Doctor of Philosophy

The role of lipocalin-2 in pneumonia associated acute respiratory distress syndrome

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Department of Medicine

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The role of lipocalin-2 in pneumonia associated acute respiratory distress syndrome

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Abstract

Introduction: Lipocalin-2 (LCN2) is a protein exressed in varius tissues and has a role in iron homeostasis, control of infection, and modulation of inflammation. The role of LCN2 in experimental acute lung injury model and its clinical significance in patients with pneumonia/acute respiratory distress syndrome (ARDS) has not been fully elucidated. The aim of study was to investigate the role of LCN2 using the experimental acute lung injury model and evaluate clinical significance of serum level of LCN2 in patients with pneumonia/ARDS.

Materials and Methods: Twenty-week-old male LCN2 knockout (KO) and wild type (WT) littermates, both in a C57BL/6 background, were used for the experiments. 1 mg/kg lipopolysaccharide (LPS) as experimental group and saline as control were injected through intratracheal method. In 24 hours later, mice were sacrificed and samples including serum, bronchoalveolar lavage fluid (BALF) and lung tissue were obtained. Mortality between LCN2KO and WT was observed along findings of BALF and lung tissue 8 days after intratracheal LPS (5mg/kg) treatment. Patients with pneumonia/ARDS who admitted to medical intensive care unit (MICU) and received invasive mechanical ventilation were included prospectively. Their serum LCN2 level s collected within 24 hours of MICU admission were measured using enzyme-linked immunosorbent assay.

Results: In LPS injected experiment, serum IL-1β and IL-6 levels were lower in LPS treated-LCN2KO mice than in WT. The recruitment of neutrophil and macrophage into alveoli was significantly lower in LPS treated-LCN2 KO mice than WT. In western blotting of lung tissue, the expressions of IL-6, phosphylated STAT3 and NF-κB were lower in LPS-treated LCN2KO mice compared to LPS-treated WT. Iron uptake of macrophages in BALF and lung tissues were lower in LPS-treated LCN2KO mice

compared to LPS-treated WT. 22.2%(2/9) LPS(5mg/kg)-treated LCN2KO mice died during 8-days while all 14 WT survived. Macrophages and neutrophils in BALF and lung were more recruited in LPS-treated LCN2KO mice while activation of NF-κB did not differ.

In 27 patients with pneumonia/ARDS, Median serum LCN2 level was 177.2 ng/ml [Interquartile range (IQR), 108.5-306.9 ng/ml], which was significantly higher than 23 normal controls [44.8 ng/ml (IQR, 30.2-64.1 ng/ml), *P* <0.001]. Median serum LCN2 level was significantly lower in patients with pneumonia/ARDS than that of non-pneumonia/ARDS [263.5 ng/ml (233.5–469.6 ng/ml), *P*=0.044]. In 27 patients with pneumonia/ARDS, serum levels of LCN2 in 14 patients with acute kidney injury (AKI) was significantly higher than 13 those without AKI [284.7 ng/ml (IQR, 171.2-427.1ng/ml) *vs* 122.8 ng/ml (IQR, 72.6-176.2 ng/ml), *P*=0.003]. There was significantly higher LCN2 levels in 16 patients with shock than those without. [284.7 ng/ml (IQR, 124.7-413.2 ng/ml) *vs* 153.5 ng/ml (IQR, 69.6-177.2ng/ml) , *P*=0.009]. Serum LCN2 level was positively correlated to SOFA score (*r*=0.467,*P*<0.014) and procalcitonin (*r*=0.523, *P*=0.007). There was no significant difference between non-survivors and survivors at 30-day mortality [255.8 ng/ml (IQR, 135.4-404.8 ng/ml) in 10 non-survivors *vs* 168.7 ng/ml (IQR, 108.1-282.2 ng/ml) in 17 survivors, *P*=0.282]. Area under the receiver operating characterstics for LCN2 to predict 30-day mortality was 0.629 [95% confidence interval (CI), 0.423-0.806, *P*=0.279].

Conclusions: LCN2 deficiency induce less inflammatory reponse at 24 hours time point after intratracheal LPS treatment. At 8 days time point, its deficiency recruit more macrophages and neutrophils and induce more inflammation response in lung and contributes to substantial mortality without NF-κB activation. Serum LCN2 is associated with organ dysfunction in patients with pneumonia/ARDS but does not predict 30-day mortality.

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Introduction

Acute respiratory distress syndrome (ARDS) is a devastating condition developing in patients with sepsis and contributes to a substantial mortality[1-4]. ARDS is a common condition to admit intensive care unit (ICU) and require invasive mechanical ventilation [5]. High mortality still remains unresolved despite of advances in critical care and research [6]. Pneumonia is known as the common risk factor for development of ARDS[5]. The key pathogenesis of ARDS is multiple and complex inflammatory responses of alveoli caused by various insults. Epithelial, immune and endothelial cells are involved in these inflammatory processes by releasing diverse inflammatory cytokines and chemokines [7,8].

Lipocalin-2 (LCN2) is a 25-kDa protein and also known as neutrophil gelatinase-associated lipocalin (NGAL), oncogene 24p3 or siderocalin[9]. LCN2 has been reported to have pleomorphic effects on infection, inflammation, or metabolism. it is upregulated in multiple tissues such as liver[10], gastrointestinal[11] or lung [12] as well as secreted from immune cells (neutrophils and macrophages). In bacterial infection, LCN2 binds to siderophore produced by bacteria and prevents host's iron to use to grow bacteria [12,13]. In addition to role of sequestrating siderophores, LCN2 modulates immune cells by release of cytokines or chemokines, migration into infected site or maturation of neutrophils in infection [14]. Elevated levels of LCN2 was associated with sepsis induced kidney injury[15-19] and pneumonia[20,21]. Upregulation of LCN2 in infectious conditions render it available both as a diagnostic biomarker and as a prognostic indicator at the range of critical illness. Kangelaris *et al* reported that the expression of gene and protein of LCN2 was risen in patients with early sepsis induced ARDS [22]. Clinical implication of serum LCN2 in sepsis associated ARDS and the roles of LCN2 in direct acute lung injury murine model still remain to be more elucidated.

We investigate role of LCN2 on the experimental acute lung injury model and

evaluated the clinical significance of serum LCN2 in patients with pneumonia/ARDS.

Material and Methods

Experimental acute lung injury mouse model

All mice were maintained in the animal facility at Gyeongsang National University (GNU). The experiments were performed in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animals. The University Animal Care Committee for Animal Research of GNU approved the study protocol (GNU-160530-M0025). Female and male LCN2-heterozygous mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). These mice were intercrossed and animals homozygous for LCN2 knock-out (KO). After genotyping at 5 weeks of age, mice were randomly divided into four groups (n = 10 per group). Twenty-week-old male LCN2 KO and wild-type (WT) littermates, both in a C57BL/6 background, were used for the experiments. Lipopolysaccharide (LPS, 1mg/kg) and saline as control were injected in intratracheal injection method. 24 hours later, the animals were sacrificed and samples including serum, BAL fluid and lung tissue were obtained. In addition, LPS (5mg/kg) and saline as control were injected in intratracheal injection method and observed until 8 days to investigate the rate of mortality.

Sample preparation

All experimental mice were anesthetized with zoletil (5 mg/kg, Virbac Laboratories, Carros, France). Blood samples were extracted from left ventricle and centrifuged. After removal of blood, bronchoalveolar lavage (BAL) was performed for measurement of cytokines and cytological analysis. For protein extraction, both lungs were isolated and frozen in liquid nitrogen and stored at -70℃. For histologic examination, lungs were slowly perfused with 4% paraformaldehyde and immersed in fixative solution overnight. Lungs were embedded in paraffin, and 5 μm sections were stained with H&E. The slides were evaluated by microscopy.

Bronchoalveolar lavage (BAL)

Twenty-four hours after LPS or saline instillation, mice were anesthetized with zoletil (5 mg/kg, Virbac Laboratories). BAL fluid (BALF) was collected by instillation and suction of 0.9% normal saline (2 x 0.8 ml) in the lungs via a tracheal cannula. The BAL fluid (BALF) was centrifuged and supernatants were collected and stored at -70℃ until used. The cell pellet was used to prepare cytospin slides for evaluation of the cells that are present in the pulmonary air spaces. The pellet was centrifuged to generate cytospin praparations (Shandon Cytospin 3 Cyto-centrifuge, Fisher Scientific, Erembodegem, Spain) of BALF cells. After air-drying, the slides were stained using Diff-Quick. Cell types in the BALF cytospins were morphologically characterized under a light microscope.

Measurement of serum and BALF cytokines level

Serum and BALF cytokines was measured using mouse IL-1β, IL-6, IL-10, LCN2, and human LCN2 (R&D Systems, MN, USA) enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' protocols.

Iron staining

BALF and lung section slides were stained with Prussian blue (Iron Stain Kit, ab150674, Abcam, Cambridge, MA, USA). The slides were visualized under a light microscope (BX51 Olympus, Tokyo, Japan), and digital images were captured and documented.

Immunohistochemistry

Deparaffinized sections from lung sections and BALF cytospin slides were placed in a solution of 0.3% H_2O_2 for 10 min. After washing, sections were treated with diluted blocking serum for 20 min. Slides were incubated overnight at 4°C in a humidified

chamber with primary antibodies (Table 1) diluted in blocking serum. Slides were incubated for 1 h at room temperature with a secondary biotinylated antibody (1:200). After washing, sections were incubated in avidin-biotin-peroxidase complex solution (ABC solution, Vector Laboratories, Burlingame, CA, USA). Sections were developed with 0.05% diaminobenzidine (DAB, Sigma) containing 0.05% H_2O_2 and were dehydrated through graded alcohols, cleared in xylene, and coverslipped with Permount (Sigma). Slides were visualized under a BX51 light microscopy (Olympus).

Double immunofluorescence

BALF cytospin slides were placed in a solution of 0.3% H_2O_2 for 10 min. After washing, sections were treated with diluted blocking serum for 20 min. Slides were incubated overnight at 4°C in a humidified chamber with primary antibodies: Ly6G (neutrophil marker) and F4/80 (macrophage marker) (Table 1) diluted in blocking serum. After washing three times with 0.1 M PBS, sections were incubated with Alexa Fluor 488-conjugated donkey anti-rabbit and 594-conjugated donkey anti-rat antibody (1:1,000, Invitrogen, Carlsbad, CA, USA). Nuclei were stained with DAPI (1:10,000; Invitrogen). The images were visualized under a BX51-DSU microscope (Olympus).

Western blot analysis

Lung tissues were cut into slices and these slices were transferred to sterile 1.5 mL microcentrifuge tubes containing 400 μL of sample buffer (7 M urea, 2 M thiourea, 4% Triton X-100, 25 mM DTT, 0.5% IPG buffer, protease inhibitor cocktail). Homogenized tissues were incubated for 1 h at 4°C, then sonicated 10 times at the interval setting 0.3. Next, samples were centrifuged for 20 min at 14,000 rpm at 4°C. Supernatants of each group were pooled and transferred to clean vials. Total extracts

and nuclear fractions were prepared from the right lung according to the protocol described by Müller et al. Lysate protein concentration was determined using bovine serum albumin (BSA) standards and a bicinchoninic acid (BCA) kit (Pierce/Thermo Scientific, Rockford, IL, USA). Equal amounts of protein (30 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were washed in Tris-buffered saline containing 0.5% Tween-20 (TBST) and incubated with the following TBST-diluted primary antibodies (Table 1). The samples were then incubated with their corresponding secondary antibodies. An enhanced chemiluminescence (ECL) western blot analysis system (Amersham Pharmacia Biotech/GE Healthcare, Piscataway, NJ, USA) was used for detection. Band densities were normalized to β-actin and p84, which were used as internal control for cytosolic and nuclear extracts, respectively.

Patients

Patients with sepsis admitted to medical ICU were prospectively screened. Sepsis-3 definition was met for all of them[1]. Among them, patients with pneumonia/ARDS meeting for the Berlin definition were finally included[2]. Their serum samples were collected within 24 hours of MICU admission. Blood samples were collected from which serum and leukocytes were separated and stored at -80°C. The study protocol was approved by the institutional review board of Gyeongsang National University Hospital, and written informed consent was obtained from all participants or their family (2017-09-022).

Clinical data regarding baseline and clinical characteristics of them were analyzed. Their laboratory data including inflammatory markers were also reviewed. Baseline [age, gender, body mass index, comorbidities] and clinical characteristics (presence of shock, acute kidney injury, and management) were assessed. The severities of illness acute physiology and chronic health evaluation (APACHE) II score, and sequential organ failure assessment score were noted. Additionally, the data of

various laboratory parameters [white cell count, hemoglobin, platelet, C-reactive protein, albumin, procalcitonin, partial pressure of carbon dioxide, and partial pressure of oxygen/fractionated inspired oxygen] were collected. Moreover, clinical outcomes (ICU and 30-day mortality) were analyzed.

Statistical analysis

Categorical variable are expressed as and compared with Chi-squared or Fisher's exact tests. Continuous variables are presented as median and interquartile range or as mean ± standard deviation, unless indicated otherwise, and compared with Mann-Whitney U or Student's t-tests. All tests of significance were two-tailed. A *P*-value of 0.05 was considered statistically significant. All data were analyzed with SPSS software version 18.0 (SPSS Inc, Chicago, IL, USA) and GraphPad 5.1 Prism software (San Diego, CA, USA).

Table 1. List of primary antibodies.

Antibody	Company	Catalog No.	Dilution	Applications	Source
F4/80	Santa Cruz	sc-377009	1:500 1:200	WB, IF	Rabbit
Ferritin	abcam	Ab75973	1:2,000	WBC	Rabbit
HO-1	Stressgen	SPA-895	1:1,000	WB	Rabbit
IL-6	Santa Cruz	sc-57315	1:1,000	WB	Mouse
LCN2	R&D	AF3508 AF1857	1:1,000, 1:200	WB, IHC	Goat
24p3R	millipore	ABC846	1:1,000	WB	Rabbit
Ly6G	abcam	ab25377	1:200	IHC	Rat
p-STAT3	Cell signaling	#9131	1:1,000	WB	Rabbit
STAT3	Cell signaling	#9139	1:1,000	WB	Mouse
NF-κBp65	Santa Cruz	sc-8008	1:1,000	WB	Mouse
p84	abcam	ab487	1:3,000	WB	Mouse
β-actin	Sigma	T5168	1:5,000	WB	Mouse

WB, western blot; IF, immunofluorescence; IHC, immunohistochemistry

Results

1. Experimental acute lung injury model

1-1. Intratracheal LPS injection up-regulates LCN2 expression in serum and lung tissue

To investigate up-regulation of LCN2 under intratracheal LPS injection, we measured and compared LCN2 levels between KCN2KO mice and WT using serologic, western blotting and immunohistochemical staining methods at 24 hours after intratracheal LPS injection. Levels of serum LCN2 increased significantly in WT mice and LCN2 was not detected in LCN2KO (Figure 1A). In lung tissue, amount of LCN2 and 24p3r, receptor for LCN2 was significantly more elevated in WT mice while that of LCN2KO mice was little identified using western blotting (Figure 1B). We also evaluated overall expression of LCN2 in alveoli and bronchiole through immunohistochemistry using anti-LCN2 antibody. As revealed by immunohistochemistry, LCN2 was more expressed around and within alveoli including neutrophils of WT mice but no expression was shown LCN2KO mice (Figure 1C). In bronchial epithelium (Figure 1D), LCN2 was strongly stained in WT and no staining was observed in LCN2KO mice.

