Inhibition of EphA2/EphrinA1 signal attenuates lipopolysaccharide-induced lung injury

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Abstract
Eph-Ephrin signalling mediates various cellular processes, including vasculogenesis, angiogenesis, cell migration, axon guidance, fluid homeostasis and repair after injury. Although previous studies have demonstrated that stimulation of the EphA receptor induces increased vascular permeability and inflammatory response in lung injury, the detailed mechanisms of EphA2 signalling are unknown. In the present study, we evaluated the role of EphA2 signalling in mice with lipopolysaccharide (LPS)-induced lung injury. Acute LPS exposure significantly up-regulated EphA2 and EphrinA1 expression. Compared with LPS + IgG mice (IgG instillation after LPS exposure), LPS + EphA2 mAb mice [EphA2 monoclonal antibody (mAb) instillation post-treatment after LPS exposure] had attenuated lung injury and reduced cell counts and protein concentration of bronchoalveolar lavage fluid (BALF). EphA2 mAb posttreatment down-regulated the expression of phosphoinositide 3-kinases (PI3K) 110γ, phospho-Akt, phospho-NF-κB p65, phospho-Src and phospho-S6K in lung lysates. In addition, inhibiting the EphA2 receptor augmented the expression of E-cadherin, which is involved in cell–cell adhesion. Our study identified EphA2 receptor as an unrecognized modulator of several signalling pathways – including PI3K-Akt-NF-kB, Src-NF-kB, E-cadherin and mTOR – in LPS-induced lung injury. These results suggest that EphA2 receptor inhibitors may function as novel therapeutic agents for LPS-induced lung injury.

Key words: acute lung injury, EphA2, EphrinA1, lipopolysaccharide.

INTRODUCTION

The Eph tyrosine kinase receptor and Ephrin ligand are cell surface-bound, and are involved in cell-to-cell communication [1,2]. The influence of Eph-Ephrin activation differs depending on cell type and environment. In addition to bidirectional signalling, the Eph receptor and Ephrin ligand function independently of each other, or in collaboration with other cell surface communication systems. Eph-Ephrin signalling contributes to several functions – including vasculogenesis, angiogenesis, cell migration, axon guidance, fluid homeostasis and repair after injury [1−3]. Several studies have focused on the complex role of Eph and Ephrin in malignancy [4,5], and according to these studies, the Eph receptor and Ephrin ligand affect multiple oncogenic signalling pathways – including MAPK/ERK, phosphoinositide 3-kinases (PI3K), E-cadherin and integrin/FAK/paxillin [4,6−8].

Recently, Eph-Ephrin signalling was found to contribute to inflammation by promoting phenotypic changes in the vascular endothelium that allow for the movement of inflammatory cells into the injured tissue [3]. Additional research is needed to explore the mechanism behind these changes, and to identify novel therapeutic approaches for acute lung injury (ALI) treatment since no effective, non-supportive treatments currently exist [9]. Some studies have reported that EphA2 signalling plays a role in lung injury and inflammation. In a bleomycin-induced lung injury model, EphA2-knockout mice showed reduced permeability...
and a lower inflammatory response compared with wild-type mice [10]. In viral and hypoxia-induced lung injuries, antagonizing EphA2 with EphA2/Fc and anti-EphA2 antibodies reduced vascular leakage and albumin extravasation [11]. However, data regarding the detailed mechanism of EphA2 signalling in lipopolysaccharide (LPS)-induced lung injury are limited.

Given the previous studies, we hypothesized that (1) the expression of EphA2 and EphrinA1 increases in LPS injured lung tissue, (2) inhibition of EphA2 signalling, even after established endotoxemia, may be useful for the treatment of lung injury and (3) cross-talk exists between EphA2 and other signalling pathways in LPS-induced lung injury.

**MATERIALS AND METHODS**

**LPS-induced lung injury model in mice**

Wild-type male C57BL/6J mice, 8–10 weeks of age weighing 20–24 g were purchased from Orient Bio. All animals were supplied with food and water and were subjected to a similar day and night light cycle.

