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Characterization of leukocyte migration pattern depending on EphA2 in bleomycin-induced lung injury

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Characterization of leukocyte migration pattern depending on EphA2 in bleomycin-induced lung injury

Directed by Professor Young-Min Hyun

The Master's Thesis
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

Eunji Park

December 2019

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This certifies that the Master's Thesis
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December 2019

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TABLE OF CONTENTS

연세대학교
YONSEI UNIVERSITY

연세대학교
YONSEI UNIVERSITY

LIST OF FIGURES

연세대학교
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ABSTRACT

Characterization of leukocyte migration pattern
depending on EphA2 in bleomycin-induced lung injury

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(Directed by Professor Young-Min Hyun)

The Eph (Erythropoietin-producing hepatoma) receptor and ephrin (Eph receptor interacting protein) expressed on endothelium affect endothelial permeability and regulate the passage of fluid and immune cells from blood into the interstitial tissue via paracellular spaces. Eph/ephrin protein expressed on immune cells modulates cell trafficking in a ligand dependent manner and Eph receptor itself mediates ligand independent promotion of cell migration and can also interact with integrins to regulate cell adhesion in cancer cell. It was known that EphA2 receptor deficiency reduces bleomycin lung injury but, whether EphA2 receptor regulates cell transmigration and retention are not certain.

In this study, acute lung inflammation was established in wild-type and EphA2

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deficient mice using bleomycin, respectively. Lung infiltrated neutrophils were detected by flow cytometry and H&E staining method. To investigate how migratory pattern of neutrophil depends on EphA2 receptor, *in vitro* migration assay was performed using isolated neutrophils from bone marrow. Neutrophil migration in naïve and inflammatory state was monitored in time-lapse manner under two-photon intravital imaging.

5 mg/kg and overdose of bleomycin induced severe expansion of alveolar septa and recruitment of immune cells in lung tissue. 3 hours after bleomycin injection, recruitment of neutrophils was significantly increased compared to naïve state but, there were no significant differences in population of infiltrated neutrophils. *In vitro* migration assay showed that EphA2 deficient neutrophils have more active migration pattern than wild-type. Bleomycin decreased migration index including track length, displacement and velocity compared to naïve state but there was no significant difference between wild-type and EphA2 deficient mice *in vivo.*

Taken together, results showed that EphA2 receptor expressed on neutrophils can modulate neutrophil migration in ligand-independent manner *in vitro* but not *in vivo* state, due to the complexity of in vivo systems and the diversity of factors that can modulate neutrophil migration simultaneously. Lung inflammation is initiated with neutrophil recruitment, so EphA2 receptor can be target for regulating neutrophil migration and inflammation process.

Key words: neutrophil, acute lung injury, ephA2, cell migration

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Characterization of leukocyte migration pattern depending on EphA2 in bleomycin-induced lung injury

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I. INTRODUCTION

Inflammation is characterized by recruitment of innate immune cells to the site of infection and injury[1,2]. As a result of the infection and tissue damage, damage-associated molecular patterns (DAMPs) are released and recognized by pattern recognition receptors such as toll-like receptors (TLRs)[2,3]. Stimulation of TLRs induces the activation of intracellular signaling pathways, including the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and mitogen-activated protein kinases (MAPK) pathways, which lead to the expression of cytokines and chemokines[4,5]. When cytokines and chemokines are released, the leukocytes that circulate in the bloodstream initiate rolling along the endothelial surface and make firm adhesion with adhesion molecules expressed on

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endothelium. The leukocytes then crawl to junctions between endothelial cells and migrate through the transmigration site[6,7]. In conclusion, several adhesion and junctional molecules that maintain endothelial integrity are related to immune cell migration[7-10]. Especially, several pro-inflammatory stimulators increase Eph/ephrin protein mRNA expression and Eph/ephrin protein expressed on the endothelium is concerned with inflammation by regulating immune cell migration[11-14].

Both EphA2 receptor and ephrinA1 are expressed on lung endothelium and the activation of the EphA2 receptor by ephrinA1 increases monolayer permeability through tight and adherence junction disruption. This causes immune cells in the blood to move to interstitial tissue via paracellular space[11,13,15,16].

Eph/ephrin protein is also expressed on immune cells, and the interaction between Eph receptor and ephrin affects cell adhesion and trafficking. Immune cells express Eph receptor and ephrin in a cell type-specific manner, and Eph/ephrin interactions modulate immune cell trafficking[17-21]. Additionally, Eph receptor itself mediates ligand independent promotion of cell migration and can also interact with integrins to regulate cell adhesion in cancer cells[22-24].