Taken together, these findings indicate that LCN2 are early and well induced in blood and lung tissue including alveoli and bronchial epithelium at 24 hours time point by intratracheal LPS treatment.

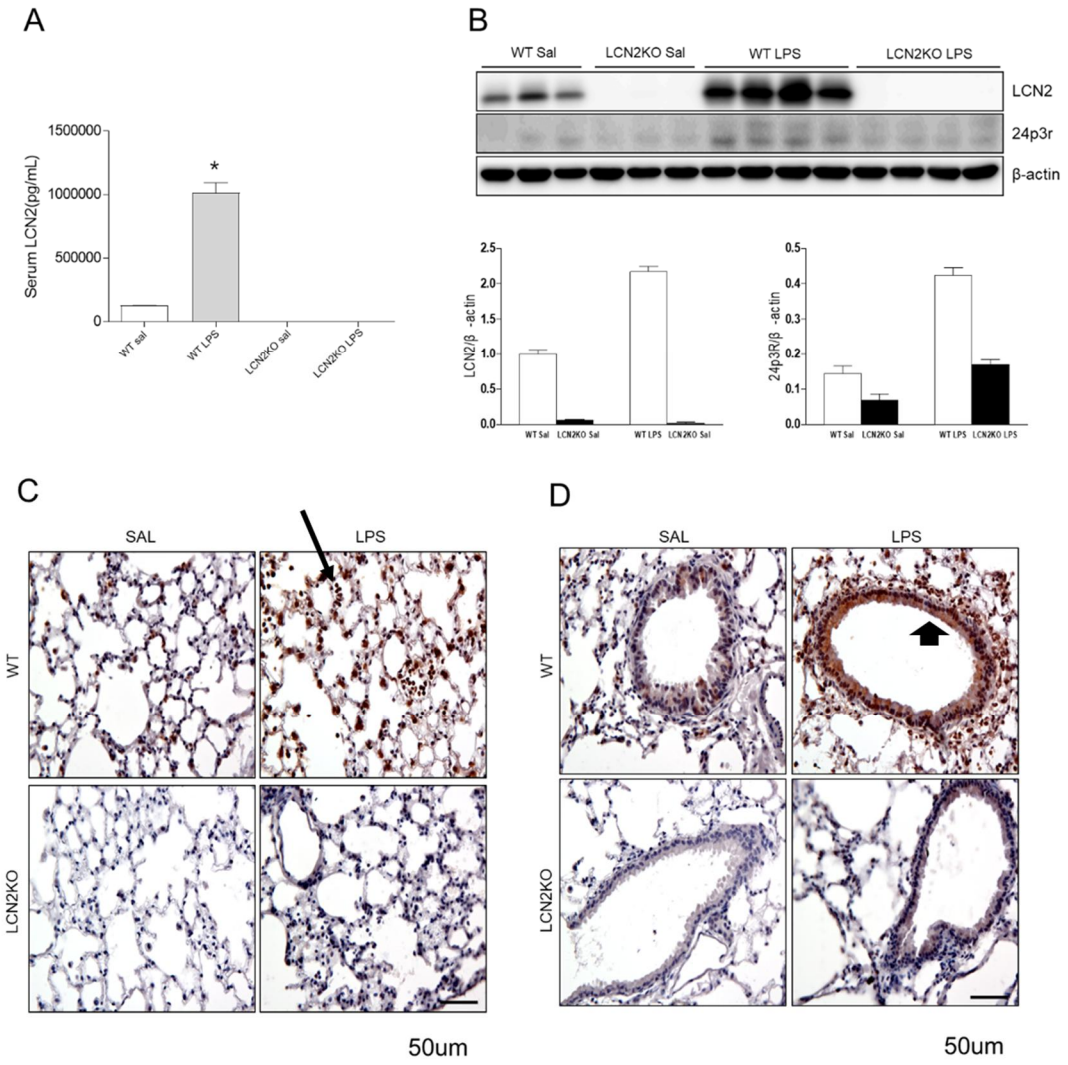

Figure 1. Effect of LPS on circulating and lung LCN2 levels in WT and LCN2KO mice. (A) Serum and (B) western blotting and quantitative analysis of LCN2 protein in the lung of LPS-treated WT and LCN2KO mice. Representative immunohistochemistry for LCN2 protein in the alveoli (C) and bronchus (D) levels. Thin arrow and thick arrow indicates neutrophil and bronchial epithelium expressing LCN2, respectively. β-actin was used as a loading control. Scale bar = 50 ㎛. Results are presented as the mean ± S.E.M. **P* < 0.05 vs. saline-treated WT mice.

1-2. Serum levels of IL-1β and IL-6 are reduced *in serum, but not BALF in intratracheal LPS acute lung injury.*

To evaluate the effect of LCN2 on production of cytokines in serum and BALF after intratracheal LPS treatment, we measured TNF-α, IL-1β and IL-6 in serum and BALF in WT and LCN2KO mice after LPS and saline as control, respectively. In serum (Figure 2A, C, E), IL-1β and IL-6 were less induced in LCN2KO mice than WT treated with intratracheal LPS. In BALF (Figure 2B, D, F), TNF-α and IL-6 levels were significantly increased in WT with LPS than WT with saline. However, the difference of elevation of these cytokines was not observed between LCN2KO and WT mice treated with LPS.

This finding suggests that LCN2 deficiency induce less production of serum pro-inflammatory cytokines at 24 hour time point after intratracheal LPS treatment.

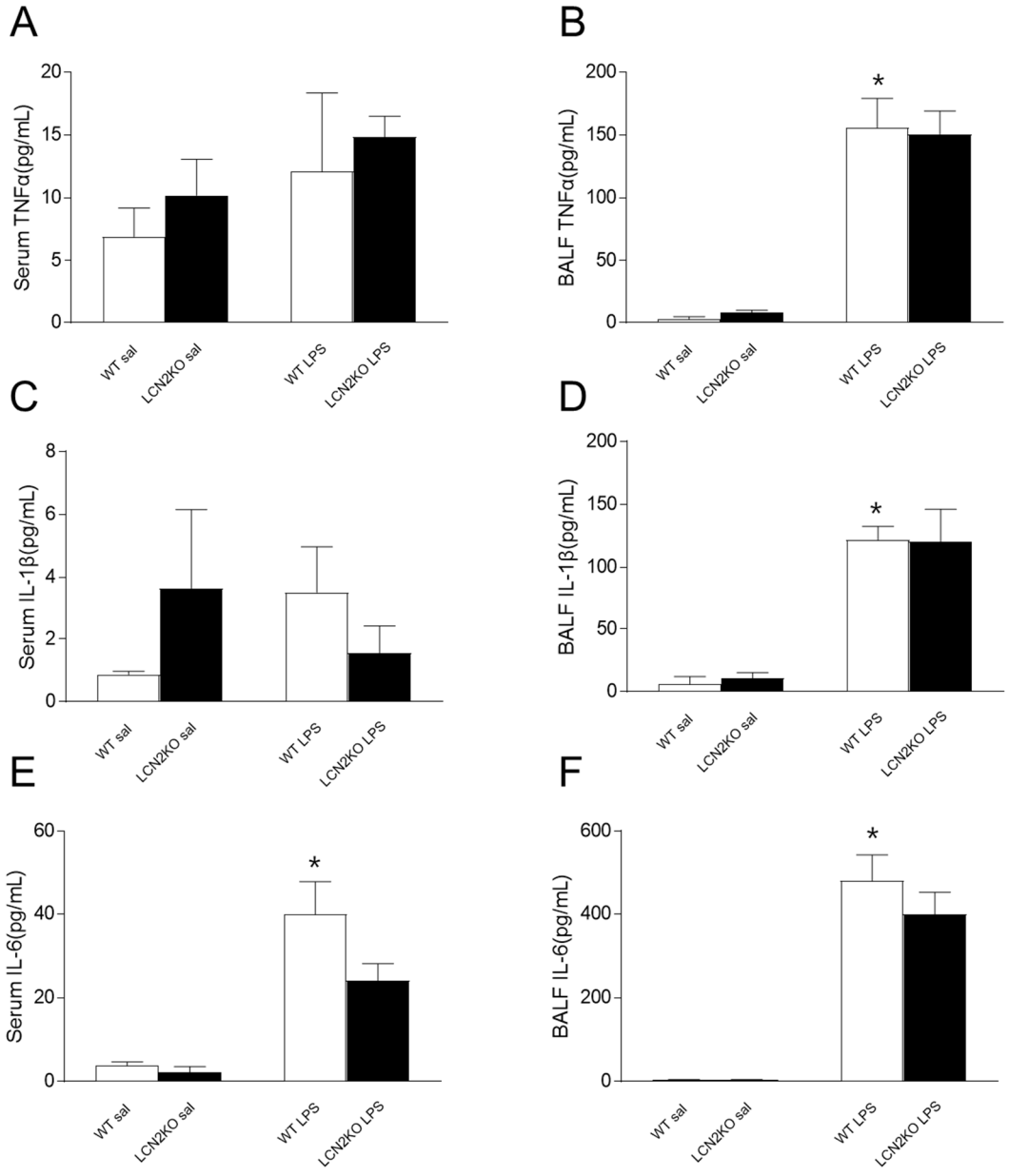

Figure 2. Effect of LPS on proinflammatory cytokines in serum and BALF of WT and LCN2 KO mice. Levels of cytokines in serum and BALF were measured and compared between LCN2KO and WT mice treated with LPS or saline as control, respectively. Induction of serum IL-1β and IL-6 were less in in LCNKO mice compared to those of WT with LPS (Figure C, E). In BALF, Levels of TNF-α, IL-1β, IL-6 were highly induced in WT treated with LPS than those with saline. Cytokines levels in BALF did not differ between LCN2KO and WT mice treated with LPS. Results are

presented as the mean±S.E.M. *P < 0.05 vs. saline-treated WT mice.

1-3. The recruitment of neutrophils and macrophages into lung are decreased in LCN2KO mice with intratracheal LPS treatment.

To examine the effect of LCN2 on recruitment of macrophages and neutrophils in BALF and alveoli, we firstly performed Diff-quick staining in BALF (Figure 3A) and added immunocytochemistry using antibody for Ly6G (lymphocyte antigen 6 complex locus G6D), which is a marker for neutrophils (Figure 3B). Ly6G was more stained in WT mice than LCN2KO mice treated with intratracheal LPS. This finding indicates that LCN2 plays on role in recruiting macrophages and neutrophils into lung when lung is damaged by intratracheal LPS instillation at 24 hours time point.

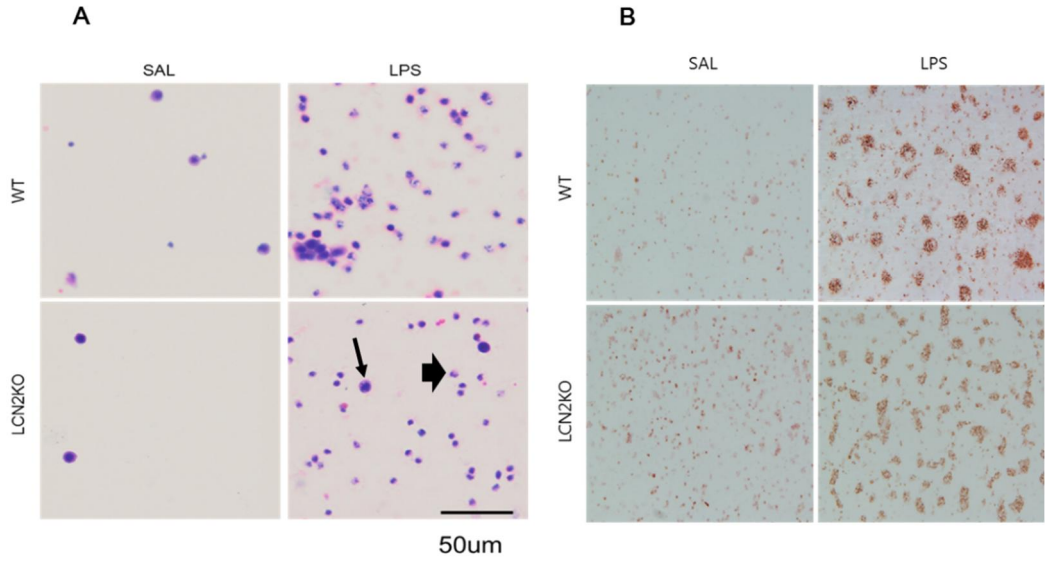

Figure 3. Effect of LCN2 deficiency on recruitment of macrophages and neutrophils in the BALF of WT and LCN2KO mice 24 hours after intratracheal LPS treatment. The recruitment of macrophages and neutrophils is less in LCN2KO with LPS than WT (A). Staining of Ly6G, marker for neutrophil is less prominent in LCN2KO with LPS than WT (B). Thin arrow and thick arrow indicates macrophage and neutrophil, respectively. Representative images of Diff-quick stained cells (A) and

immunocytochemistry for Ly6G (B) in BALF. Scale bar = 50 or 200㎛.

To examine difference of recruitment of macrophages in lung tissue, we observed lung microscopically after H&E staining and counted macrophages in lung (Figure 4 A, B). We found that numbers of macrophages were decreased in LCN2KO mice than WT at 24 hours time point after intratracheal LPS treatment. To test whether recruitment of macrophages in lung was different between LCN2KO and WT mice as shown in neutrophils, western blotting for F4/80 protein, well-known as a marker for macrophage, was performed. The expression of F4/80 protein was lower in LCN2KO mice than that of WT with intratracheal LPS treatment (Figure 4C). To clarify the recruitment of macrophages and neutrophil more specifically, we perfomed immunofluorescence (Figure 5). At immunofluorescence, Ly6G, marker for neutrophil and F4/80, marker for macrophage was less brightened in LCN2KO mice compared to WT 24 hours after intratracheal LPS treatment.