Twenty-four mice were randomly divided into three groups: (A) control group with IgG posttreatment, (B) LPS-induced lung injury group with IgG posttreatment, and (C) LPS-induced lung injury group with EphA2 monoclonal antibody (mAb) posttreatment.

The treatments were administered intranasally. Mice were lightly anesthetized by isoflurane inhalation (Abbott Laboratory), and were held in a supine position with the head elevated. The administration solution was gradually released into the nostril with the help of a microsyringe from Hamilton Com. The rate of release was adjusted to allow the mouse to inhale the solution without forming bubbles.

*Escherichia coli* LPS (E. coli 0127: B8; Sigma) (40 μg/g) in 50 μl PBS was administered by intranasal (i.n.) inhalation. For posttreatment, 4 μg of either mouse IgG (catalogue 37355, Abcam) or monoclonal EphA2 antibody (catalogue 233720, R&D Systems) were intranasally administered 6 and 12 h after LPS treatment. The control group was given 50 μl of sterile PBS followed by two doses of IgG (at 6 and 12 h after PBS treatment, i.n., 4 μg).

**Isolation of bronchoalveolar lavage cells, lung tissue and cell counts**

Twenty-four hours after LPS/PBS inoculation, all mice were humanely killed by lethal overdose of ketamine and xylazine. BAL was performed through a tracheal cannula using two 1 ml aliquots of sterile saline. The BAL fluid was centrifuged (4 °C, 1500 g, 10 min) and the supernatant was stored at 80 °C for further analysis.

The cell pellet was reconstituted in 100 μl PBS and used to quantitatively and qualitatively analyse the cells. Total cell numbers in each sample were determined using a hemocytometer (Marienfield) according to the manufacturer’s protocol. Slide chambers were prepared by inserting the slide into the frame with the Poly-l-lysine coating up and clamping with clips on either side. A 90 μl aliquot of each sample was transferred into the slide chambers, which were then inserted into a cytocentrifuge with the slide facing outward. The slides were centrifuged at 600 rpm for 6 min, then removed from the cytocentrifuge and dried prior to staining. Diff-Quick (Sysmex Corporation) staining was used. The slides were immersed in three Diff Quick fluids (Fixative, Solution I, Solution II) for 5 s and rinsed with purified water.

The protein content of the BAL supernatant was measured using the Coomassie Brilliant Blue G-250 technique (Quick StartTM Bradford Protein Assay). Twenty-five microliters of each sample and 200 μl of working reagent were pipetted into a microplate well and mixed thoroughly on a plate shaker for 30 s. After incubation for 30 min at 37 °C, the plate was cooled and the absorbance read at 562 nm in a spectrophotometer.

**Lung tissue harvest and histologic examination**

The right lung was isolated and stored at −80 °C prior to protein extraction, after flushing the pulmonary vasculature with saline under low pressure. The left lung was inflated via the tracheotomy with low-melting point agarose (4%) in PBS at 25 cm H2O pressure and until the pleural margins became sharp. The lungs were then excised and fixed overnight in 10% formaldehyde in PBS and embedded in paraffin for sectioning at 5 μm thickness. Left lung sections were stained with H&E and subjectively evaluated by light microscopy. The histopathology was reviewed in a blinded manner by two qualified investigators. Five easily identifiable pathologic processes were scored using the weighted scale presented in the official ATS workshop report [12]. Lung sections were processed for immunohistochemistry using anti-rabbit EphA2 (catalogue PA5-14574, Thermo Fisher Scientific) or anti-mouse EphA2 (catalogue 233720, R&D Systems), and anti-goat EphrinA1 (catalogue PA5-19397, Thermo Fisher Scientific) antibodies. A peroxidase-based assay was performed using diaminobenzamide (DAB) as the chromogen.