Bleomycin (BLM), a chemotherapeutic antibiotic, was used to induce acute lung inflammation in this study. Lung inflammation and fibrosis have been shown to be major adverse drug effects of bleomycin in human cancer therapy[25,26]. Bleomycin causes DNA double-strand bond breaks in lung tissue and promotes production of ROS and therefore induces neutrophil and other innate immune cell recruitment to injured sites[27,28]. Acute lung inflammation induced by bleomycin is similar to acute lung injury (ALI), characterized by disruption of alveolar-

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capillary membrane, increase of permeability, edema formation and infiltration of neutrophils[29-31]. In the lung of mice injected with bleomycin, expression of EphA2 receptor and ephrinA1 was increased. EphA2 receptor not only increased permeability, but also regulated production of chemokines that recruit immune cells to injured lung. Furthermore, EphA2 deficient mice showed decreased influx of neutrophils and were protected from lung injury[15].

Neutrophils are the first to be recruited from the blood into sites of infection and are most abundant in the blood. Neutrophils release pro-inflammatory mediators, employ strategies of phagocytosis, and generate reactive oxygen species (ROS)[32,33]. Neutrophils also have EphA2 receptor, but the role of EphA2 receptor expressed on neutrophils is unclear. In a recent study, EphA2 receptor expressed on neutrophils was shown to serve as a receptor for *Candida albicans*, and downstream signaling of EphA2 receptor was important for antifungal activity[34]. However, the role of EphA2 receptor in neutrophil migration and whether EphA2 receptor affects cell transmigration and retention in acute lung inflammation or not are unknown.

Here, we examined the effect of EphA2 receptor on neutrophil migration and characterization of leukocyte migration patterns depending on EphA2 in bleomycin-induced lung injury using *in vitro* migration assay and intravital imaging technique.

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II. MATERIALS AND METHODS

1. Bleomycin treatment

Bleomycin (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used for inducing acute lung inflammation to mice. To determine concentration of bleomycin for study, C57BL/6J mice was anesthetized with zoletil (30 mg/kg)-rumpun mixture, which was diluted 1:10 (v:v) in PBS by intraperitoneal injection (IP). And 2 mg/kg, 3 mg/kg, 4 mg/kg, 5 mg/kg and 6 mg/kg of bleomycin was inserted by intratracheal injection (IT) respectively. Lung tissue collection and intravital imaging were performed 7 days after injection and bleomycin concentration appropriate for the experiments (3 mg/kg) was determined. Alveolar septa thickness was assessed by measuring the shortest length of a straight line that crossed the alveolar wall using Volocity (PerkinElmer, Waltham, Massachusetts, USA)[35].

3 mg/kg of bleomycin was inserted by intratracheal injection and C57BL/6J wild-type mice was used as control. The following method was appropriate for 6~8 week and 20~25 g male mice. All animal studies are approved by the Animal Care and Use Committee of the Yonsei University College of Medicine.

2. Two-photon intravital imaging

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Two-photon intravital imaging was performed 3 hours after bleomycin injection using each group of animals treated by method of Ⅱtr. Mice was anesthetized with zoletil-rumpum mixture, which was diluted 1:10 in PBS. 10 mg/kg of FITC dextran (Invitrogen, Carlsbad, California, USA) and 25 mg/kg of Texas red dextran (Invitrogen, Carlsbad, California, USA) were injected respectively via intravenous to label blood flow. And 0.1 mg/kg of PE anti-mouse Gr1 (BioLegend, San Diego, California, USA) and 0.25 mg/kg of FITC anti-mouse Gr1 (BioLegend, San Diego, California, USA) were used respectively for labeling neutrophils.

Mice intubation was proceeded prior to lung surgery. 20 G x 1.16 intravenous catheter was used for mice intubation. To prevent the detachment of cannula from the ventilator, the connection area was fixed with surgical tape and suture. Ventilator supplied oxygen to mice continuously and mice was fixed on heating pad using surgical tape to maintain a normal body temperature. After skin removal, tissue between ribs was cut and lung chamber was fixed prior to imaging (figure 1).