Taken together, these findings indicate that LCN2 has a role to recruit neutrophils and macrophages into lung at 24 hours time after intratrcheal LPS treatment.

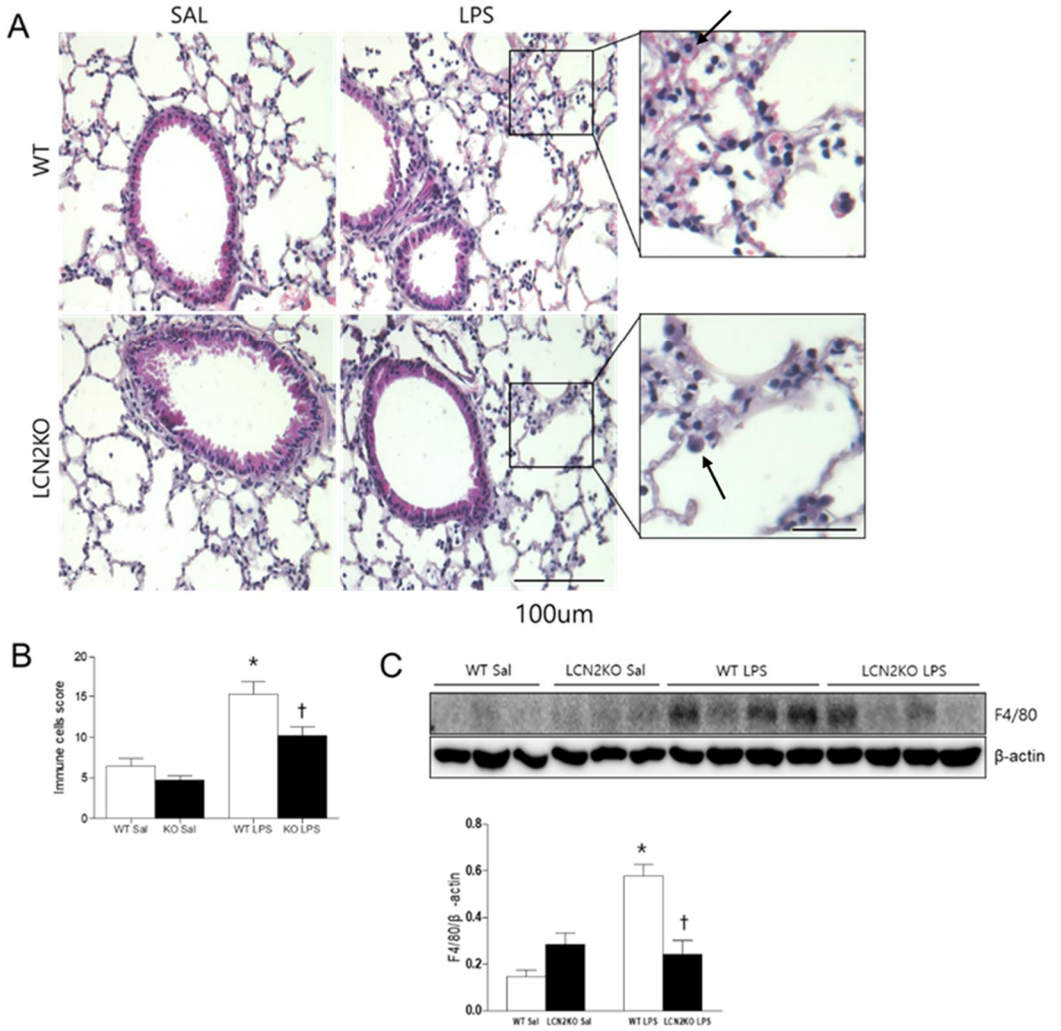

Figure 4. Effect of LCN2 deficiency on macrophage infiltration in the lung of WT and LCN2KO mice 24 hours after intratracheal LPS treatment. (A) and (B), macrophages are less observed and numbers of it are decreased in LCN2KO mice compared to WT 24 hours after intratracheal LPS treatment. (C), F4/80, marker for macrophage are decreased in LCN2KO mice compared to WT. (A) Representative H&E-stained lung sections. (B) The number of macrophages in the alveoli. (C) Western blot and quantitative analysis for F4/80, a marker for macrophage. Arrow indicates macrophage. β-actin was used as a loading control. Results are presented as the mean±S.E.M. *P < 0.05 vs. saline-treated WT mice. †P < 0.05 vs. LPS-treated WT mice. Scale bar = 100㎛.

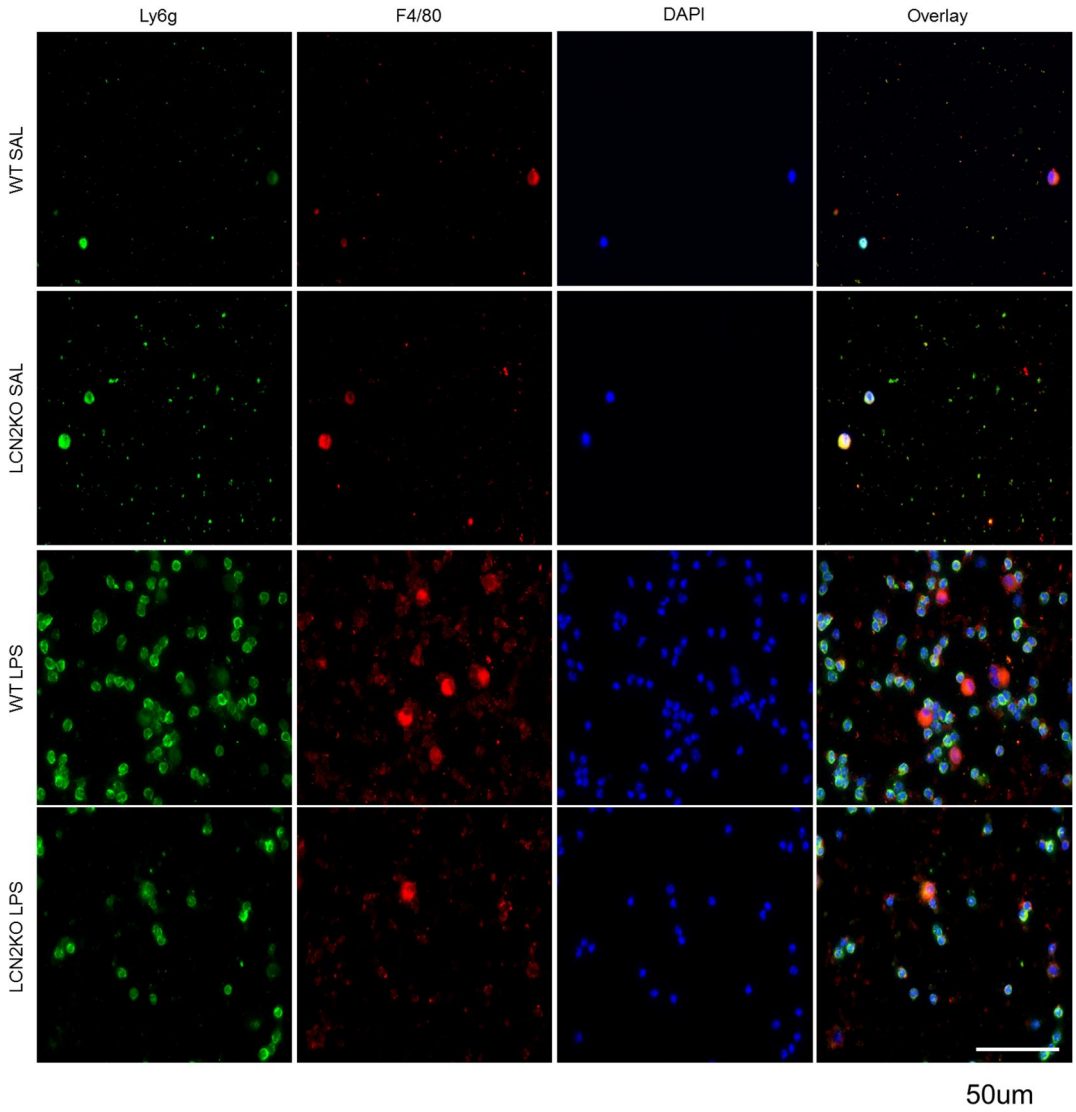

Figure 5. Effect of LCN2 deficiency on neutrophil and macrophage infiltration in the BALF of LPS-treated WT and LCN2KO mice. Representative images of **i**mmunofluorescence for Ly6G and F4/80. Ly6G for neutrophil and F4/80 for macrophage were less stained in LCN2KO mice than WT at 24 hour time point after intratracheal LPS treatment. Scale bar = 50㎛.

1-4. LCN2 deficiency induce less expression of nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) *in lung tissue.*

LPS stimulates toll-like receptor 4 in epithelial cells and immune cells and then down-

stream pathway is activated including NF-κB-signal transducer and STAT3 loop. Both NF-κB and STAT3 loop induce LCN2 production. To examine the effect of LCN2 on NF-κB and IL-6-STAT3 signals, we investigated IL6-STAT3 and NF-κB activation in lung tissue using western blotting (Figure 6). The expressions of IL-6, STAT3 and phospholylated STAT3 was lower in LCN2KO mice than those of WT at 24 hours time point after intratracheal LPS treatment. Regarding NF-κB activation (Figure 7), The expressions of total and nuclear NF-κB were lower in LCN2KO mice than WT mice at 24 hours time point after intratracheal LPS treatment.

Taken together, these findings indicate that LCN2 deficiency less activates NF-κB and IL-6/p-STAT3 signaling pathway in lung at 24 hours time point after intratracheal LPS treatment.

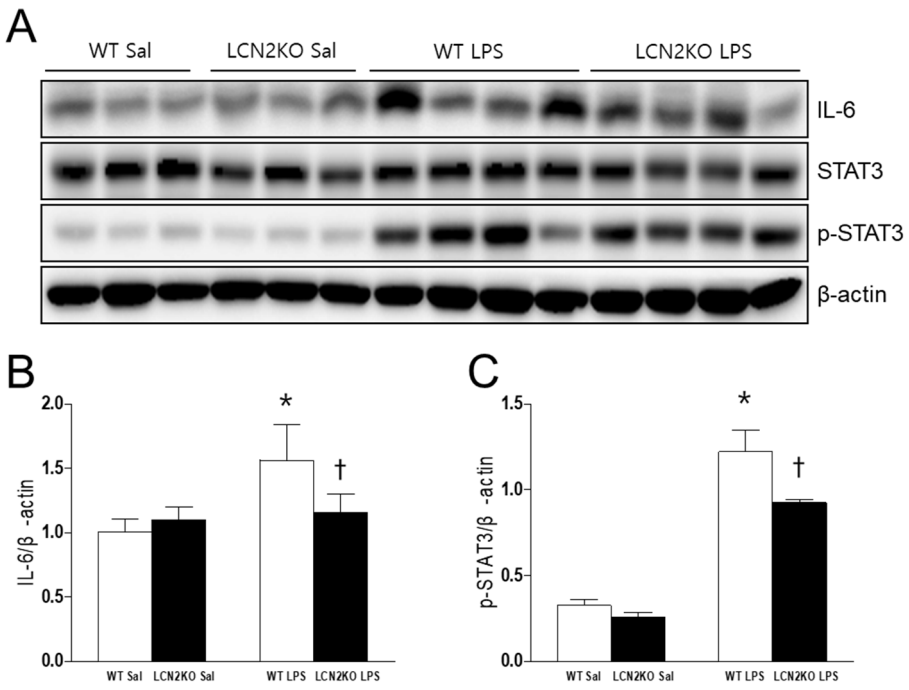

Figure 6. Effect of LCN2 deficiency on IL-6/phospholylated STAT3 signaling pathway in the lung of LPS-treated WT and LCN2KO mice. Western blotting (A) and quantitative analysis for IL-6 (B) and p-STAT3 (C). Western blotting shows that expressions of IL-6, STAT3 and p-STAT3 were decreased in LCN2KO mice compared to WT at 24 hour time point after intratracheal LPS treatment (A). In

quantitatie analysis, IL-6 (B) and p-STAT3 (C) were lower in LCN2KO than WT. β-actin was used as a loading control. Results are presented as the mean ± S.E.M. *P < 0.05 vs. saline-treated WT mice. †P < 0.05 vs. LPS-treated WT mice.

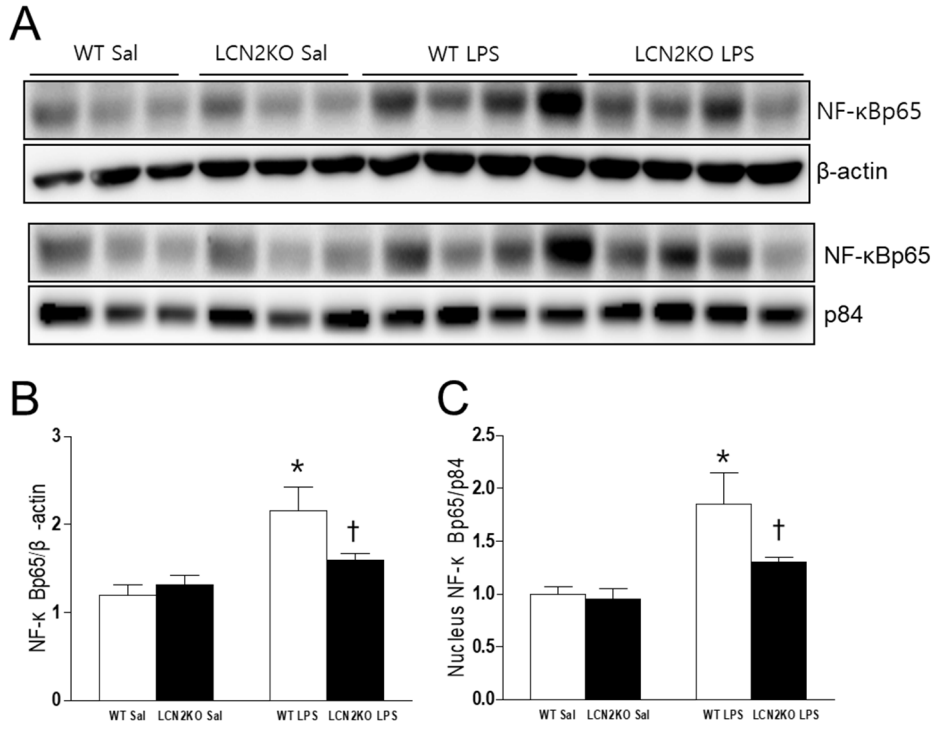

Figure 7. Effect of LCN2 deficiency on NF-κBp65 expression in the lung of LPS-treated mice. Western blotting (A) and quantitative analysis for total NF-kB (B) and nuclear NF-κB (C). Expressions of total and nuclear NF-κB are lower in LCN2KO mice compared to WT at 24 hour after intratracheal LPS treatment. β-actin and p84 were used as loading controls. Results are presented as the mean ± S.E.M. *P < 0.05 vs. saline-treated WT mice. †P < 0.05 vs. LPS-treated WT mice.

