Neutrophil Infiltration is Partially Inhibited by EC-18 in the LPS-Induced Acute Lung Injury

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#2212

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Abstract

Acute lung injury (ALI) is an acute respiratory failure and linked closely to neutrophil accumulation. It can lead to acute respiratory distress syndrome (ARDS). Mouse model of ALI was established by lipopolysaccharide (LPS) administration. LPS, an outer membrane of gram negative bacteria, is considered as immune stimulator via recognition as pathogen-associated molecular patterns (PAMP). ALI and neutrophil infiltration were readily induced by intranasal injection of LPS. In this study, we investigated whether EC-18 (PLAG) treatment attenuates LPS-induced ALI.

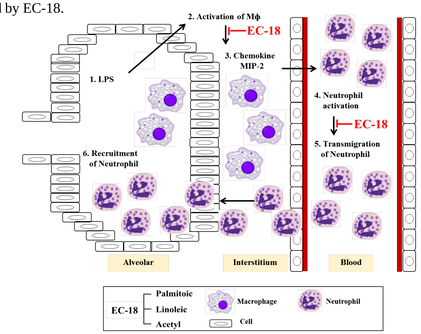
EC-18 (PLAG, 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol) is an immune modulator in the allergic asthma response through modulation of the balance between Th1 and Th2. To analyze the role of EC-18 in LPS-induced ALI mice, Balb/c mice were divided into three separate groups: **control, LPS treated, and LPS/EC-18 co-treated** (n=7 per group). 25 mg/kg of LPS was administered by an intranasal route, and 250 mg/kg of EC-18 was orally administered. Mice were sacrificed after 16 h, and various samples were collected. Bone marrow cells, whole blood cells, cells in lung and bronchoalveolar lavage fluid (BALF) were analyzed using complete blood count (CBC) assay.

As results, the number of neutrophil in the bone marrow was decreased in the LPS treated group, and the circulating neutrophil, neutrophil in the lung and BALF were significantly increased in the LPS treated group. **Neutrophils in the lung and BALF were dramatically decreased in the LPS/EC-18 co-treated group**. Also, Evans blue staining of the lung indicated that capillary permeability was enhanced in the LPS injected mice, and this permeability was lessened in the EC-18/LPS co-treated group as much as that of control.

These findings suggested that **EC-18 could effectively block neutrophil transmigration into the lung and vesicular leakage**. Consequently, EC-18 could be utilized as a potential therapeutic agent for acute and chronic inflammation related disease like ALI.

Introduction

Acute lung injury (ALI) is a severe respiratory inflammation with an increased permeability of the alveolar-capillary barrier. Neutrophil migration into the lung is the critical step in the early progression of ALI. Neutrophil recruitment into the lung is occurred by the massive pro-inflammatory cytokines and chemokines, and leads to lung edema, endothelial and epithelial injury. ALI is still associated with a high mortality, and a specific therapy is not available. There are several factors involved in neutrophil migration into the lung of ALI model mouse. **MIP-2 (CXCL2)** is secreted by resident macrophages in the lung, and it has the chemotactic effect for neutrophil recruitment toward inflammation sites. **S100A8** and **S100A9**, which are known as inflammatory protein complex, exist in high amounts in the cytoplasm of neutrophils. They are able to regulate neutrophil activation and migration into the target tissue. **IL-6** is a critical pro-inflammatory cytokine that contributes to the initiation and extension of the inflammatory response. Furthermore, IL-6 autocrine signaling loop shows the enhanced self-amplification through STAT3 phosphorylation. They play an important role in host defense, however, the excessive production of these factors can cause the severe inflammation such as acute lung injury and are controlled by several signal factors. **STAT3** activation in the lung is associated with acute phage of lung injury. STAT3 is commonly phosphorylated by IL-6, but also by TLR4->MyD88->p38 MAPK->STAT3 pathway. In this study, we investigated whether EC-18 has the therapeutic potential in LPS injected mouse model and examined what molecule and the signal pathway was regulated by EC-18.



The suggested ALI progression in LPS introduced mouse.

Serial cascade is supposed to be happened from LPS infection to recruitment of neutrophil in the alveolar like as ① LPS introduction via nasal injection will induce ② activation of resident Mϕ in the interstitium and ③ release of DAMP and chemokines including CXCL-1,2,8. Then ④ neutrophil will activate and stat to ⑤ transmigrate toward alveolar space. And ⑥ neutrophil will recruited into alveolar and involve in the activity of tissue destruction.

Results

1. EC-18 attenuated the LPS-induced ALI..

The effect of EC-18 on the vascular leakage was investigated by Evans blue leakage assay because Evans blue dye extravasation into the tissue was used as an index of increased vascular permeability and neutrophil transmigration. In LPS administrated mice, the image of lung tissues showed the strong inflammation through a large amount of Evans blue accumulation. LPS with EC-18 or dexamethasone co-treated mice were decreased Evans blue accumulation in lungs.