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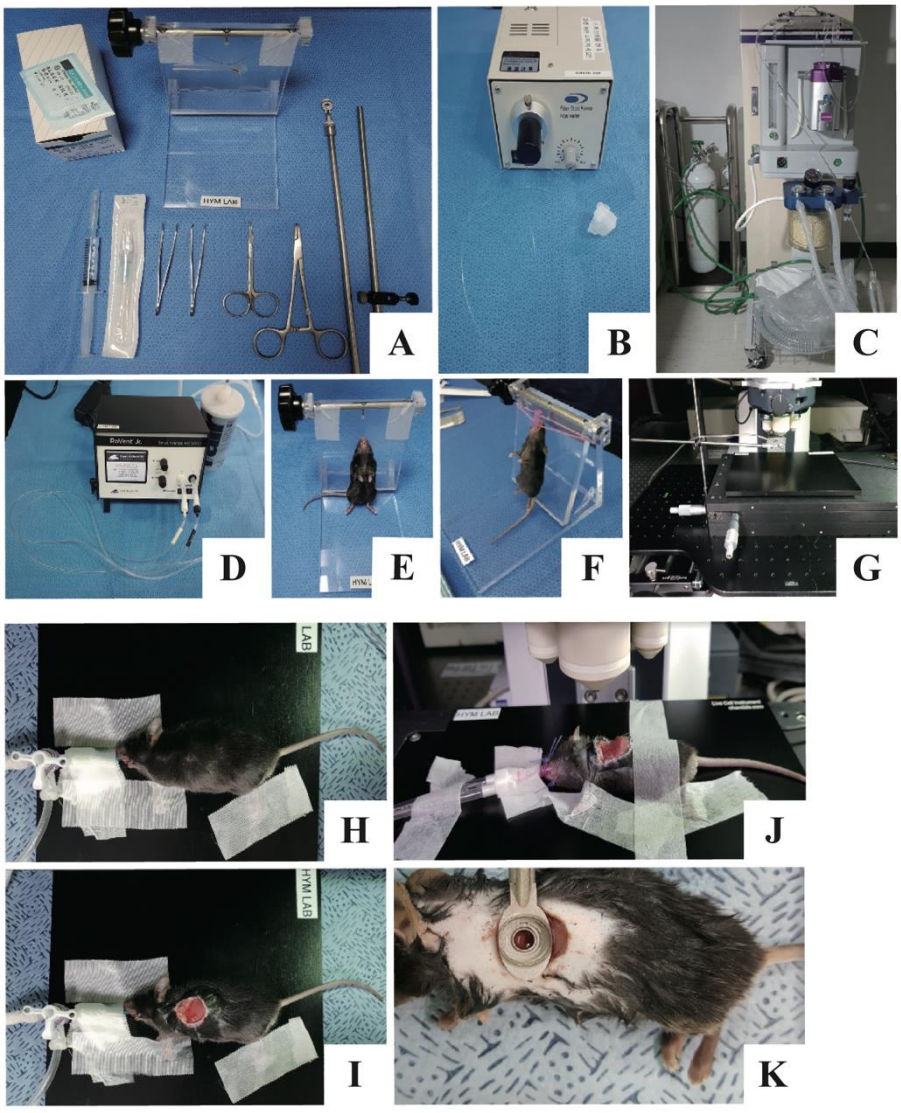

Figure 1. Lung imaging setup. (A) Surgical tools. (B) Intubation tools (LED source). (C-D) Ventilator. (E-F) Intratracheal injection, IT. (G) Imaging chamber. (H-K) Fixation of the mice on chamber with surgical tape.

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3. Flow cytometry

Flow cytometry was performed 3 hours after bleomycin injection using each group of animals treated by method of Ⅱ.1 to identify percent and the number of lung infiltrated neutrophils. Mice was anesthetized with zoletil-rumpum mixture, which was diluted 1:10 in PBS. Mice lung was collected after perfusion and tissue was cut into small pieces in 1 ml of HBSS medium. Tissue was incubated with 0.5 mg/ml of 2 X collagenase (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 0.1 M $CaCl_2$ and 1 mg/kg of DNase in HBSS medium at 37℃, 200 rpm for 45 minutes. 1 ml of heat inactivated FBS (Atlas Biologicals, Fort Collins, Colorado, USA) was added to incubated tissue and tissue was grinded on 70 μm strainer. Tissue was centrifuged at 10℃, 300 g for 5 minutes. After supernatant was removed, pellet was suspended with 1 ml of ACK lysis buffer (Gibco, Waltham, Massachusetts, USA) and incubated for 1 minutes. Cells were centrifuged at 10℃, 300 g for 5 minutes and supernatant was removed. Cells were suspended with 1 ml of FACS buffer (PBS containing 0.5% BSA and 0.1% azide) and counted using hemocytometer. 1 x 10^6 cells were suspended with 500 μl of FACS buffer and 0.2 μg of Fc blocking antibody (Ultra-LEAF™ Purified anti-mouse CD16/32 Antibody, Biolegend, San Diego, California, USA) was added. After incubation at 4℃ for 20 minutes, Cells were added with 0.5 μg of APC anti-mouse CD11b, PE anti-mouse Ly6C and FITC anti-mouse Ly6G and incubated at 4℃ for 20 minutes. Cells were centrifuged at