**ELISA**

Keratinocyte chemoattractant (KC/CXCL1), macrophage inflammatory protein (MIP-2), interleukin-1β (IL-1β), interleukin 6 (IL-6) and tumour necrosis factor α (TNF-α) levels in whole-lung homogenates were measured using quantified ELISA kits (MCYTOMAG-70K, MILLIPLEX MAG Mouse Cytokine/Chemokine kit from Millipore) according to the manufacturer’s directions.

**Western blotting**

The frozen right lungs were mechanically disrupted using a homogenizer in 600 μl of homogenization buffer (PRO-PREPTM Extraction solution iNtRON BIOTECHNOLOGY) per 10 mg tissue. Cell lysis was induced by incubation for 20–30 min on ice or in the freezer at −20°C. The samples were centrifuged at 13,000 g for 30 min at 4 °C. Equal amounts of protein were separated by SDS/PAGE and transferred to a nitrocellulose membrane before immunoblotting with primary antibodies as indicated. Membranes were incubated with anti-rabbit or anti-mouse IgG HRP-conjugated secondary antibodies, and developed using Super-Signal West Pico chemiluminescence detection kit.
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Results

LPS-induced lung injury increases the expression of lung EphA2 and Ephrin A1

As measured by Western blotting, the expression of EphA2 and EphrinA1 protein in lung tissue increased after LPS treatment (EphA2: 12.61-fold, \( P < 0.001 \), EphrinA1: 7.26-fold, \( P < 0.001 \), Figure 1), compared with PBS treatment. The increased expression of EphA2 and EphrinA1 in the lung was inhibited by EphA2 mAb posttreatment (EphA2: \( P = 0.002 \), EphrinA1: \( P < 0.001 \)). To determine where in the lung EphA2 and EphrinA1 were expressed, immunohistochemistry was performed. Immunostaining for EphA2 and EphrinA1 in the lung tissue from PBS-treated control animals showed weakly detectable expression in the endothelial and epithelial bronchial cells, as well as stronger staining in alveolar septum. In contrast, LPS-injured mice demonstrated marked increases in EphA2 and EphrinA1 staining in type II pneumocytes and alveolar macrophages around inflamed areas with thickened septae (Figure 2).

We performed the additional experiment for control group in which mice is intranasally treated with mouse monoclonal EphA2 Ab after PBS exposure (PBS + EphA2 mAb). The expression of EphA2 protein was not different between PBS + EphA2 mAb group and PBS + IgG group (not shown, included in Supplementary Figure S1). In the H&E staining, PBS + EphA2 mAb group showed mild capillary congestion and RBC extravasation compared with PBS + IgG group, however, inflammatory cells were scanty in both groups (included in Supplementary Figure S2).

EphA2 antagonism attenuates LPS-induced lung injury

After finding that LPS-induced lung injury is associated with increased expression of EphA2 and EphrinA1 in the lung, we
Figure 1 EphA2 and EphrinA1 protein levels increase in LPS-induced lung injury and decrease after EphA2 mAb posttreatment

Western blotting (upper panel) and graphical representations of densitometry (lower panels, n = 8 per group) are shown. Values are presented as means ± S.D. *P < 0.05.

sought to determine whether EphA2 contributes to permeability and inflammation by evaluating the effects of an EphA2 mAb posttreatment.

As shown in Figure 3, LPS caused lung injury and oedema, as demonstrated by a significant increase in the total protein concentration (LPS + IgG: 0.90 ± 0.34 mg/ml, PBS + IgG: 0.17 ± 0.05 mg/ml, P < 0.001) and total cell count (LPS + IgG: 2948 × 10^4 ± 2996 × 10^4, PBS + IgG: 21 × 10^4 ± 14 × 10^4, P = 0.015) in BALF. The protein concentration and total cell count in BALF of PBS + EphA2 mAb group were significantly lower than those of LPS + IgG group (total protein concentration: 0.39 ± 0.31 mg/ml; P = 0.034, total cell count: 348 × 10^4 ± 269 × 10^4; P = 0.016). The EphA2 mAb posttreatment resulted in a significant reduction in the total protein concentration (LPS + EphA2 mAb: 0.23 ± 0.15 mg/ml, LPS + IgG: 0.90 ± 0.34 mg/ml, P = 0.002) and total cell count (LPS + EphA2 mAb: 157 × 10^4 ± 107 × 10^4, LPS + IgG: 2948 × 10^4 ± 2996 × 10^4, P = 0.019) in BALF compared with IgG posttreatment after LPS instillation. In addition, histologic examination of the lungs of mice that received EphA2 mAb as well as LPS revealed a significant decrease in lung injury score compared with mice that received LPS and IgG posttreatment (LPS + EphA2 mAb: 40.9 ± 10.5, LPS + IgG: 68.5 ± 10.7, P = 0.001).