1-5. Expression of Heme oxygenase (HO)-1 is decreased in LCN2KO mice.

To examine the effect of LCN2 on expression of HO-1, a rate limiting enzyme for heme metabolism, we perfomed western blotting for HO-1 (Figure 8). The expression of HO-1 was significantly lower in LCN2KO mice than WT at 24 hours time point after intratracheal LPS treatment. In addition, we also evaluated the expression of ferritin,

which stores iron and act as acute phase protein in infection. Expression of ferritin was significantly higher in mice with LPS than control swith saline. There was no significant difference of expression of ferritin between LCN2KO and WT mice at 24 hours time point after intratracheal LPS instillation.

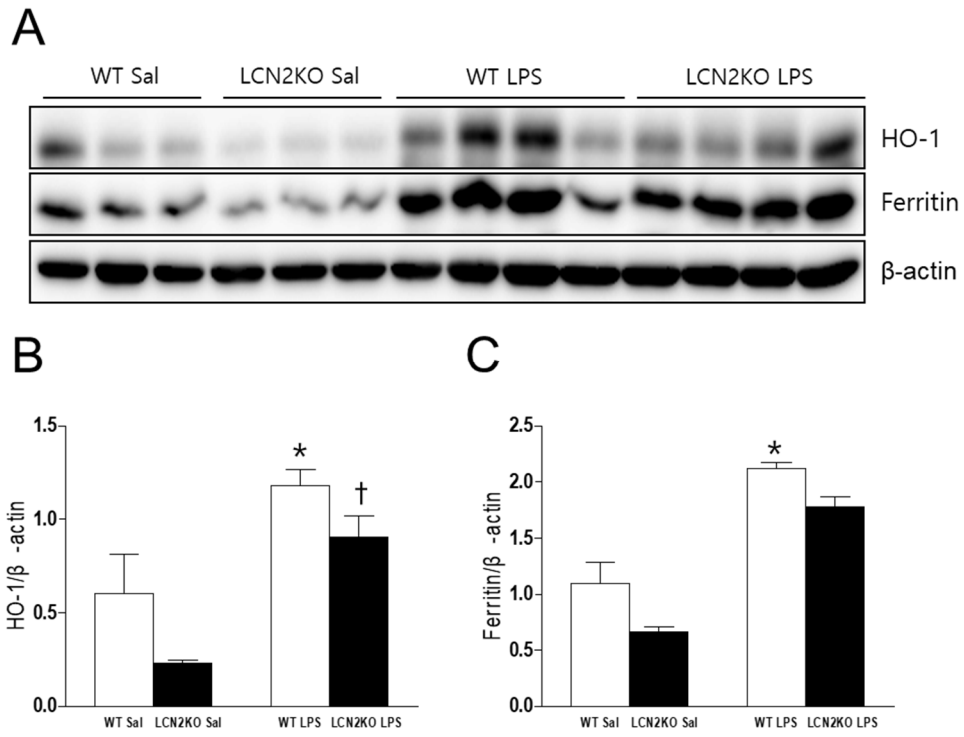

Figure 8. Effect of LCN2 deficiency on HO-1 and ferritin expressions in the lung of LPS-treated WT and LCN2KO mice. Western blotts (A) and quantitative analysis of expression HO-1 (B) and ferritin (C) in lung tissues. Expressions of HO-1 are decreased in LCN2KO mice compared to WT at 24 hour after intratracheal LPS treatment (A, B) while expression of ferrin was not significantly different (A,C). β-actin was used as a loading control. Results are presented as the mean ± S.E.M. **P* < 0.05 vs. saline-treated WT mice. †*P* < 0.05 vs. LPS-treated WT mice.

1-6. Iron uptake of macrophages is reduced in LCN2KO mice.

To exaimine the effect of LCN2 on iron uptake of alveolar macrophage in acute lung injury, we perfomed iron staining on macrophages from BALF and lung tissue (Figure 9). In both BALF (Figure 9A) and lung tissue (Figure 9B), The degree of iron staining within macrophages was lower in LCN2KO mice than WT at 24 hours time point after intratracheal LPS treatment. We also compared the number of iron uptake macrophages and the proportion of iron uptake macrophages among total macrophages in lung. Total number and proportion of iron uptake macrophages were lower in LCN2KO mice than WT (Figure 9C,D).

These finding indicates that the uptake of iron is less induced in macrophage in LCN2KO mice than WT under acute lung injury at 24 hours time point after intratracheal LPS treatment.

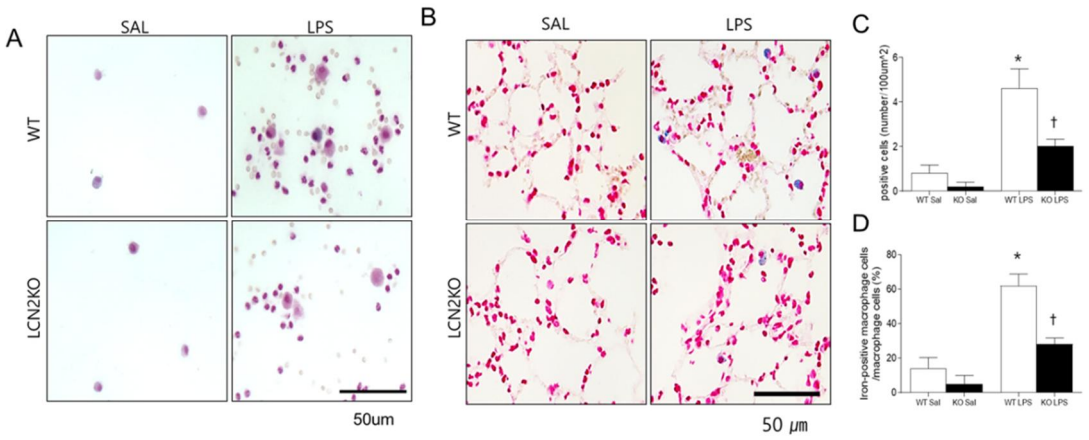

Figure 9. Effect of LCN2 deficiency on iron-positive macrophage infiltration in the BALF and lung of LPS-treated WT and LCN2KO mice.

(A), in BAL, number of iron-containing macrophages and degree of iron staining were decreased in LCN2KO mice compared to WT at 24 hour after intratracheal LPS treatment. (B, C, D), in alveoli, number of iron-containing macrophages and degree of iron staining were also decreased in LCN2KO mice compared to WT at 24 hour after intratracheal LPS treatment. Representative iron staining images in BALF (A)

and alveoli (B). Cell count for positive iron staining (C, D). Results are presented as the mean ± S.E.M. *$P < 0.05$ vs. saline-treated WT mice. †$P < 0.05$ vs. LPS-treated WT mice. Scale bar = 50㎛.

1-7. Persistent LCN2 deficiency increases mortality rate at 8-day after intratracheal LPS treatment

Based on less induction of pro-inflammatory cytokines, recruitment of neutrophils and macrophages in LCN2KO mice at 24-hour after intratracheal LPS treatment, we hypothesize that the early reduced inflammatory response under LCN2 deficiency improves survival in LCN2KO mice. To prove our hypothesis, we gave 5mg/kg LPS through intratracheal method in LCN2KO (n=9) and WT mice (n=14) to observe difference of mortality until 8 days period. Two of nine (22.2%) LCN2KO mice died during 8-day period while all WT mice survived at 8 days period after intratracheal LPS treatment (Figure 10).

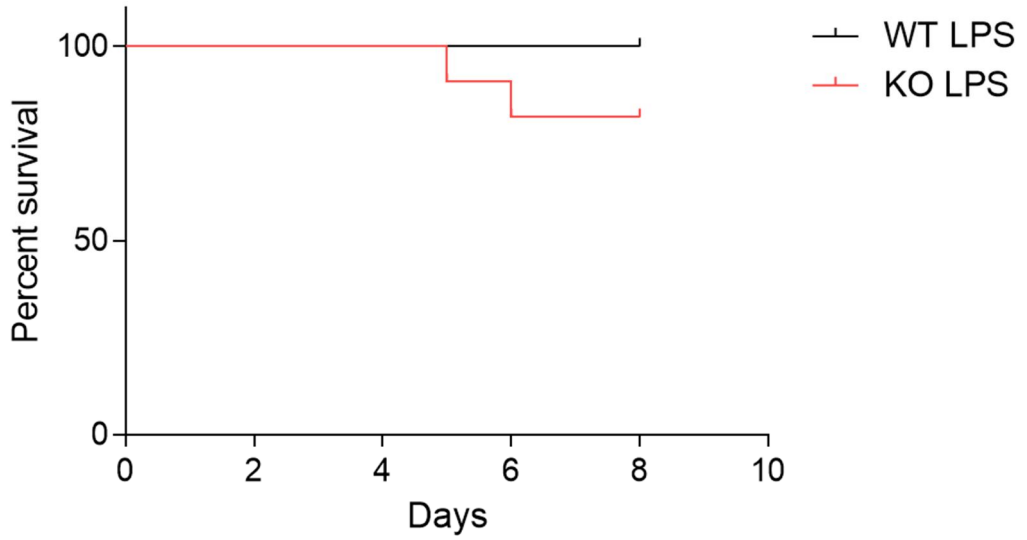

Figure 10. Effect of LCN2 deficiency on survival at 8 days in LPS (5mg/kg)-treated WT and LCN2KO mice. After intratracheal LPS (5mg/kg) instillation, 22.2% LCN2KO mice died 5 days later while WT survive until 8 days.

1-8. Persistent LCN2 deficiency is associated with more inflammation and recruitment of macrophages and neutrophils but does not induce more expression of IL-6 and NF-κB signaling pathway in lung tissue.

To elucidate the mechanism of different mortality in LPS-treated LCN2KO mice compared to WT, we firstly investigated whether the recruitment of macrophage and neutrophil in BALF at 8 days was different compared to 24 hours finding of less recruitment after intratracheal LPS treatment. In BALF, more macrophages and neutrophils were recruited in LCN2KO mice compared to WT at 8 days after intratracheal LPS treatment (Figure 11A). We also performed immunofluorescence of to observe recruitment of macrophage and neutrophil in BALF. Immunofluorescent staining of both Ly6g, marker for neutrophil and F4/80, marker for macrophage were more enhanced in LCN2KO mice compared to WT at 8days after intratracheal LPS treatment (Figure 11B). In addition, we observed the microscopic changes of lung and quantitive expression of IL-6 and NF-κB using western blotting. There was more inflammatory change and macrophage infiltration in alveoli in LCN2KO mice than WT (Figure 12A). There was no significantly difference of expression of IL-6 and NF-κB between LCN2KO mice and WT at 8 days after intratracheal LPS treatment (Figure 12B).

Taken together, these findings indicate that LCN2 deficiency induces more recruitment of macrophages and neutrophils into lung at 8 days without different activation of IL-6 and NF-κB signaling pathway. Other activating pathway contributiong to recruitment of more macrophages and neutrophils with inflammation and death in LCN2KO mice are needed to investigate in LPS-treated intratracheal lung injury model.

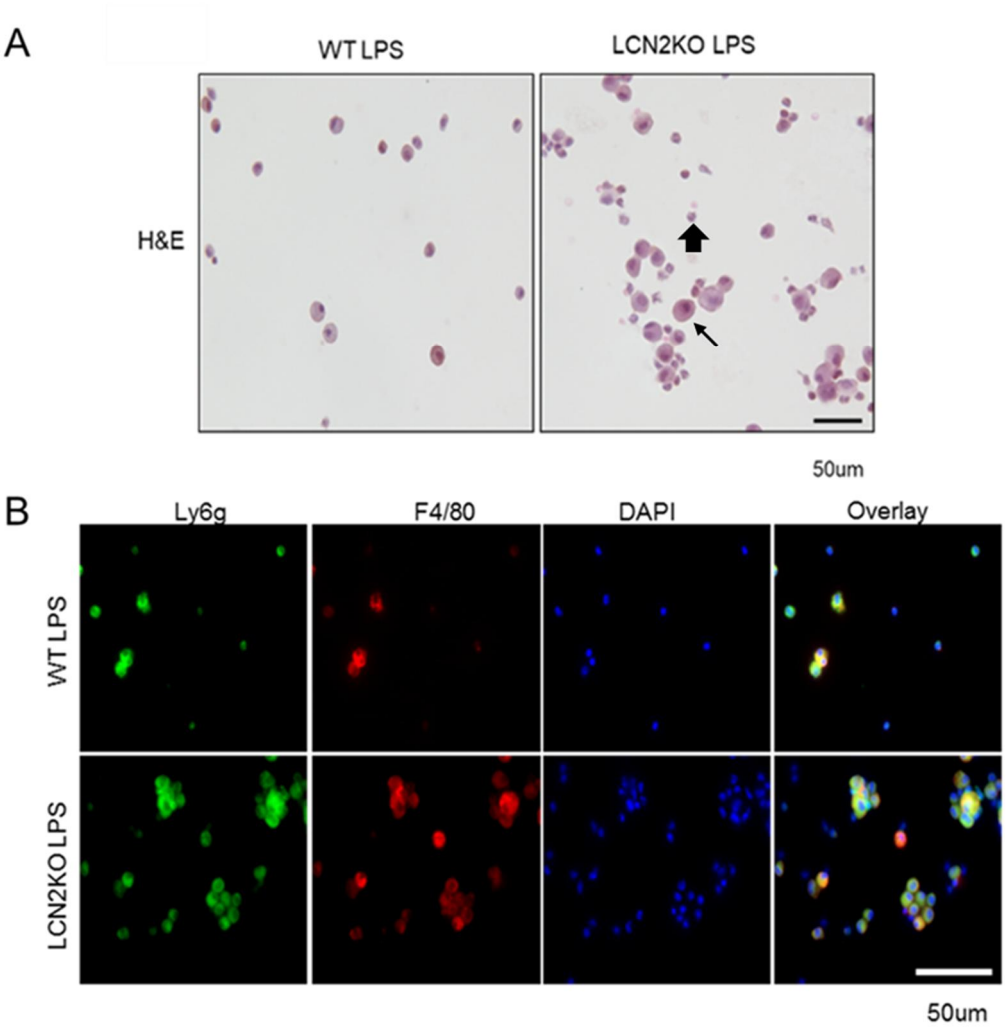

Figure 11. Effect of LCN2 deficiency on recruitment of neutrophil and macrophage in the BALF of WT and LCN2 KO mice 8 days after intratreacheal LPS (5mg/kg) treatment. In H&E staining (A), more macrophages and neutrophils were recruited in LCN2KO mice compared to WT 8 days after intratracheal LPS (5mg/kg) treatment. At immunofluorescence (B), Ly6g, marker for neutrophil and F4/80, marker for macrophages were prominently stained in LCN2KO mice compared to WT8 days after intratracheal LPS (5mg/kg) treatment. Thin arrow and thick arrow are macrophages and neutrophils, respectively. Scale bar = 50㎛.