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4℃, 1,500 rpm for 3 minutes and washed with 500 μl of FACS buffer twice. Pellet was suspended with 500 μl of FACS buffer and flow cytometry was performed with BD LSR II.

4. Hematoxylin & Eosin staining

Hematoxylin & Eosin (H&E) staining was performed 3 hours after bleomycin injection using each group of animals treated by method of Ⅱ.1 to observe lung infiltrated neutrophils and disruption of lung structure. Mice was anesthetized with zoletil-rumpum mixture, which was diluted 1:10 in PBS and mice lung was collected after perfusion. Lung was fixed in 4% paraformaldehyde (Biosesang, Sungnam, Republic of Korea) for 24 hours. Lung was embedded in paraffin and sectioned to a thickness of 5 μm.

5. Cell migration assay analysis

In vitro neutrophil migration assay and two-photon intravital imaging results were analyzed using Volocity and Imaris (Oxford Instruments, Abingdon-on-Thames, United Kingdom) software. The number of neutrophils was counted and velocity, displacement, track length and meandering index were used as migration index.

6. Neutrophil isolation

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Wild-type and EphA2 deficient mice were sacrificed in CO_2 chamber and hip joint was isolated. Muscle and tissue surrounding bone were removed and both end of femur were cut. Bone marrow was isolated from mice femur using RPMI 1640 containing 10% FBS, 1% PSA and 2 mM EDTA. After centrifugation at 4℃, 1,500 rpm for 3 minutes, supernatant was removed and cells were suspended with 10 ml of PBS. Cells were filtered with 70 μm strainer and centrifuged at 4℃, 1,500 rpm for 3 minutes. Supernatant was removed and incubated at room temperature (RT) for 5 minutes with 2 ml of ACK lysis buffer. After centrifugation at 4℃, 1,500 rpm for 3 minutes, cells were suspended in 500 μl of Leibovitz's medium containing 10% FBS and 1% PSA and stained with 1 μM CMTPX (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 37℃ for 15 minutes. Cells were centrifuged at 4℃, 1,500 rpm for 3 minutes and washed with 500 μl of PBS twice. Neutrophils were isolated from CMTPX stained bone marrow using EasySep™ Mouse Neutrophil Enrichment Kit (Stemcell, Vancouver, Canada) according to provided protocol.

7. *In vitro* neutrophil migration assay

In vitro neutrophil migration assay was performed to analyze neutrophil migration characterization depending on EphA2 receptor.

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Confocal dish was coated with 200 μl of 10 μg/ml fibronectin (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and incubated at 37 ℃ for 1 hour. Fibronectin was removed and then confocal dish was washed with PBS twice.

Neutrophils (2 x 10^5 cells) isolated from bone marrow of wild-type and EphA2 deficient mice were suspended in 200 μl of phenol red free RPMI 1640 containing 10% FBS and 1% PSA respectively. Cells were incubated on confocal dish with 1 μM fMLP at 37℃ for 5 minutes. Confocal dish was washed with phenol red free RPMI 1640 containing 10% FBS and 1% PSA twice and floating cells were removed. Confocal dish was filled with 200 μl of phenol red free RPMI 1640 containing 10% FBS and 1% PSA and 1 μM fMLP was added. Nikon Ti2 microscope was used for *in vitro* migration assay imaging and live cell chamber supplied 37℃ temperature and mixed gas during imaging. The duration of imaging was 30 minutes and interval was 10 seconds.

8. Statistical analysis

All experiments were replicated at least three times. Statistical results from experiments were expressed as mean ± standard deviation and t tests, One-way ANOVA and Two-way ANOVA in GraphPad Prism 7.0 were used for statistical analysis.