The expression of inflammatory cytokines decreased after EphA2 monoclonal antibody posttreatment in the LPS-induced lung injury model

As shown in Figure 4, concentrations of cytokines (IL-1β, IL-6, KC, MIP-2 and TNF-α) in the lungs were significantly higher in mice with IgG posttreatment after LPS exposure when compared with mice with IgG posttreatment after PBS exposure.

The decreased expression of IL-1β, KC and MIP-2 was detected in the lung tissue of mice with EphA2 mAb posttreatment compared with mice with IgG posttreatment after LPS exposure. The expression of IL-6 and TNF-α decreased in the lung tissue of mice with EphA2 mAb posttreatment compared with mice with IgG posttreatment after LPS exposure, but was not significantly different.

LPS up-regulates PI3K-Akt-NF-κB signalling and Src-NF-κB signalling via EphA2 signalling

Given the protective effect of EphA2 antagonism in LPS-induced lung injury, we wondered whether inhibiting EphA2-EphrinA1
signalling would affect the existing signalling pathways. To answer this question, the expression of various proteins in lung tissue was measured in the PBS + IgG, LPS + IgG and LPS + EphA2 mAb groups.

As shown in Figure 5A, compared with the PBS + IgG group, the LPS + IgG group showed significantly increased PI3K 110γ and Akt phosphorylation (PI3K 110γ: 7.43-fold, \( P = 0.027 \), phospho-Akt: 1.7-fold, \( P = 0.020 \)). After EphA2 mAb posttreatment, the expression of PI3K 110γ and phosphorylation of Akt by LPS challenge were reduced (PI3K 110γ: \( P = 0.039 \), phospho-Akt: \( P = 0.013 \)). This result shows that EphA2 signalling may be involved in LPS-induced activation of Akt through a PI3Kγ-dependent step.

LPS also induced a 2.5-fold increase in Src phosphorylation in the lung (\( P = 0.009 \)), which was inhibited by EphA2 mAb (2-fold decrease: \( P = 0.005 \), Figure 5B). The phosphorylation

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Figure 2 Immunostaining of EphA2 and EphrinA1 in mouse lungs
(a) Increased EphA2 immunostaining after LPS exposure compared with the control group (PBS + IgG) is inhibited by EphA2 mAb posttreatment. (b) The increased EphrinA1 immunostaining after LPS exposure is reduced by EphA2 mAb posttreatment. Scale bar: 50 \( \mu \)m.

Figure 3 EphA2 mAb posttreatment attenuates LPS-induced lung injury
(a) Total BALF protein concentration. (b) Total BALF cell counts. (c) Lung injury scores (\( n = 8 \) per group). *\( P < 0.05 \).

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Figure 4  IL-1β, IL-6, KC, MIP-2 and TNF-α levels in lung tissue lysates

Cytokine levels, measured by an ELISA, were reduced in mice with EphA2 mAb posttreatment compared with those with IgG posttreatment in LPS-induced lung injury (n = 8 per group). Values are presented as means ± S.D. *P < 0.05.