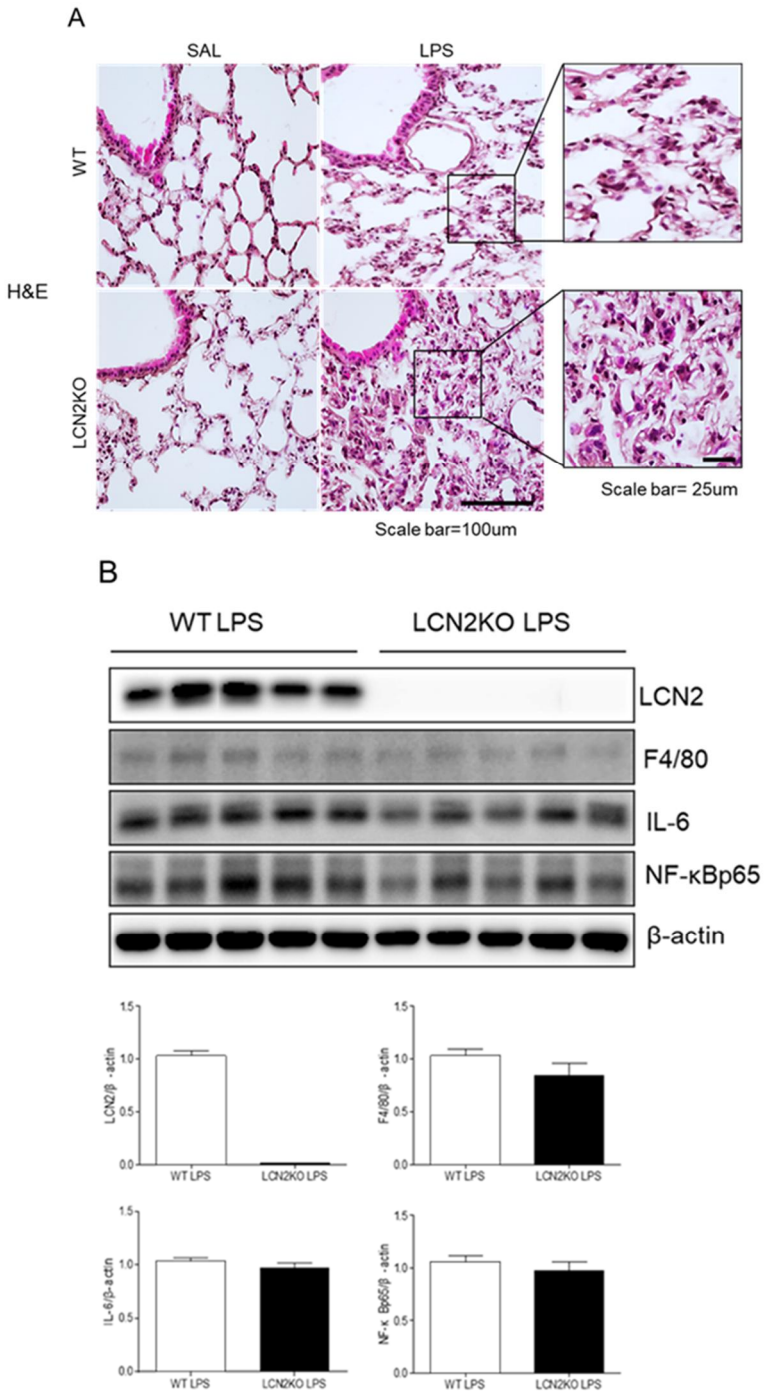

Figure 12. Effect of LCN2 deficiency on lung inflammation and macrophages infiltration in WT-and LCN2 mice 8 days after LPS (5mg/kg) treatment. (A),Microscopically inflammation and macrophages infiltration were more seen in lung of LPS-treated LCN2KO mice compared to WT. Representative microscopic imaging

of lung sections in LPS-treated WT and LCN2KO mice. (B), western blotting and quantitative analysis of LCN2, F4/50, IL-6 and NF-κBp65 expressions in the lung. There were no difference of F4/80, marker for macrophage, IL-6 and NF-κBp65 between LCN2KO and WT mice at 8 days after intratracheal LPS treatment. β-actin was used as a loading control. Results are presented as the mean ± S.E.M. Scale bar = 25 or 100㎛.

2. Human data

2-1.The baseline and clinical characteristics of patients with pneumonia/ARDS

To examine clinical significance of LCN2 in patients with pneumonia/ARDS, we reviewed clinical data from electronic medical records of them. Baseline and clinical charaicteristics are presented in Table 2 and compared with those with non-pneumonia/ARDS. In patients with pneumonia/ARDS, median age was 78 years old and 74% was male. Septic shock and acute kidney injury were concomittantly presented in 59.3% and 51.9%, respectively. 30-day and ICU mortality rates were 33.3% and 37%, respecitively. Compared to patients with non-pneumonia/ARDS, those with pneumonia/ARDS received significantly less renal replacement therapy. There was a lower trend of the frequency of DM and SOFA score toward patients with pneumonia/ARDS. There were no sigficant difference of other characteristics between pneumonia and non-pneumonia/ARDS.

2-2. Serum Levels of LCN2 in patients with pneumonia/ARDS is elevated and differs according to AKI and shock.

Median serum LCN2 level was 177.2 ng/ml [Interquartile range (IQR), 108.5-306.9 ng/ml], which was significantly higher than 23 normal healthy controls [44.8 ng/ml (IQR, 30.2-64.1 ng/ml), *P* <0.001]. Median serum LCN2 level was significantly lower

in patients with pneumonia/ARDS than that of non-pneumonia/ARDS [263.5 ng/ml (233.5–469.6 ng/ml), *P*=0.044] (Figure 13).

Table 2. Comparision of characteristics between pneumonia and non-pneumonia ARDS

Variables	Pneumonia/ARDS N=27	Non-pneumonia/ARDS N=10	*P*-value
Age, years	78 (60–83)	62.5 (57.8–78.8)	0.319
Male gender	20 (74)	7 (70)	1
BMI, kg/m^2	21.3 (17.9–25.4)	23.2 (20.1–24.9)	0.371
DM	5 (18.5)	5 (50)	0.094
CKD	2 (7.4)	3 (30)	0.110
CLD	2 (7.4)	1 (10)	1
CVD	7 (25.9)	1 (10)	0.404
COPD	6 (22.2)	0 (0)	0.162
APACHEII score	24 (16–30)	22.5 (17.5–29.3)	0.801
SOFA score	11 (7–13)	14.5 (10–16)	0.084
Septic shock	16 (59.3)	6 (60)	1
AKI	14 (51.9)	7 (70)	0.461
RRT	5 (18.5)	6 (60)	0.038
WBC, x 10^3/mm^3	13.2 (10–23)	15.7 (13.9–20.3)	0.511
Hb, g/dl	11 (9.7)	11.6 (9.6–13.3)	0.674
PLT, x 10^3/mm^3	209 (135–283)	211 (124.3–381.3)	0.933
CRP, mg/dl	15.9 (8.3–23.9)	14.5 (10.6–25)	0.724
PCT, ng/ml	0.95 (0.22–23.7)	1.72 (0.52–59.2)	0.602
ICU mortality	10 (37)	5 (50)	0.708
30-day mortality	9 (33.3)	5 (50)	0.454

Data are expressed as median (interquartile range) or number (%). BMI, body mass index;

DM, diabetes mellitus; CKD, chronic kidney disease; CLD, chronic liver disease; CVD, cerebrovascular disease; COPD, chronic obstructive pulmonary disease; APACHE, acute physiology and chronic health evaluation; SOFA, sequential organ failure assessment; AKI, acute kidney injury; RRT, renal replacement therapy; WBC, white blood cell; Hb, hemoglobin; PLT, platelet; CRP, C-reactive protein; PCT, procalcitonin; ICU, intensive care unit.

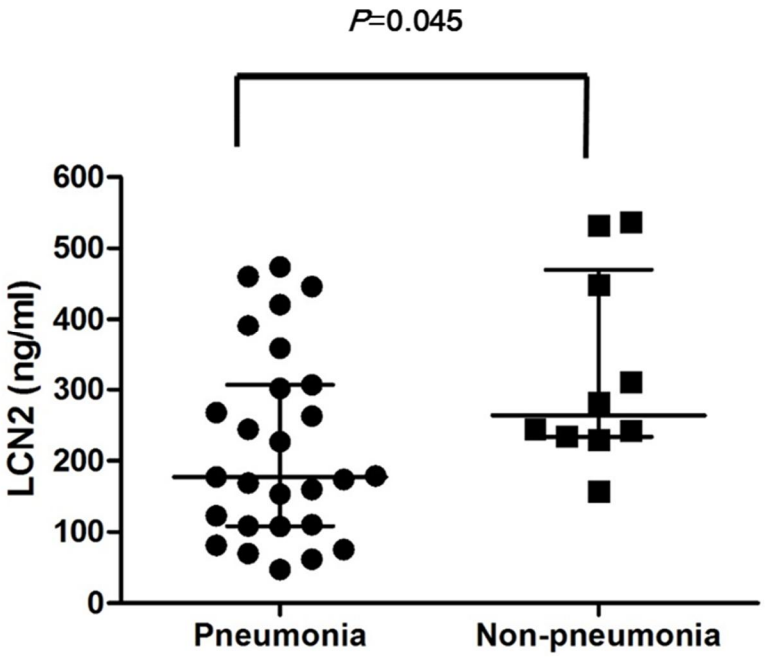

Figure 13. Comparisons of serum LCN2 levels between pneumonia and non-pneumonia/ARDS

To evaluate the difference of serum LCN2 levels according to concomittant clinical

presentations in 27 patients with pneumonia/ARDS, we performed the subgroup analysis according to AKI and shock status. Among 27 patients with pneumonia/ARDS, the level of serum LCN2 in 14 patients with AKI was significantly higher than 13 those without AKI [284.7 ng/ml (IQR, 171.2-427.1ng/ml) *vs* 122.8 ng/ml (IQR, 72.6-176.2 ng/ml), *P*=0.003] (Figure 14A). There was significantly higher LCN2 levels in 16 patients with shock than those without. [284.7 ng/ml (IQR, 124.7-413.2 ng/ml) *vs* 153.5 ng/ml (IQR, 69.6-177.2ng/ml) , *P*=0.009] (Figure 14B).

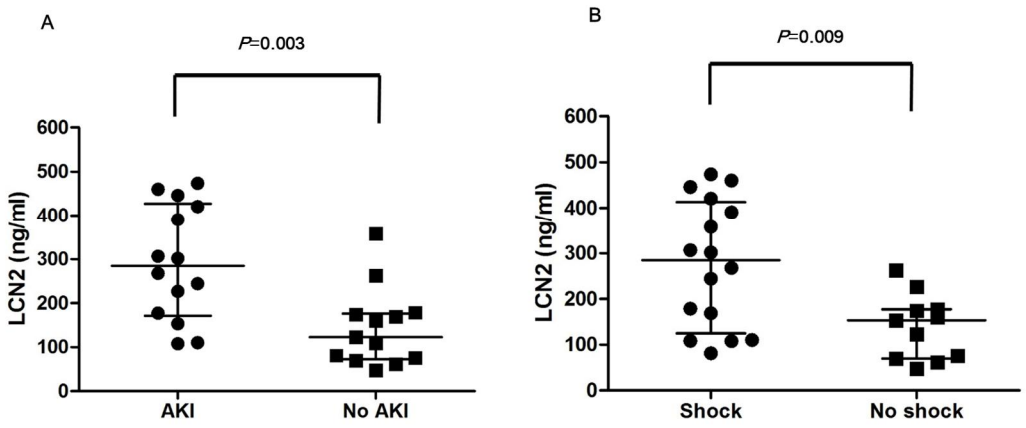

Figure 14. Comparison of serum LCN2 levels between AKI vs no AKI (A) and shock and no shock (B) in patients with pneumonia/ARDS.

2-3. Serum LCN2 level is correlated with SOFA score and procalcitonin in patients

with pneumonia/ARDS

To examine which severity of illness scores and laboratory variables are correlated with serum LCN2 levels, we performed correlation analysis between serum LCN2 and APACHE II, SOFA score, WBC, CRP, procalcitonin and lactate in 27 patients with pneumonia/ARDS (Figure 15). serum LCN2 level was positively correlated to SOFA score (r=0.467,P<0.014) and procalcitonin (r=0.523, P=0.007).

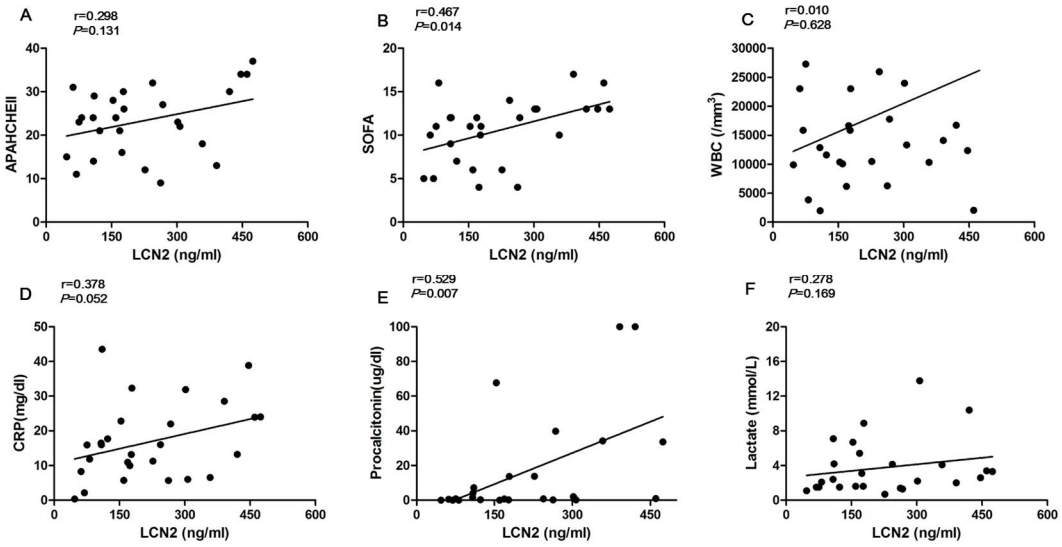

Figure 15. Correlation of serum LCN2 with APACHE II (A), SOFA score (B), WBC (C), CRP (D), procalcitonin (E), lactate (F) in 27 patients with pneumonia/ARDS.