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III. RESULTS

1. Higher concentration of bleomycin induced disruption of lung structure

To determine bleomycin dose for study, C57BL/6J mice were treated with different concentration of bleomycin respectively. 7 days after injection, H&E straining and intravital imaging were performed. 25 mg/kg of Texas red dextran and 0.25 mg/kg of FITC anti-mouse Gr1 were injected via intravenous to label blood flow and neutrophils respectively.

H&E staining results showed that more than 5 mg/kg dose of bleomycin make alveolar septa thick and induce recruitment of immune cells (Figure 2A, C). Intravital imaging results showed that higher concentration of bleomycin increased disruption of alveoli structure (Figure 2B).

Several replication results showed that 4 mg/kg and overdose of bleomycin were lethal to mice, therefore 3 mg/kg dose of bleomycin was selected for study.

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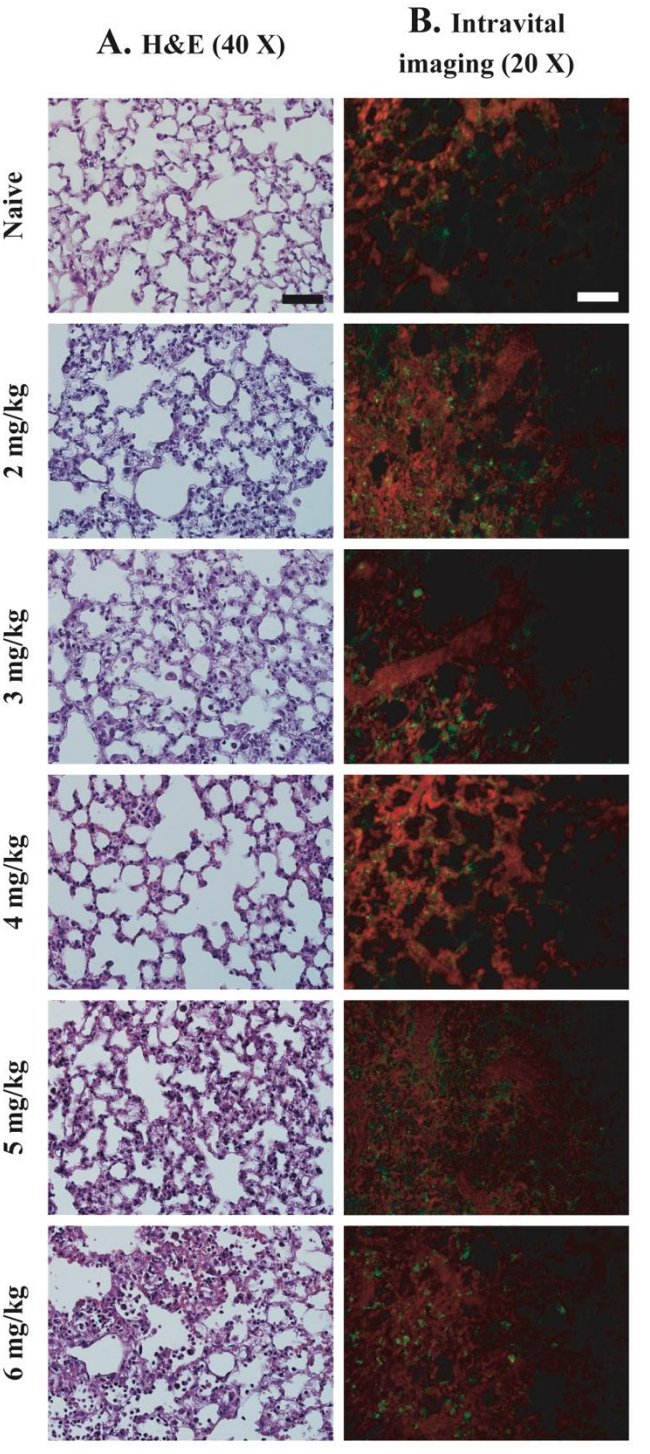