Figure 5  The expression of PI3Kγ, Akt, Src and NF-κB protein in mouse lung lysates after LPS exposure and EphA2 mAb posttreatment

(A) PI3Kγ and Akt protein levels increase in LPS-induced lung injury and decrease after EphA2 mAb posttreatment. Western blotting (left panel) and graphical representations of densitometry (right panels, n = 8 per group) are shown. Values are presented as means ± S.D. *P < 0.05. (B) Src and NF-κB protein levels increase in lung lysates after LPS exposure and decrease after EphA2 mAb posttreatment. Western blotting (left panel) and graphical representations of densitometry (right panels, n = 8 per group) are shown. Values are presented as means ± S.D. *P < 0.05.
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Figure 6 S6K protein levels increase in lung lysates after LPS exposure and decrease after EphA2 mAb posttreatment. On the other hand, E-cadherin protein levels decrease in lung lysates after LPS exposure and increase after EphA2 mAb posttreatment. Western blotting (A) and graphical representations of densitometry (B) are shown. Values are presented as means ± S.D. *P < 0.05 (n = 8). (C) Immunofluorescence staining of E-cadherin in mouse lungs. Lung slides were labelled with an anti-E-cadherin antibody and a FITC-conjugated secondary antibody (C). Scale bar: 50 μm.

of NF-κB p65 increased significantly in the lung after LPS exposure compared with PBS exposure (P = 0.006). There was significant difference in the phosphorylation of NF-κB p65 between EphA2 antagonist posttreatment and IgG posttreatment after LPS exposure (P = 0.045, Figure 5B). This finding indicates that EphA2 may play a critical role in activating downstream signalling pathways, such as the PI3Kγ-Akt-NF-κB and Src-NF-κB signalling pathways.

LPS up-regulates mTOR signalling via EphA2 activation

The phosphorylation of Erk1/Erk2 was increased 6.76-fold in lung tissue after LPS exposure (P = 0.016). But, EphA2 mAb posttreatment did not significantly decrease the phosphorylation of Erk1/Erk2 after LPS exposure (P = 0.082). The phosphorylation of S6, a major target of mTOR, was increased 2.18-fold in lung tissue after LPS exposure (P = 0.004), and this increase was inhibited by EphA2 mAb posttreatment (P = 0.003, Figure 6).

LPS down-regulates E-cadherin via EphA2 activation

LPS exposure reduced the expression of E-cadherin (P = 0.001) and EphA2 mAb posttreatment significantly restored E-cadherin protein expression (P = 0.006, Figure 6). E-cadherin expression was confirmed by immunofluorescence analysis (Figure 6C). Although E-cadherin expression was strong in the control group, it was down-regulated after LPS exposure. Inhibition of EphA2 signalling by EphA2 mAb treatment blocked the LPS-induced down-regulation of E-cadherin expression in lung tissue. These results demonstrate that LPS-induced EphA2 expression may partly regulate the expression of E-cadherin and influence adherens junction and epithelial hyperpermeability.

The expression of EphA2 and Ephrin in human BALF is up-regulated in patients with infections

EphA2 and EphrinA1 protein expression was measured in BALF of human adults (Figure 7). EphA2 levels were significantly elevated in adults with pulmonary infection compared with control subjects and patients with ILD (pulmonary infection: 0.14 ± 0.16 ng/ml, control: 0.03 ± 0.02 ng/ml; P = 0.010, ILD: 0.06 ± 0.06 ng/ml; P = 0.004). The EphrinA1 levels of adults with pulmonary infection were higher than those of the other two groups (pulmonary infection: 0.63 ± 0.67 ng/ml, control: 0.01 ± 0.01 ng/ml; P = 0.001, ILD: 0.13 ± 0.10 ng/ml; P < 0.001), and EphrinA1 levels of adults with ILD were higher than the control subjects (P = 0.001).

DISCUSSION

EphA2 signalling has been studied in angiogenesis, cell migration, fluid homeostasis and vascular assembly during the early stages of development [1,2]. The interaction between Eph receptor and the Ephrin ligand results in the modification of cytoskeletal proteins and cell surface receptors [1,2]. Previous studies have found that actin cytoskeleton rearrangement may be a key event preceding the regulation of inflammatory responses in various cell populations [16–19]. The disruption of endothelial cell junctions by EphA2 signalling allows the passage of fluid, proteins and inflammatory cells into injured tissues [3,20]. Taken together, the current available evidence suggests that EphA2 signalling may be an important mediator of inflammation and injury [10,11,21,22].