2-4. Comparison of serum LCN2 levels between non-survivors and survivors at 30-

day and area under the receiver operating characteristic (AUROC) for 30-day mortality.

During 30-day, 37%(10/27) patients with pneumonia/ARDS died. To evaluate difference of serum LCN2 levels, we compared serum LCN2 level between non-survivors and survivors at 30-day. In 27 patients with pneumonia/ARDS (Figure 16A), there was no significant difference between 10 non-survivors and 17 survivors at 30-day mortality [255.8 ng/ml (IQR, 135.4-404.8 ng/ml) *vs* 168.7 ng/ml (IQR, 108.1-282.2 ng/ml), *P*=0.282]. To evaluate whether serum LCN2 predicts 30-day mortality in patients with pneumonia/ARDS, we performed receiver operating characteristics (ROC) curve analysis for LCN2 and compared the area under the ROC (AUROC) to the severity of illness score such as APACEH II and SOFA score well-known as prognostic index (Figure 16B). AUROC for LCN2 was 0.629 [95% confidence interval (CI), 0.423-0.806, *P*=0.279]. AUROCs for APACHE II and SOFA score were 0.774 [95% CI, 0.573-0.911, *P*=0.006] and 0.815 [0.619-0.937, *P*<0.001].

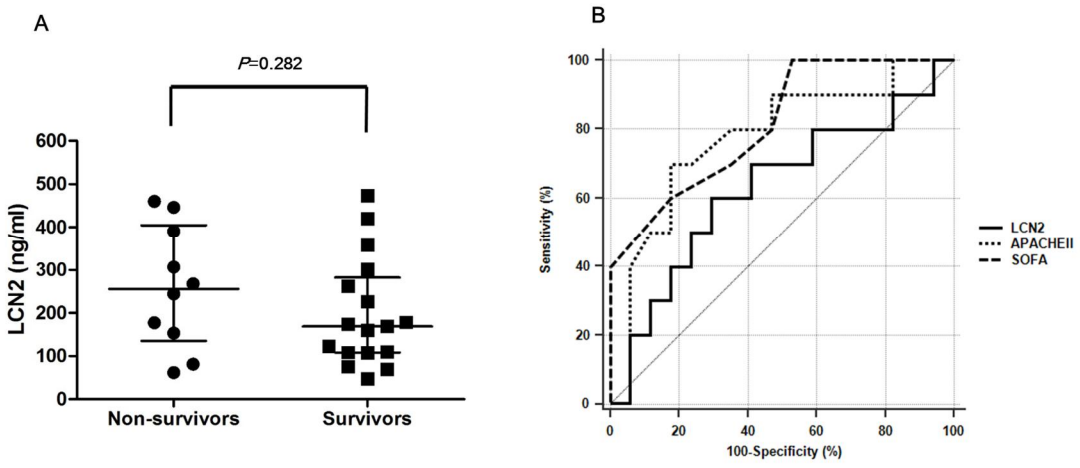

Figure 16. Comparison of serum LCN levels between non-survivors and survivors (A) and receiver operating characteristic curves for 30-day mortality (B) between LCN2 and severity of illness score in patients with pneumonia/ARDS.

Discussion

In the present study, serum proinflmmatory cytokines and recruitment of macrophage and neutrophil in BALF and lung tissue, protein expression of inflammatory proteins in lung tissue were less induced in LCN2KO mice compared WT mice at initial 24 hours time point after intratracheal LPS treatment. In addition, decreased inflammatory response induced less HO-1 induction and iron uptake of macrophage. In contrast, mortality rate was higher in LCN2KO mice than WT in 8 days time point. In BALF and lung tissue, more macrophages and neutrophils were recruited along with inflammation in lung in LCN2KO mice. In western blotting, there was no difference of expression of IL-6 and NF-κB between LCN2KO and WT in lung tissue. In human, serum level of LCN2 was elevated in patients with pneumonia/ARDS than normal healthy controls. The elevation of serum LCN2 level was less induced in patients with pneumonia/ARDS than that of non-pneumonia/ARDS.The level of serum LCN2 was significantly higher in case of concomitant AKI or shock in patients with pneumonia/ARDS. The level of serum LCN was moderately correlated to SOFA score and blood procalcitonin. The level of serum LCN2 did not differ between non-survirvor and survivors at 30-day and seems not be a marker to predict 30-day mortality.

ARDS is a fatal condition encountered in ICU.[5] The mortality rate is still high in spite of advances of ventilator strategies and adjunctive therapies in critical care and research[6]. The main pathogenesis is diverse inflammatory responses in alveoli induced by hetereoneous causes, which respiratory epithelial, immune or endothelial cells involves [7,8]. Pneumonia is the common risk factor for development of ARDS[5]. Respiratory epithelial, immune or endothelial cells are activated by various pathogens including bacteria, virus, fungi etc. and secret many cytokines and chemokines leading to inflammation of lung. Exudative phase of ARDS is characterized by innate immune cell-mediated damage of the alveolar endothelial and epithelial barriers and accumulation. Resident alveolar macrophages secrete proinflammatory cytokines, leading to neutrophil or macrophages recruitment as well as activation of alveolar cells

to promote and sustain inflammatoion and tissue injury. Recruited neutrophils release many inflammatory materials resulting in aveolar epithelial damage. Considering substantial inflammation in exudative phase, It is important to identify the role of proteins to involve in proinflammatory response induced by alveolar macrophages and neutrophils.

LCN2 is a 25-kDa protein composed of 178 amino acids and also known as neutrophil gelatinase-associated lipocalin (NGAL), oncogene 24p3 or siderocalin and originally found in neutrophil [9,23]. It is localized on the long arm of chromosome 9, (9q34.11) in a cluster of at least three lipocalins. Its expression has been found in several organs including liver [10], gastrointestinal [11] or lung [12]. It is also secreted by respiratory epithelial and immune cells [9,24,25]. Its function is multipotent in various organs (Figure 17)

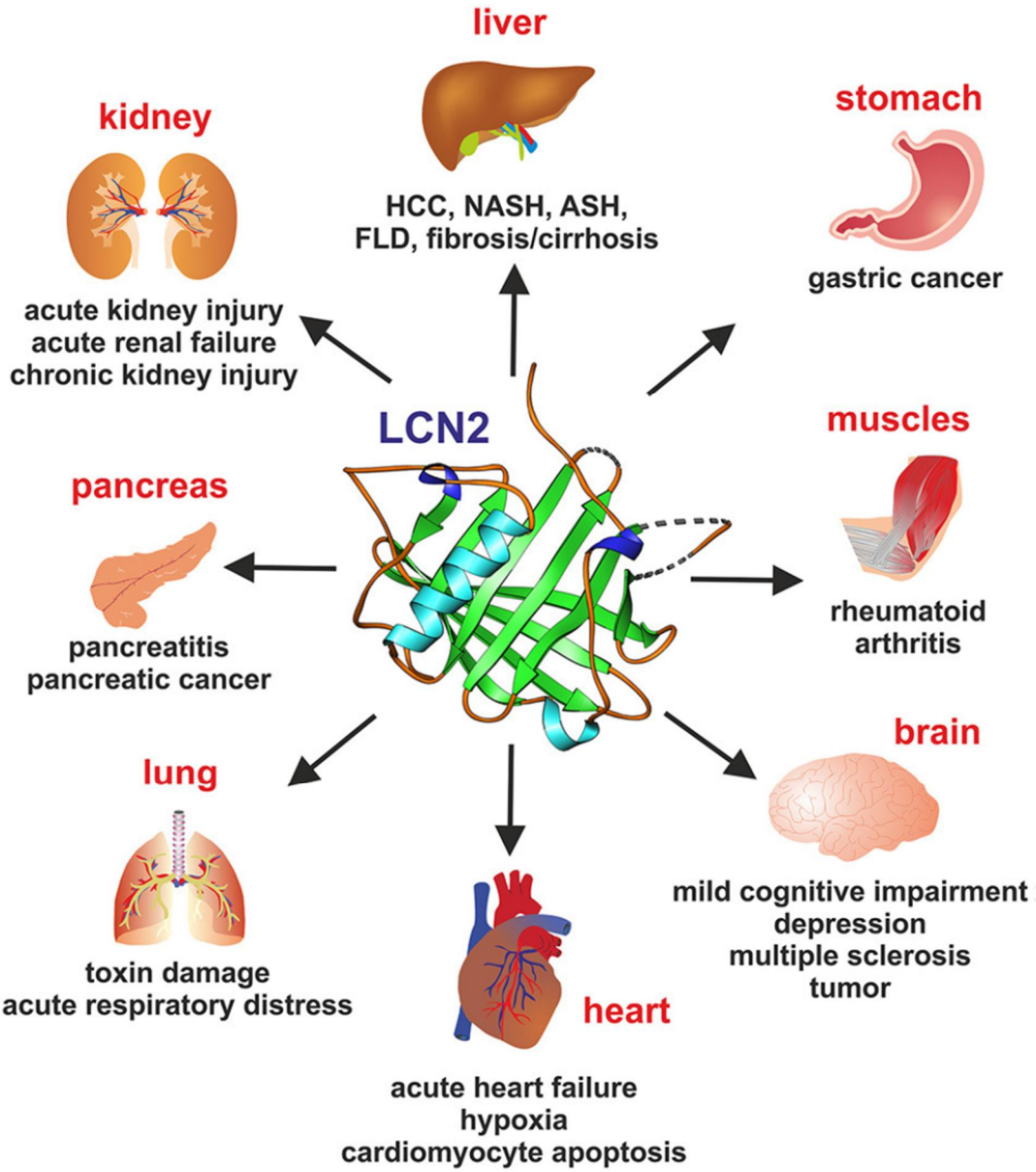

Figure 17. LCN2 in organ damage. (Reference from Asimakopoulou et al[10].)

Several experimental models has been used to investigate pathogenesis of LCN2 in inflammation and infection.Table 3 summarizes studies about LCN2 in experimental inflammatory or infectous models. LPS release from bacteria is a strong stimulator to secret LCN2 in liver and lungs.[12] LPS activates toll-like receptor in epithelial and immunce cells, which activate NF-κB-STAT3 signaling. The activation of NF-κB induces pro-inflammatory cytokines such as IL-6, IL-1β, or TNF-α as well as LCN2.

Proinflamamtory cytokines also stimulate upregulation of LCN2 gene and secret LCN2 from immune cells and epithelial cells. The role of LCN2 for infectious or inflammatory condition remain controversial. It depends on status of experimental models, types of insult such as bacteria inself or LPS and injection method or injected dose of them. Flo et al demonstrated that LCN2 bound to siderophores produced by bacteria and limited bacterial growth by preventing them using host's iron[13]. Srinivasan *et al*.[26] demonstrated that oxidative stress was increased via delayed hypoferemia of inflammation in LCN2KO mice which indicate that LCN2 has an antioxidant effect by regulating iron homeostasis. LCN2 also affects neutrophils and macrophage. LCN2 deficiency alters neutrophil homeostasis, reducing the migration of neutrophils[14] and impairs neutrophil function[27]. In macrophages, expression of inflammatory cytokines are decreased and migration and phagocytosis is also impaired[14]. Gupta *et al.*[28] demonstrated that upregulation of LCN2 by mesenchymal stem cells partly contributed to bacterial clearance in murine *Escherichia coli*. With this finding, LCN2 show therapeutic potential proteins in bacterial lung infection. In contrast to protective role of LCN2 against infection as previously noted, Warszawska *et al.*[21] showed that in their pneumococcal pneumonia mice models, LCN2 induced IL-10 from macrophage and deactivated macrophages, characterized by downregulation of proinflammatory cytokines and upregulation of IL-10. These deactivation of macrophages resulted in an impaired immune response, bacterial clearance of *S.pneumonia* and increased mortality.

In unestablished role of LCN2 by experimental infection or inflammation models induced by LPS or bacteria itself, we aim to focus on the effect of LCN2 deficienc on the localized lung inflammatory status under intratracheal LPS instillation induced acute lung injury mouse model. In the present study, levels of serum inflammatory cytokines, number and recruitment of immune cells in BALF, expression of inflammatory proteins in lung tissue were less induced in LCN2KO mice than those of WT at 24 hours time points after intratracheal LPS treatment. These findings indicate that LCN2 deficiency attenuates inflammatory response at 24 hours time point after

intratracheal LPS treatment. The expression of HO-1 and uptake of iron in macrophages was less induced in LCN2KO, which also reflect less inflammatory reponse. In the controversial role of LCN2 by experimental models, our findings weight for the pro-inflammatory inducer as LCN2 at 24 hours time point after intratracheal LPS instillation induced acute lung injury mouse model.

We examined that attenuation of inflammatory response under LCN2 deficiency at 24 hours time point could improve survival in LCN2KO mice than WT. In contrast to attenuation of inflammation at 24 hours time point, mortality rate was higher in LCN2KO mice than WT ultil 8 day time point. In BALF, more recruitment of neutrophils and macrophages were found in LCN2KO mice than WT, which as opposed to less recruitment of them at 24 hour time point after intratracheal LPS treatment. In lung tissue, microscopically more inflammation and macrophages infiltration were also seen in LCN2KO mice compared to WT. Considering no difference of expression of IL-6 and NF-κB in lung tissue, the activation of NF-κB pathway may not contribute to increased mortality and more recruitment of macrophages and neutrophils in LCN2KO mice at 8 days time point.

These different phenomenon at two different time points is not fully understood. The plausiable explanations are are that 1) relative low dose of LPS at 24 hours times point may induce less lung inflammation despite of usual dose of induction of direct acute lung injury, 2) The persistence of endotoxin in alveoli activates other inflammatory pathways to induce inflammation at late phase which results in organ damages and death in LCN2KO mice. Further research involved in molecular pathway is needed to explain these different role of LCN2 at the different time interval.

Table 3. Summary of experimental LCN2 findings.