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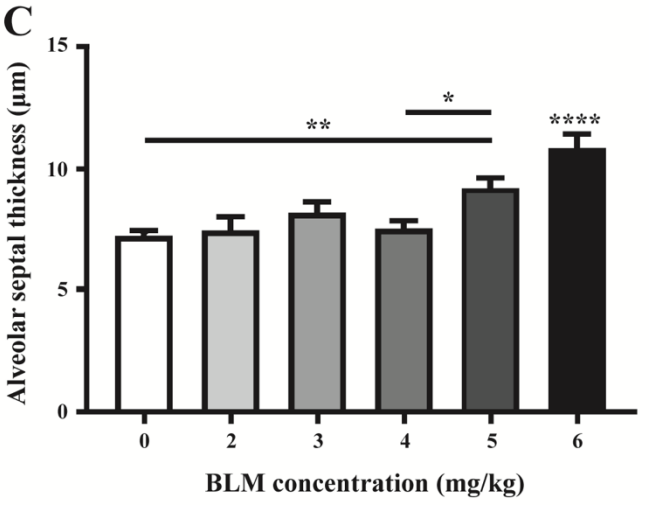

Figure 2. H&E staining and intravital imaging results depending on concentration of bleomycin. (A) Histological change depending on the concentration of bleomycin after 7 days of intratracheal injection (Scale bar=50 um). Higher concentration of bleomycin induced the accumulation of immune cells. (B) Intravital imaging results depending on the concentration of bleomycin. Higher concentration of bleomycin induced disruption of alveolar structure (Scale bar=50 um). (C) Higher concentration of bleomycin induced the expansion of alveolar septa. *, p value < 0.05; **, p value < 0.01; ****, p value < 0.0001

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2. After bleomycin injection, populations of lung infiltrated neutrophils were not different between wild-type and EphA2 deficient mice.

Flow cytometry was performed to verify the number of lung infiltrated neutrophil depending on EphA2 receptor 3 hours after bleomycin intratracheal injection.

The gating strategy is shown as Figure 3A. Total lung cells were first gated on a forward scatter (FSC)/side scatter (SSC) plot. Granulocytes were gated on the SSC/CD11b+ and neutrophils were separated according to CD11b+Ly6C^{mid}Ly6G+. 3 hours after bleomycin injection, both wild-type and EphA2 deficient mice showed increase of neutrophil percent and number compared to naïve state but percent and the number of infiltrated neutrophils in lung tissue were not different between wild-type and EphA2 deficient mice (Figure 3B-C).

Data were analyzed using FlowJo software and percent of the neutrophil was gated on whole lung cells.

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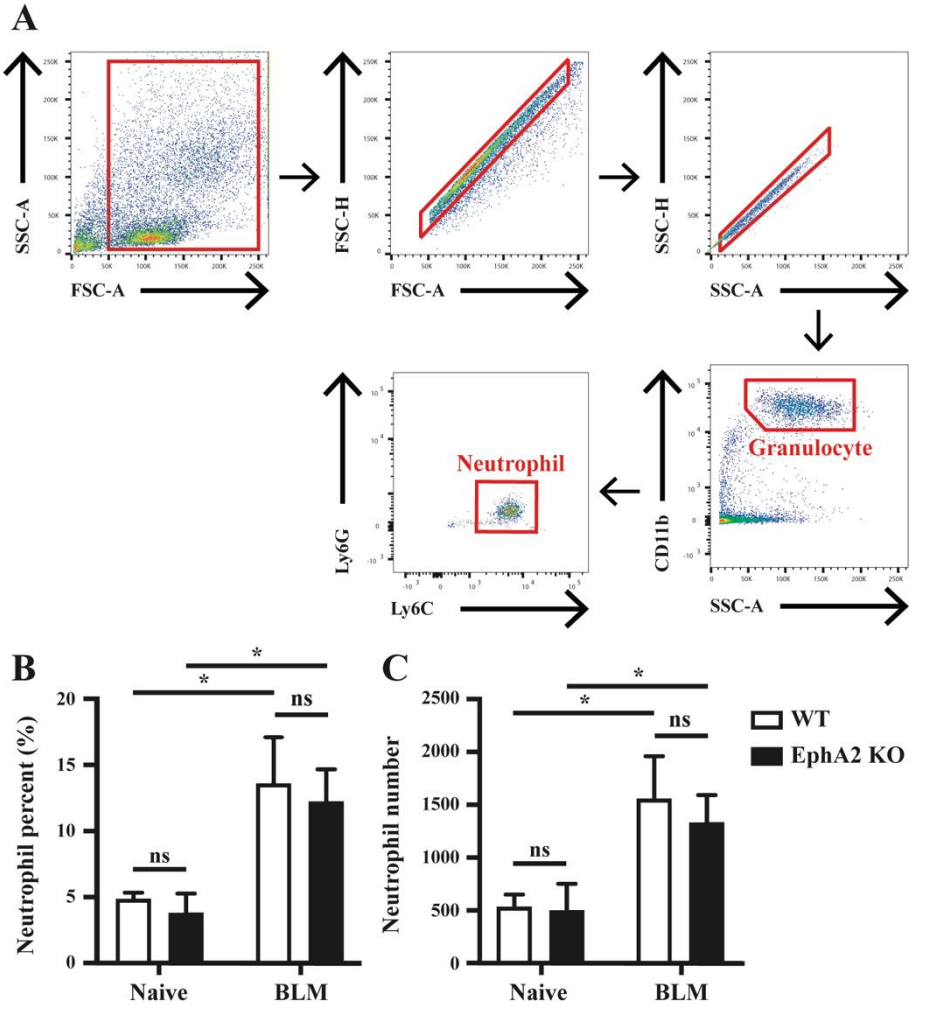