Our studies demonstrate that EphA2 contributes to the pathogenesis of LPS-induced lung injury. Both the expression of the EphA2 receptor and the EphrinA1 ligand were increased in LPS-injured lung tissue. In addition, blocking of the activation of the EphA2 receptor by EphA2 mAb treatment ameliorated the permeability and inflammatory responses associated with ALI. The recruited inflammatory cells and lung injury score after LPS exposure decreased in mice with EphA2 mAb posttreatment compared with those with IgG posttreatment (Figure 3). In the present experiment, we demonstrate that EphA2 signalling has an
important role in regulating endotoxin-induced inflammatory cytokines including IL-1β, KC and MIP-2 (Figure 4).

These results are consistent with previous studies. In rats exposed to viral respiratory infection and hypoxia, EphA2 expression was markedly up-regulated, and EphA2 antagonism reduced vascular leakage in lung injury [11]. Similarly, in bleomycin-induced lung injury, EphA2-KO mice were protected from protein extravasation and inflammatory responses [10]. Both studies demonstrated that EphrinA1 stimulation of the lung endothelial EphA2 receptor leads to the disruption of endothelial adherens junctions and increased permeability. Similarly, our results suggest that LPS induces an increase in ligand-mediated activation of EphA2.

However, Ivanov et al. [23] demonstrated counter-directed changes in the expression regulation of the EphA2 receptor and EphrinA1 ligand in lung tissue in phase 2 (90 min post-LPS) of LPS injection, contrary to our data. The reasons for the different results may be the different experimental durations used in these studies. In the present study, mice were killed 24 h after LPS exposure; however, Ivanov et al. killed mice after only 90 min. Different temporal expressions of EphA2 and EphrinA1 may be involved in the regulation of the cellular events underlying different stages of systemic inflammation.

The cascade of multiple signalling pathways in the mechanism of ALI is complex and remains unclear. Despite recent advances, ALI still accounts for significant morbidity and mortality in critically ill patients [24,25]. Therefore, investigating the molecular and cellular signalling pathways that mediate ALI is important for the development of specific effective therapies.

We found several potential mechanisms through which EphA2 signalling may contribute to the development of LPS-induced lung inflammation and injury (Figure 8).

The activation of Akt through the PI3K-dependent pathway leads to increased nuclear translocation of NF-κB, which regulates proinflammatory cytokine production in endotoxemia-associated ALI [26]. The role of the PI3K-Akt pathway in modulating NF-κB activation has been demonstrated in numerous cell populations – including neutrophils, epithelial cells and fibroblasts [26–29]. In the present study, antagonizing EphA2 expression by EphA2 mAb posttreatment after LPS exposure resulted in down-regulation of the expression of PI3K-Akt-NF-κB compared with the LPS-only group. The results suggest that LPS-induced up-regulation of the PI3K-Akt-NF-κB pathway is mediated by EphA2 signalling.
Lee et al. [30] and Severgnini et al. [31] reported that Src tyrosine kinases mediate the activation of NF-κB in LPS-induced lung injury, and that selective Src tyrosine kinase inhibitors may be a potential therapeutic agent. Our data showed that EphA2 antagonism significantly reduced Src phosphorylation and NF-κB activation. These findings suggest the involvement of EphA2 signalling in LPS-induced NF-κB activation via Src tyrosine kinase as an upstream pathway.

Intratracheally administered LPS induces epithelial injury and barrier integrity dysfunction in a murine model of ALI [32–34]. He et al. [35] reported that LPS induces epithelial barrier dysfunction through the regulation of E-cadherin intracellular trafficking. Nasreen et al. [36] demonstrated that EphA2 and EphrinA1 expression levels were increased in bronchial airway epithelial cells exposed to tobacco smoke, and this may be an important event preceding down-regulation of E-cadherin expression and MAPK-dependent hyperpermeability.