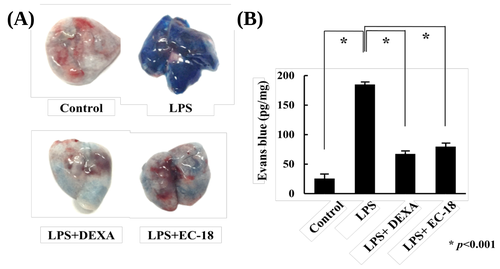


Figure 1. Mice were divided into four separate groups: control, LPS treated, LPS/dexamethasone co-treated, and LPS/EC-18 co-treated (n=7 per group). LPS (25 mg/kg, Sigma) was administrated via intranasal route, and EC-18 (250 mg/kg) or dexamethasone (3 mg/kg, Sigma) were orally administered. (A) Evans blue (50 mg/kg, Sigma) diluted in 250 μl of PBS was intravascularly injected 30 min before sacrifice. 16 h after LPS treatment, mice were anesthetized with 2,2,2-Tribromoethanol (150 mg/kg, Sigma) and perfused with PBS. (B) Evans blue dye extracted from lungs and quantified. * p<0.001.

2. EC-18 attenuated neutrophil migration into the lung .

LPS challenge significantly increased the neutrophil infiltration into BALF compared to the control. Neutrophils in LPS/EC-18 or LPS/dexamethasone treated groups more retained in blood compared to LPS treated group. LPS/EC-18 and LPS/dexamethasone co-treated group were significantly decreased neutrophil migration in BALF. LPS induced neutrophil population up to 86.27 % of leukocyte and EC-18 significantly reduced to 32.17 %. EC-18 (55.87 %) relatively induced the lymphocyte population compare to LPS (3.13 %) administrated mice. These findings suggest that EC-18 significantly suppress LPS-induced ALI progression.

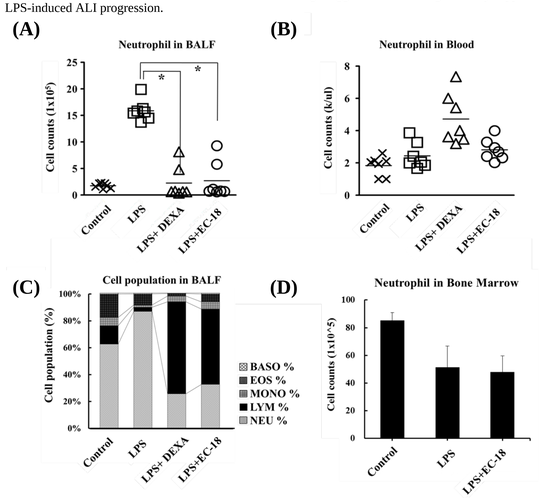


Figure 2. Mice were treated as described in figure 1. Cells in the bronchoalveolar lavage fluid (BALF) were flushed out by intratracheal injection using PBS. Neutrophils in BALF (A) and Blood (B) were counted with Complete Blood Cell Count. (C) BALF cells were also counted with Complete Blood Cell Count (CBC, Shenzhen Mindray Bio-medical Electronics) and cell population was analyzed. (D) Mice were divided into three separate groups: control, LPS treated, and LPS/EC-18 co-treated (n=5 per group). LPS (25 mg/kg) was administered via an intranasal route, and EC-18 (250 mg/kg) was orally administered. After 16 h, mice were anesthetized with 2,2,2-Tribromoethanol (150 mg/kg) and bone marrow cells were harvested from femurs and tibias. Bone marrow-derived cells were counted with CBC analysis.* p<0.001.

3. EC-18 down-regulated the level of pro-inflammatory cytokines in BALF and Serum.

MIP-2 mRNA expression was increased in LPS administration compared to control. EC-18 showed no difference on MIP-2 mRNA expression in the lung. However, the mRNA expressions of MIP-2, IL-6, S100A8 and S100A9 in BALF cells were significantly downregulated by EC-18 treatment. MIP-2 and IL-6 were significantly increased in BALF and serum by LPS administration and EC-18 markedly decreased the secretion of MIP-2 and IL-6. From these data, EC-18 effectively suppressed the anti-inflammatory cytokines production in inflammatory region through blocking neutrophil transmigration.