Figure 3. Flow cytometry gating strategy and population of lung infiltrated neutrophil after bleomycin injection. (A) Flow cytometry gating strategy. (B-C) 3 hours after bleomycin injection, both wild-type and EphA2 deficient mice showed increase of neutrophil percent and number compared to naïve state, but there were non-significant results in population of lung infiltrated neutrophils between wild-type and EphA2 deficient mice. *, p value < 0.05

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3. After bleomycin injection, recruitment of immune cells was observed in lung tissue.

3 hours after bleomycin injection, H&E staining was performed to observe histological change of lung and infiltration of immune cells between wild-type and EphA2 deficient mice. Significant disruption of alveolar structure and expansion of alveolar septa were not observed, but both WT and EphA2 deficient mice showed increased recruitment of immune cells compared to naïve state.

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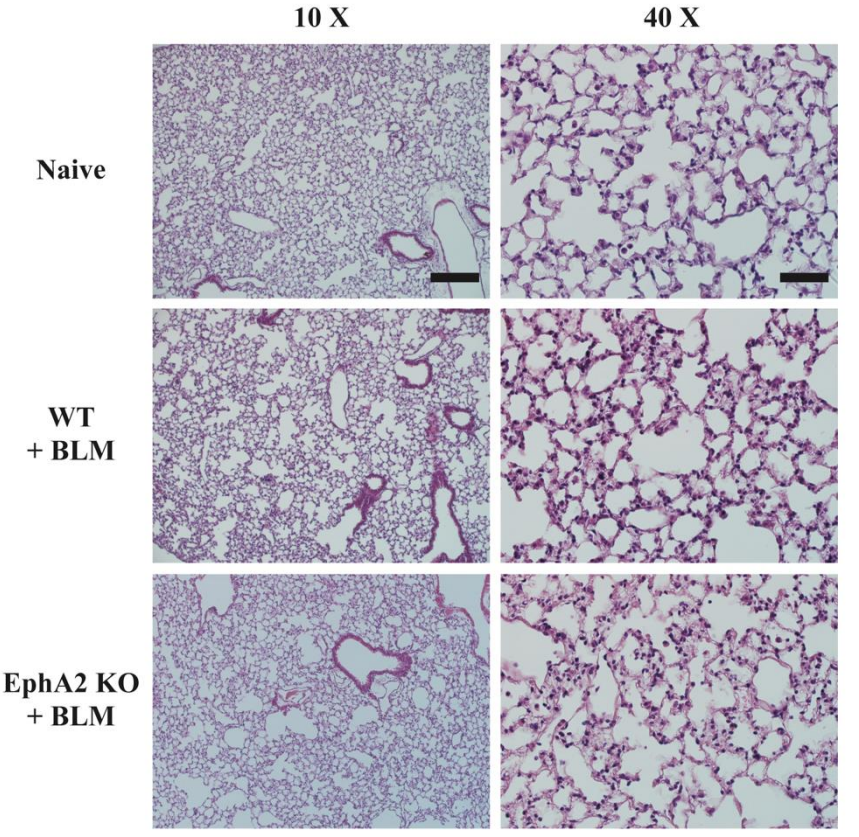

Figure 4. Histological change after bleomycin injection. 3 hours after bleomycin injection, disruption of alveolar structure and expansion of alveolar septa were not observed, but both WT and EphA2 deficient mice showed recruitment of immune cells compared to naïve state (Scale bar=200 μm in 10 X images and Scale bar=50 μm in 40 X images)**.**

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4. Track length and velocity were increased in EphA2 deficient neutrophils than wild-type *in vitro*.