Our study demonstrated that EphA2 mAb enhance the pulmonary epithelial barrier integrity through E-cadherin accumulation in LPS-induced lung injury. Also, immunofluorescence study supported the possibility of enhancing E-cadherin with EphA2 mAb. Further studies are needed to determine whether up-regulated E-cadherin expression and reduced protein leakage after EphA2 mAb posttreatment is associated with reduced Erk phosphorylation or activated PKC pathway, as reported by previous studies [35,36].

Previous studies have shown that LPS exposure activates mTOR signalling in inflammatory cells and lung tissues [37,38]. Our results showed that the phosphorylation of S6, a downstream target of mTOR, increased after inhaled endotoxin administration and was reduced after inhibition of EphA2 signalling.

Several studies have used rapamycin to dissect the role of mTOR in ALI [38–40]. Feilhaber et al. [39] showed that the inactivation of mTOR attenuates MyD88-dependent processes (i.e. NF-κB, TNF-α and neutrophil recruitment), but enhances MyD88-independent signalling (i.e. STAT1 and apoptosis), leading to lung injury and apoptosis. Similarly, Wang et al. [40] reported that rapamycin reduced the level of inflammatory mediators but did not change permeability and mortality in LPS-induced lung injuries. Considering that in the present study EphA2 contributed not only to changes in inflammatory responses but also to permeability, signal transduction pathways other than mTOR signalling may constitute a complex network around EphA2 signalling.

In human data, adults with pulmonary infections had higher EphA2 and EphrinA1 levels in the BALF when compared with adults with ILD and the control group. This result is in line with the mouse experiment, which showed that LPS exposure leads to an elevation in EphA2 and EphrinA1 levels. However, the sample size in the present study was small and causality cannot be demonstrated. The clinical significance of the elevated EphA2 and EphrinA1 levels in adults with pulmonary infection need to be further investigated.

Our study includes a couple of limitations. As the number of mice used in the experiment is small (n = 8), particular attention was needed whilst interpreting the statistical results. Further experiments are needed to accurately identify the relationship between signal pathways concerning EphA2 signalling in LPS-induced lung injury. Also, the possibility of clinical application of EphA2 mAb as a therapeutic target for ALI should be proven through several ex vivo and in vivo animal models.

In conclusion, we demonstrated that EphA2 signalling contributes to permeability and inflammation in LPS-induced lung injuries, and that it may regulate several signalling pathways including the PI3K-Akt-NF-κB, src-NF-κB, E-cadherin and mTOR pathways. In the mouse experiment, the inhibition of EphA2 expression by EphA2 mAb administration attenuated lung injury. The clinical potential of an EphA2 signalling inhibitor should be evaluated.

**CLINICAL PERSPECTIVES**

- Stimulation of the EphA receptor increases vascular permeability and inflammatory response in lung injury. The aim of the present study was to evaluate the effect of EphA2 receptor inhibitors in vivo and to investigate the cross-talk between EphA2 and other signalling pathways in LPS-induced lung injury.
- The results of the present study show EphA2 receptor is an essential modulator of several signalling pathways including PI3K-Akt-NF-κB, Src-NF-κB, E-cadherin and mTOR in LPS-induced lung injury.
- These findings suggest that targeting EphA2 receptor may be a novel strategy in LPS-induced lung injury.

**AUTHOR CONTRIBUTION**

Ivor Douglas, Mi Hwa Shin, Young Sam Kim and Moo Suk Park conceived and designed the study. Kyung Soo Chung, Eun Young Kim, Ji Ye Jung and Young Ae Kang analysed and interpreted the data. Ji Young Hong, Se Kyo Kim and Joon Chang drafted the paper.

**FUNDING**

This work was supported by the ‘Kiturami’ Faculty Research Assistance Program of Yonsei University College of Medicine for 2012 [grant number 6-2012-0149].

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Received 3 December 2015/19 August 2016; accepted 22 August 2016
Accepted Manuscript online 22 August 2016, doi: 10.1042/CS20160360