression were mediated by MAPK signaling.

The present study is limited by its *in vitro* design; therefore, further *in vivo* analyses are required to confirm the role of MAPKs and MIP-2 in mediating the effects of PFC in ALI. In addition, the effects of PFC on the downstream mediators of MAPK signaling were not assessed. Finally, although both PFC and MAPK inhibitors individually suppressed LPS-induced MIP-2 expression, the mechanism by which PFC reduced MIP-

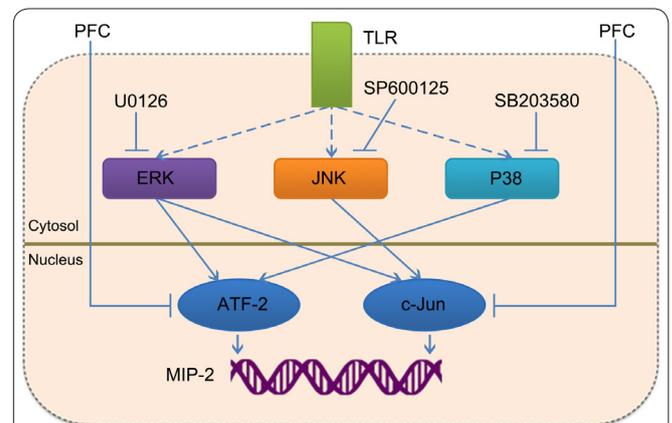


Figure 7. PFC inhibits LPS-induced MIP-2 expression and activation of ATF-2 and c-Jun in A549 pulmonary epithelial cells. LPS-mediated TLR activation induces MAPK signaling and subsequent ATF-2 and c-Jun activation and/or nuclear translocation, resulting in increased MIP-2 expression. MAPK inhibitors suppress MIP-2 expression. PFC suppresses induction of MIP-2 expression by LPS.

2 expression was not directly explored. Thus, it remains possible that PFC suppresses LPS-induced MIP-2 expression by blocking the activation of other signaling pathways, including NF- κ B (28,29).

In conclusion, the present study examined the hypothesis that PFC modulates LPS-induced MAPK signaling and MIP-2 expression and secretion. We showed here that MIP-2 mRNA expression and secretion was increased in A549 cells following LPS stimulation, which was ameliorated by PFC. These effects may be mediated by the suppression of TLR-mediated MAPK signaling and subsequent ATF-2 or c-Jun activation and/or nuclear translocation by PFC (Figure 7). ERK, JNK, and p38 inhibitors also suppressed LPS-induced MIP-2 mRNA expression in A549 cells. Further *in vivo* studies will confirm our findings and may suggest a new potential clinical treatment for pulmonary inflammation in ALI.

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