Year/author	models	stimulator/ injection method	Findings
2004 Flo *et al*[13].	WT and Lcn2 KO mice	*E.coli*, intraperitoneal method	LCN2 production is induced through TLR4. LCN2 limits E.coli growth by iron sequestration through binding siderophore. LCN2 KO mice showed accelerated lethality.
2006 Berger *et al*,[33]	WT and Lcn2 KO mice	*E.coli*, intraperitoneal method	LCN2 KO mice showed significant accelerated lethality due to loss of iron sequestration
2007 Sunil *et al*.[34],	WT C57BL/6	LPS from *E.coli*, intravenous or intraperitoneal method	LPS upregulates expression of LCN2 mRNA and protein in lungs and alveolar macrophage and type II cells
2009 Chan *et al*.[35]	WT and Lcn2 KO mice	*K.pneumonia*, intratracheal method	LCN2 is up-regulated in the lung following K.pneumonia infection. LCN2 KO mice impairs lung bacterial clearance. Lung reconstitution of LCN2 in LCN KO significantly decreased bacterial burden and lung injury
2010 Wu *et al*.[36]	WT and Lcn2 KO mice	*E.coli*, intratracheal method	LCN2 expression is induced in bronchial epithelium and type II pneumocytes. LCN2 KO mice showed significantly higher mortality. Higher bacterial numbers were observed in lung s of LCN2 KO mice
2012 Srinivasan *et al*[26]	WT and Lcn2 KO mice	LPS from *E.coli*, intraperitneal method	All LCN2 KO mice died within 8 day LPS injection. Serum TNF-α and IL-18 levels were elevated in LCN2 KO mice. LCN2 KO mice showed increased LPS-induced immune cell apoptosis. LCN2 KO mice

			showed delayed hypoferremia and increase of oxidative stress

Table 3. Summary of experimental LCN2 findings. (continued)

Year/author	models	stimulator/ injection method	Findings
2012 Gupta *et al.*[22]	WT C57BL/6	Mesenchymal stem cells (MSCs), *E.coli*, intratracheal method	Treatment with WT MSCs improved 48 h survival and lung injury compared with control mice. WT MSCs enhance bacterial clearance from alveolar space. LCN2 production in upregulated with MSC treatment
2013 Warszawska *et al.*[21]	WT and Lcn2 KO mice	*S.pneumonia*, intranasal method	LCN2 deactivates macrophages in pneumocccal pneumonia infected mice. LCN2 attenuated the early inflammatory response and impaired bacterial clearance in an IL-10-dependent manner. LCN2 deficiency enhanced survival of mice suffering from pneumococcal pneumonia.
2019 Wang *et al.*[13],	WT and Lcn2 KO mice RAW 264.7 macrophages	*E.coli*, in vitro infection, intragastric and intraperitoneal injection	LCN2 deficiency alters neutrophil homeostasis and reduces the migration of neutrophil. Serum and peritoneal lavage TNF alphal levels were significantly lower in LCN2 KO mice. IL-6, IL-1beta and TNF-alpha were significantly decreased in LCN2 macrophages.

Broad expression of LCN2 in various tissues and cell types and feasibility of detection of it make it potential marker for both diagnostic biomarker and prognostic indicator at the range of critical illness. Table 4 summarizes studies about the significance of

LCN2 in several clinical conditions.In AKI, several studies showed that elevation of LCN2 in plasma or urine was a use marker to predict development and progression of AKI in critically ill patients.[15-19] There has been reported about clinical utility of plasma LCN2 in pneumonia. Yeh *et al*.[20] showed that LCN2 was associated with severity in patients with community acquired pneumonia. Kim *et al* [37]showed that plasma LCN2 measured at emergency department was correlated to severity of pneumonia and Independent predictor of hospital mortality. Recent retrospective cohort study performed by Min *et al*[38] reported that plasma LCN2 is the potential marker for predicting ICU admission and in-hospital mortality. Warszawska *et al.*[21] showed in their human data that LCN2 levels in BALF and plasma were significantly enhanced in patients with confirmed bacterial pneumonia irrespective of gram positive or gram negative pathogen. LCN2 level in BALF was approximately 10-fold higher than in plasma. They observed a tendency for a worse outcome during severe pneumonia with gram-positive bacterial in patients in the ICU with elevated LCN2 levels in BAL while more survival in pneumonia patients with pneumonia caused by gram negative bacteria and elevated LCN2 levels of BALF. Wang *et al*.[30] and Hong *et al*[39] showed that serum LCN2 in sepsis was an independent predictors of 28-day mortality and supported the progsnotic value of plasma LCN2.

The role of LCN2 in ARDS has not been fully elucidated. Kangelaris *et al*[22]*.* demonstrated that mRNA levels of LCN2 involved in initial neutrophil response were upregulated in patients with early sepsis-induced ARDS and they reported that correlation of LCN2 gene expression and plasma protein levels. They also found that the association between expressed LCN2 and ARDS was independent of enrollment creatinine, indicating that highly expressed LCN2 may play a direct role in ARDS pathogenesis. Peumonia is one of causes developing sepsis and the common risk factor for development of ARDS in ICU. The clinical significance of serum LCN2 in patients with pneumonia/ARDS who admitted to ICU has not been fully elucidated. To examine whether LCN2 has a diagnostic or prognostic role in pneumonia/ARDS, we measured serum LCN2 in 27 patients with pneumonia/ARDS and analyzed the value

of serum LCN2 with clinical data. In the current study, the level of serum LCN2 was elevated in patients with pneumonia/ARDS compared to normal healthy subjects but lower than 10 those with non-pneumonia/ARDS. The difference of serum LCN2 concentration between pneumonia and non-pneumonia origin implies that LCN2 secretion is differently induced from sources of infection. Level of serum LCN2 was significantly higher in critical condition including AKI or shock. Further research is needed about which sourse of infection induce LCN2 production predominant and potential organ specific inflammatory marker. In the current study, the level of LCN2 was correlated to SOFA score and blood procalcitonin. Considering elevation of LCN2 in AKI or shock , SOFA score used in sepsis may be associated with serum LCN2.[1] In clinical practice, blood procalcitonin has been used as one of inflammatory markers to differentiate between infection and non-infection and investigated as a marker to assess treatment response.[31,32] Regarding the correlation between LCN2 and procalcitonin was reported in the previous study, serum LCN2 may be a useful marker to distinguish infection from non-infectious contidition. Previous study about plasma LCN in pneumonia reported that plasma LCN2 was independent factor for 28 day mortality. In the present study, there was no significant difference of serum LCN2 between non-survivors and survivors with pneumonia/ARDS at 30-day. Small number of patients with pneumonia/ARDS admitted to ICU were included in contrast to previous studies, which results in no significance of serum LCN2 in 30-day morality. Further research including large number of patients with pneuomonia/ARDS will be needed to prove the prognostic marker of serum LCN2. The role of LCN2 in pneumonia/ARDS is described in Figure 18.

Table 4 . Summary of clinical correlation with LCN2.

Year	Population	Disease	Study design	Findings
2010 Cruz et al.[15]	ICU patients (n=307)	Acute kidney injury	prospective observational cohort	Plasma LCN2 is higher in patients with AKI and good diagnostic marker for AKI development plasma LCN2 is correlated with severity of AKI
2010 Constantin et al.[16],	ICU patients (n=88)	Acute kidney injury	prospective observational cohort	Plasma LCN2 is early marker of AKI in critically ill patients
2011 de Geus et al.[18]	ICU patients (n=632)	Acute kidney injury	prospective observational cohort	plasma and urine LCN2 predict development of severe AKI .plasma and urine LCN2 predict the initiation of renal replacement therapy within first 7 days of ICU stay
2010 Martensson et al.[17]	ICU patients with sepsis	Acute kidney injury	Retrospectiv e cohort	Urine LCN2 is useful in predicting AKI
2014 Matsa et al.[19]	ICU patients (n=194)	Acute kidney injury	Prospective obsevational cohort	plasma and urine LCN2 measured at admission can predict an occurrence of AKI for up to 72 hours of ICU stay
2013 Yeh et al.[20]	Hospitalize d patients (n=61)	CAP	Prospective observational cohort	Plasma LCN2 levels was higher in patients with CAP than normal health. Plasma LCN2 leveles was correlated

				with severity of index of CAP (PSI and CURB-65)

Table 4 . Summary of clinical correlation with LCN2. (continued)

Year	Population	Disease	Study design	Findings
2013 Warszawska et al.[21]	Patients with invasive mechanical ventilation (n=64)	Pneumonia	Prospective observational cohort	LCN2 levels in BALF and plasma were significantly enhanced in patients with confirmed bacterial pneumonia LCN2 levels in BALF were approximately 10-fold higher than in plasma
2016 Kim et al.[37]	Patients with CAP in ER (n=362)	Pneumonia	Prospective observational cohort	Correlation between LCN2 and severity of pneumonia Independent predictor of hospital mortality
2020 Min et al.[38]	Patients with pneumonia (n=241)	Pneumonia	Retrospective Observational cohort	Plasmal LCN2 is a useful marker for predicting ICU admission and in-hospital mortality.
2014 Wang et al.[30]	Septic patients in ER (n=480)	Sepsis (>70% pneumonia)	Prosepective observational cohort	Serum LCN2 was higher not only in non-survivors than survivors. Serum LCN2 is an independent predictors of 28-day mortality
2015 Hong et al.[39]	Septic patients in ER (n=470)	Sepsis	Prospective observational cohort	Plasma LCN2 is a useful biomarker for assessing the severity of disease and a good predictor of 28-day mortality.
2015 Kangelaris et al.[22]	ICU patients (n=57)	Sepsis associated ARDS	Prospective observational cohort	LCN2 gene expression was upregulated in patients with sepsis-associated ARDS

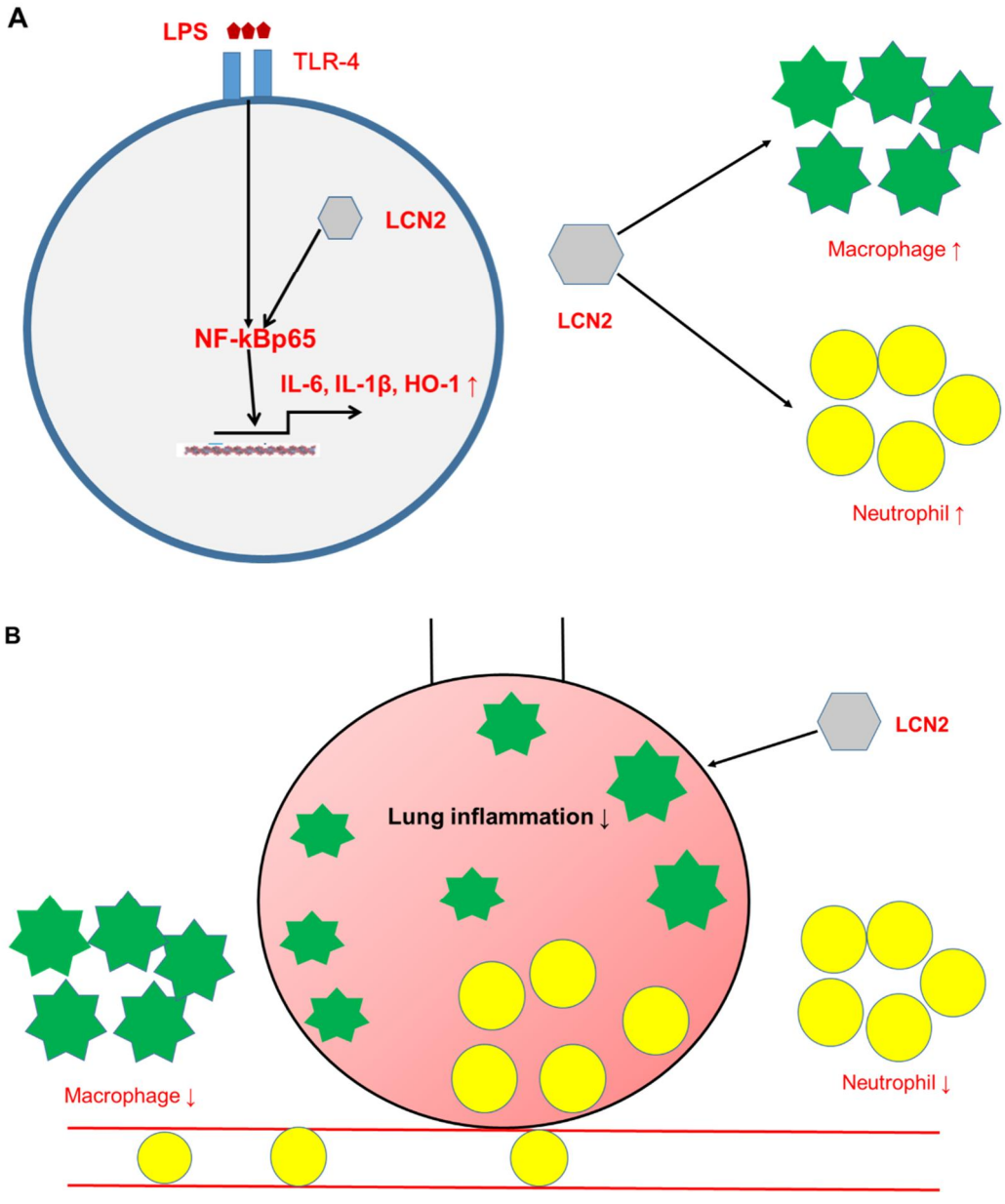

Figure 18. Schematic description of role of LCN2 at 24 hours (A) and 8 days in intratracheal LPS lung injury (B). (A), at 24 hours time point after intratracheal LPS

treatment, LCN2 induce more proinflammatory cytokines and recruitment of macrophages and neutrophils. (B), at 8 days, LCN2 reversely reduce more recruitment of macrophages and neuthophils and microscopic lung inflammation and improved survival

Our analyses have several limitations. First, in humans, we only measured serum LCN2 and the levels of LCN2 in BALF was not measured and correlation of serum and BALF LCN2 was not evaluated. Second, the role of LCN2 according to pathogen was not evaluated. In mouse model, bacteria were not used to develop acute lung injury, which may limits generalization. Third, serum samples were obtained in early stage of sepsis and serial change could not be investigated, so monitoring markers for treatment response is not evaluated. Fourth, we only observed the increased mortality and more recruitment of macrophages and neutrophils at the late phase. The activation of NF-κB was not different between LCN2KO and WT mice. The alternative pathway was not investigated to induce increased mortality and more recruitment of macrophages and neutrophils in similar NF-κB activation.Fifth, different induction of inflammatory reponse in LCN2 deficiency between early and late phase of acute lung injury is not poorly understood in the current study. Sixth, the recombinant LCN2 was not administered in LCN2 KO mice to investigate therapeutic agents.

Conclusion

In summary, LCN2 deficiency attenuates inflammatory response at the early phase after intratracheal LPS treatment. In contrast, LCN2 deficiency contributes to high mortality and hyperinflammation in LCN2KO mice at the late phase without change of NF-κB activation. Serum LCN2 was elevated in patients with pneumonia/sepsis and significantly higher in AKI or shock. Serum LCN2 only is not a good marker to predict prognosis in patients with pneumonia/ARDS admitted to ICU.