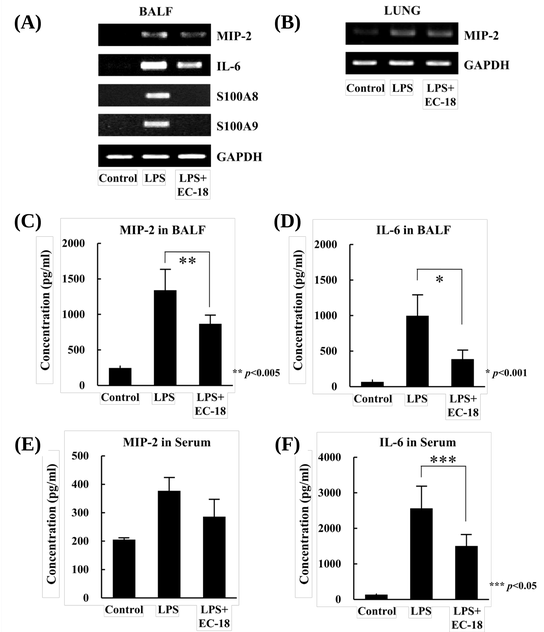


Figure 3. Mice were treated with LPS, LPS plus EC-18 as described in figure 1. (A) MIP-2, IL-6, S100A8 and S100A9 mRNA levels in BALF cells were analyzed by conventional RT-PCR after 16 h. (B) The extracted lungs were homogenized and mRNA levels of MIP-2 was analyzed by conventional RT-PCR. After 16 h LPS challenge, BALF cells (C, D) and serum (E, F) were collected to measure the secreted MIP-2 and IL-6 by using each ELISA kit (R&D Systems). Significantly different from LPS alone * $p<0.001$, ** $p<0.005$ and *** $p<0.05$.

4. EC-18 inhibited neutrophil activation via blocking of STAT3 phosphorylation.

The increase of myeloperoxidase (MPO) activity reflects neutrophil accumulation in the lung. MPO activity was substantially increased in LPS treated group and significantly decreased in LPS/EC-18 co-treated group. STAT3 is phosphorylated in inflammatory response and plays a critical role in the inflammation and STAT3 activation was neutrophil dependent. STAT3 was phosphorylated by LPS challenge in the lung and EC-18 downregulated the phosphorylation of STAT3.

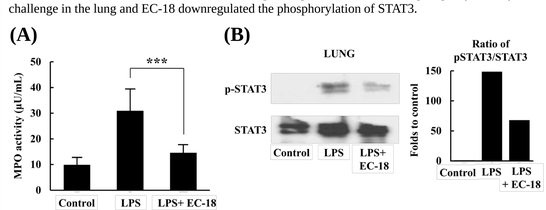


Figure 4. Mice were treated with LPS, LPS plus EC-18 as described in figure 1. (A) Mice were sacrificed 16 h after LPS administration and lungs were homogenized with 0.1 % NP-40 (Sigma). After centrifugation, the supernatants were analyzed by MPO activity assay kit (Abcam). (B) STAT3 phosphorylation was determined by western blot analysis in lung tissues. The primary antibodies were anti-phospho-STAT3 (Cell Signaling) and anti-total STAT3 (Cell Signaling). Result from blots were represented as densitometry analysis. *** $p<0.05$.

5. EC-18 inhibited LPS-induced the recruitment of inflammatory cell into lung.

LPS treatment group was shown the enormous inflammatory cell on lung tissue compare to control group. LPS/EC-18 co-treated mice were significantly attenuated inflammatory cell infiltration in the alveolar space at the control level and showed the normal alveolar morphology. More precisely, the massive amounts of neutrophil transmigration from blood into lung was blocked by EC-18 treatment

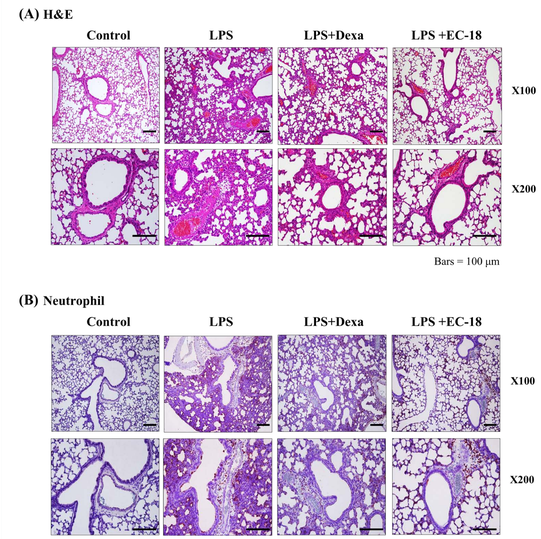
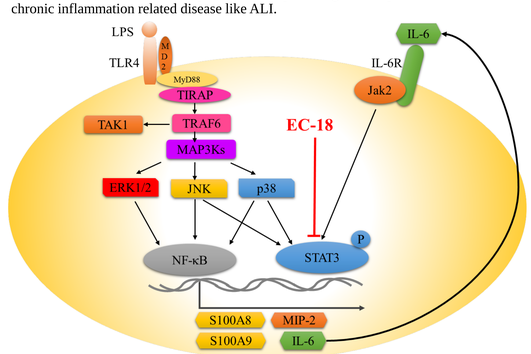


Figure 5. Mice were treated as described in figure 1. Histological examination of lung tissues was performed 16 h after LPS administration. (A) The lung sections were stained with hematoxylin and eosin (B) For immunohistochemical staining, the sections were incubated with 1:100 dilution of primary antibody, rat anti–mouse neutrophil (NIMP-R14, Thermo Fisher Scientific Inc.) at 4°C overnight. The staining indicated neutrophil expressions. Representative images of lung section are presented. Scale bars, 100 μm.

Conclusion

- EC-18 (PLAG) effectively blocked the neutrophil transmigration into the lung alveolar.
- EC-18 could be utilized as a potential therapeutic agent for prevention of acute and chronic inflammation related disease like ALI.



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