References

1. Singer M, Deutschman CS, Seymour CW, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA 2016;315:801-10.
2. Ranieri VM, Rubenfeld GD, Thompson BT, et al. Acute respiratory distress syndrome: the Berlin Definition. JAMA 2012;307:2526-33.
3. Mikkelsen ME, Shah CV, Meyer NJ, et al. The epidemiology of acute respiratory distress syndrome in patients presenting to the emergency department with severe sepsis. Shock 2013;40:375-81.
4. Auriemma CL, Zhuo H, Delucchi K, et al. Acute respiratory distress syndrome-attributable mortality in critically ill patients with sepsis. Intensive Care Med 2020.
5. Bellani G, Laffey JG, Pham T, et al. Epidemiology, Patterns of Care, and Mortality for Patients With Acute Respiratory Distress Syndrome in Intensive Care Units in 50 Countries. JAMA 2016;315:788-800.
6. Fan E, Brodie D, Slutsky AS. Acute Respiratory Distress Syndrome: Advances in Diagnosis and Treatment. JAMA 2018;319:698-710.
7. Matthay MA, Zemans RL, Zimmerman GA, et al. Acute respiratory distress syndrome. Nat Rev Dis Primers 2019;5:18.
8. Thompson BT, Chambers RC, Liu KD. Acute Respiratory Distress Syndrome. New Engl J Med 2017;377:562-72.
9. Kjeldsen L, Johnsen AH, Sengelov H, Borregaard N. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. J Biol Chem

1993;268:10425-32.

10. Asimakopoulou A, Weiskirchen S, Weiskirchen R. Lipocalin 2 (LCN2) Expression in Hepatic Malfunction and Therapy. Front Physiol 2016;7:430.
11. Lu F, Inoue K, Kato J, Minamishima S, Morisaki H. Functions and regulation of lipocalin-2 in gut-origin sepsis: a narrative review. Crit Care 2019;23:269.
12. Sunil VR, Patel KJ, Nilsen-Hamilton M, Heck DE, Laskin JD, Laskin DL. Acute endotoxemia is associated with upregulation of lipocalin 24p3/Lcn2 in lung and liver. Exp Mol Pathol 2007;83:177-87.
13. Flo TH, Smith KD, Sato S, et al. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. Nature 2004;432:917-21.
14. Wang Q, Li S, Tang X, Liang L, Wang F, Du H. Lipocalin 2 Protects Against Escherichia coli Infection by Modulating Neutrophil and Macrophage Function. Front Immunol 2019;10:2594.
15. Cruz DN, de Cal M, Garzotto F, et al. Plasma neutrophil gelatinase-associated lipocalin is an early biomarker for acute kidney injury in an adult ICU population. Intensive Care Med 2010;36:444-51.
16. Constantin JM, Futier E, Perbet S, et al. Plasma neutrophil gelatinase-associated lipocalin is an early marker of acute kidney injury in adult critically ill patients: a prospective study. J Crit Care 2010;25:176.e1-6.
17. Martensson J, Bell M, Oldner A, Xu S, Venge P, Martling CR. Neutrophil gelatinase-associated lipocalin in adult septic patients with and without acute kidney injury. Intensive Care Med 2010;36:1333-40.
18. De Geus HR, Bakker J, Lesaffre EM, le Noble JL. Neutrophil gelatinase associated lipocalin at ICU admission predicts for acute kidney injury in adult patients. Am J Respir Crit Care Med 2011;183:907-14.
19. Matsa R, Ashley E, Sharma V, Walden AP, Keating L. Plasma and urine neutrophil gelatinase-associated lipocalin in the diagnosis of new onset acute kidney injury in critically ill patients. Crit Care 2014;18:R137.

20. Yeh YH, Chang JL, Hsiao PC, et al. Circulating level of lipocalin 2 as a predictor of severity in patients with community-acquired pneumonia. J Clin Lab Anal 2013;27:253-60.
21. Warszawska JM, Gawish R, Sharif O, et al. Lipocalin 2 deactivates macrophages and worsens pneumococcal pneumonia outcomes. J Clin Invest 2013;123:3363-72.
22. Kangelaris KN, Prakash A, Liu KD, et al. Increased expression of neutrophil-related genes in patients with early sepsis-induced ARDS. Am J Physiol Lung Cell Mol Physiol 2015;308:L1102-13.
23. Kjeldsen L, Bainton DF, Sengelov H, Borregaard N. Identification of neutrophil gelatinase-associated lipocalin as a novel matrix protein of specific granules in human neutrophils. Blood 1994;83:799-807.
24. Cowland JB, Sorensen OE, Sehested M, Borregaard N. Neutrophil gelatinase associated lipocalin is up-regulated in human epithelial cells by IL-1 beta, but not by TNF-alpha. J Immunol 2003;171:6630-9.
25. Guo H, Jin D, Chen X. Lipocalin 2 is a regulator of macrophage polarization and NF-kappaB/STAT3 pathway activation. Mol Endocrinol 2014;28:1616-28.
26. Srinivasan G, Aitken JD, Zhang B, et al. Lipocalin 2 deficiency dysregulates iron homeostasis and exacerbates endotoxin-induced sepsis. J Immunol 2012;189:1911-9.
27. Schroll A, Eller K, Feistritzer C, et al. Lipocalin-2 ameliorates granulocyte functionality. Eur J Immunol 2012;42:3346-57.
28. Gupta N, Krasnodembskaya A, Kapetanaki M, et al. Mesenchymal stem cells enhance survival and bacterial clearance in murine Escherichia coli pneumonia. Thorax 2012;67:533-9.
29. Rhodes A, Evans LE, Alhazzani W, et al. Surviving Sepsis Campaign: International Guidelines for Management of Sepsis and Septic Shock: 2016. Critical Care Med 2017;45:486-552.

30. Wang M, Zhang Q, Zhao X, Dong G, Li C. Diagnostic and prognostic value of neutrophil gelatinase-associated lipocalin, matrix metalloproteinase-9, and tissue inhibitor of matrix metalloproteinases-1 for sepsis in the Emergency Department: an observational study. Crit Care 2014;18:634.
31. Wirz Y, Meier MA, Bouadma L, et al. Effect of procalcitonin-guided antibiotic treatment on clinical outcomes in intensive care unit patients with infection and sepsis patients: a patient-level meta-analysis of randomized trials. Crit Care 2018;22:191.
32. Gregoriano C, Heilmann E, Molitor A, Schuetz P. Role of procalcitonin use in the management of sepsis. J Thorac Dis 2020;12:S5-s15.
33. Berger T, Togawa A, Duncan GS, et al. Lipocalin 2-deficient mice exhibit I ncreased sensitivity to Escherichia coli infection but not to ischemia-reperfusion injury. Proc Natl Acad Sci USA 2006;103:1834-9.
34. Sunil VR, Patel KJ, Nilsen-Hamilton M, Heck DE, Laskin JD, Laskin DL. Acute endotoxemia is associated with upregulation of lipocalin 24p3/Lcn2 in lung and liver. Exp Mol Pathol 2007;83:177-87.
35. Chan YR, Liu JS, Pociask DA, et al. Lipocalin 2 is required for pulmonary host defense against Klebsiella infection. J Immunol 2009;182:4947-56.
36. Wu H, Santoni-Rugiu E, Ralfkiaer E, et al. Lipocalin 2 is protective against E. coli pneumonia. Respir Res 2010;11:96.
37. Kim JW, Hong DY, Lee KR, Kim SY, Baek KJ, Park SO. Usefulness of plasma neutrophil gelatinase-associated lipocalin concentration for predicting the severity and mortality of patients with community-acquired pneumonia. Clin Chim Acta 2016;462:140-5.
38. Min JH, Lee H, Chung SJ, et al. Neutrophil Gelatinase-Associated Lipocalin for Predicting Intensive Care Unit Admission and Mortality in Patients with Pneumonia. Tohoku J Exp Med 2020;250:243-51.
39. Hong DY, Kim JW, Paik JH, et al. Value of plasma neutrophil gelatinase-

associated lipocalin in predicting the mortality of patients with sepsis at the emergency department. Clin Chim Acta 2016;452:177-81.

국문 초록

Lipocalin-2(LCN2)는 다양한 조직과 면역세포에 분비되는 단백질로서 철의 대사, 급성감염, 염증반응에 관련되는 등 다양한 역할을 하는 것으로 알려져 왔다. 패혈증연관 급성호흡곤란증후군은 높은 사망률을 보이고 있는 중환자실에서 흔히 보는 조건으로, 폐포내 상피세포 및 면역세포들로 인해 다양한 염증반응이 발생한다. 폐렴은 급성호흡곤란증후군 발생에 흔한 위험인자로, 폐렴연관 급성호흡곤란증후군에서 LCN2의 역할에 대해서는 명확하게 규명이 되어 있지 않았다. 이에 LCN2 결핍 마우스 모델에서 기도 내로 Lipopolysaccharide (LPS)를 투여하여 혈청, 기관지세포액, 폐 조직의 단백질 발현에 대해 알아보고자 하였다. 그외 LPS 투여 8일째 사망률 및 대식세포, 호중구의 동원 차이, 염증의 차이 등을 관찰하였다. 또한 폐렴 연관 급성호흡곤란 증후군 환자에서 혈청 LCN2 수치 및 이와 연관된 변수들에 대해 알아 보고자 하였다. 마우스 모델에서 LPS 24시간 이후에 측정한 혈청 IL-1β, IL-6수치는 LCN2 결핍 모델에서 낮았다. 기관지세포액 및 폐 조직에서 호중구 및 폐 대식세포의 동원 정도가 LCN2 결핍 마우스에서 낮았다. 폐 조직의 western blot 분석에서, IL-6, STAT3, NF-κB 발현 정도는 LCN2 결핍 마우스 모델에서 낮았다. 조직에서의 HO-1 효소 또한 LCN2 결핍 마우스에 낮았다. LPS 투여 8일째 때 LCN2 결핍 마우스 9마리 중 2마리는 죽었던 반면 대조군에서는 모두 생존하였다. 기관지세포액에서 대식세포 및 호중구의 동원 정도 폐조직에서 염증 유발 및 대식세포의 침윤이 LCN2 결핍 마우스에서 더 많았다. 폐조직의 western blot 분석에서 IL-6와 NF-κB의 발현 정도는 차이가 없었다. 폐렴 연관

급성호흡곤란 증후군 환자에서 혈청 LCN2 수치 (177.2 ng/ml) 는 정상 대조군 (44.8 ng/ml)과 정상 대조군(104.8 ng/ml) 보다 유의하게 높았으나, 비폐렴연관 급성호흡란증후군 환자 (263.5 ng/ml, *P*=0.044)보다는 낮았다. LCN2는 급성 신손상과 쇽이 동반된 경우 유의하게 높았다. SOFA 점수 (r=0.467, *P*<0.014)와 procalcitonin (r=0.523, *P*=0.007) 수치와 유의한 양의 상관관계를 보였다. 30일에 사망환자와 생존환자의 혈청 LCN2 농도간에는 유의한 차이가 없었고, 30일 사망을 예측하지 못하였다.

이번 연구에서 기도내 LPS 투여 마우스 모델에서 LCN2의 결핍으로 24시간에 폐내에 염증이 낮아짐을 확인하였다. 하지만, 8일째 사망률은 LCN2 결핍 마우스에서 높았고, 사망률과 폐포내 염증은 LCN2 결핍 마우스에서 더 높았다. 폐렴연관 급성 호흡곤란 증후군 환자에서 혈청 LCN2 농도는 상승은 하나 급성신손상 및 쇽이 있는 경우 유의할 뿐, 예후를 예측하는 인자로서의 역할은 보이지 않았다.

감사의 글

2007년 서울아산병원에서 내과 전공의 2년차 여름 휴가를 다녀와서, 호흡기내과를 지원하였었고, 호흡기내과 중 중환자 쪽에 관심을 가지게 되어, 내과 전공의 4년차때 석사 지도 교수님이신 고윤석 교수님, 임채만 교수님, 홍상범 교수님 지도하에 석사 학위를 받았었습니다. 2013년 서울아산병원 호흡기내과 임상강사로 입사하여, 호흡기내과 교수님들과 근무하면서 호흡기내과 만의 장점에 대해 배우게 되었습니다. 호흡기내과 임상강사 2년차인 2014년도에 울산대학교 대학원 박사과정을 시작하였습니다. 당시 홍상범 교수님께 지도교수님을 부탁 드렸었고, 흔쾌히 허락을 해주셔서 박사학위 과정에 첫걸음을 나갈 수 있었습니다. 호흡기내과 임상강사 2년을 마친 후, 모교인 경상대학교병원에서 근무할 수 있는 기회가 주어져 내려와 근무를 시작하였고, 현재도 근무 중에 있습니다. 모교병원에서 근무 기회를 주신 이종덕 교수님과 경상대병원 호흡기-알레르기내과 교수님들께 이 자리를 빌어 감사 드립니다. 박사 과정 수료 후, 학위 논문에 대한 연구에 대해 고민이 많았었습니다. 다행히 이종덕 교수님 소개로 경상대학교 의과대학교 해부학교실 노구섭 교수님과 연구를 진행하게 되었습니다. 해부학교실은 의과대학 때부터 해부학교수님들과 인사 드리면서 개인적으로 좋아했던 교실이었습니다. 노구섭 교수님께서는 의과대학교 다닐 때부터 해부학교실 조교 선생님으로 계셨고, 개인적으로 동아리 선배님으로, 모교 병원 근무 전부터 알고 지내던 분이었습니다. 노구섭 교수님과 대학원 선생님의 도움으로 1년 전부터 연구를 진행하게 되어 연구결과가 나왔고, 지도교수님의 홍상범 교수님의 허락으로 이번 학기에 학위 심사를 받을 수 있게 되었습니다. 코로나 19사태로 화상회의를 통한 발표였지만, 이번 학기에 논문 심사를 받을 수 있게 해주신, 심사위원장이신 임채만 교수님, 석사

지도교수님이신 고윤석 교수님, 박사 지도교수님이신 홍상범 교수님, 허진원 교수님, 그리고 노구섭 교수님께 진심으로 감사 드립니다. 여러 교수님들의 도움이 없었다면 연구를 마무리 할 수 없음을 이번 박사 학위 논문을 준비 하면서 많이 느끼게 되었습니다. 다시 한번 도움을 주신 교수님들께 감사의 인사를 드립니다. 이 학위논문을 발판으로 더 많은 연구에 정진을 하도록 하겠습니다.

마지막으로 호흡기내과에 제가 일을 하도록 묵묵히 지켜주고, 박사학위를 무사히 마치게 해준 사랑하는 아내 고민경, 아들 유지한에도 고마움을 전합니다.