In vitro neutrophil migration assay was performed to verify the effect of EphA2 receptor on neutrophil migration. Neutrophil was incubated on fibronectin-coated confocal dish and migration assay was proceeded for 30 minutes under the condition that 37 ℃ temperature and mixed gas was supplied.

Volocity software provided migration pattern of neutrophils as shown in figure 5A, B and analyzed neutrophil migration as number.

As a result, when there was fMLP stimulation only, significant differences in migration track length and velocity were observed between wild-type neutrophils and EphA2 deficient neutrophils (Figure 5C-F).

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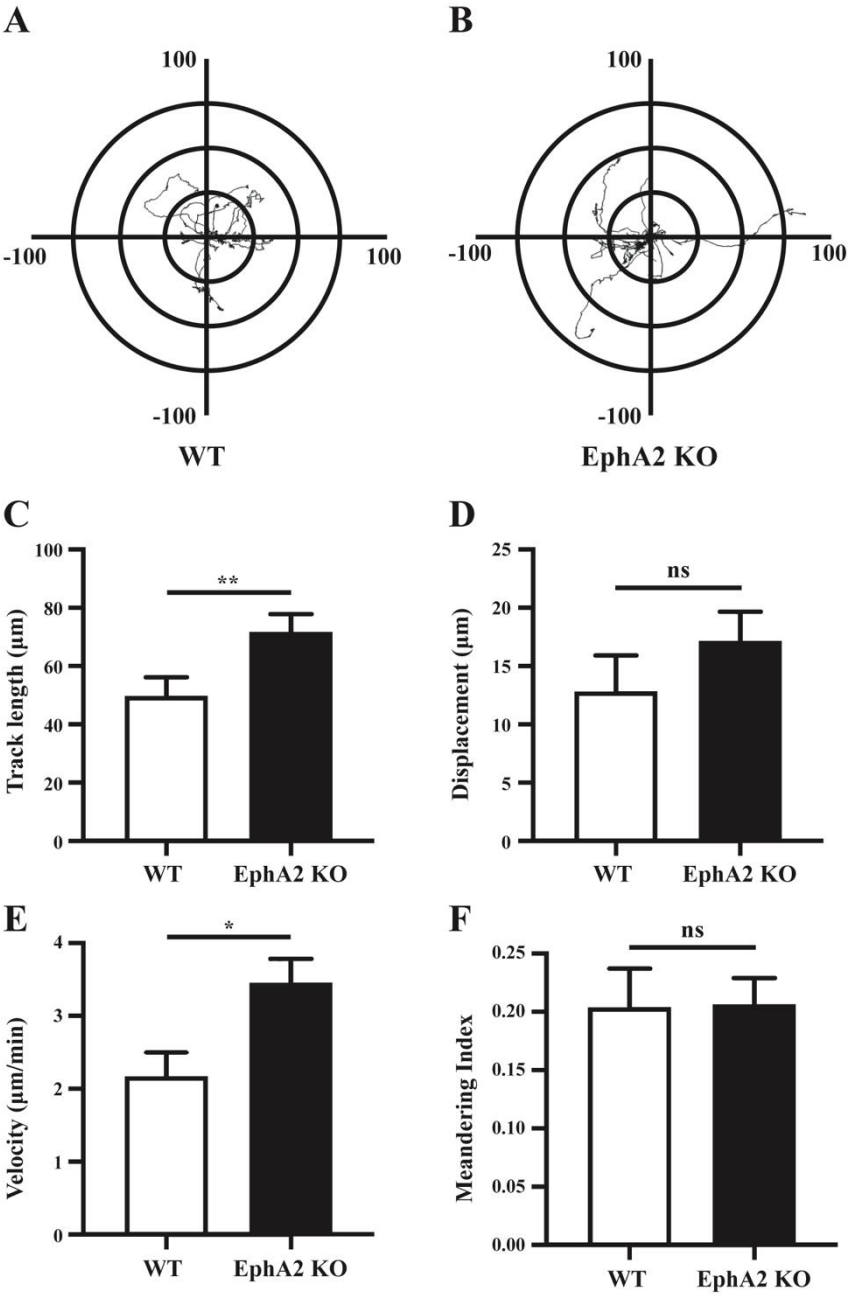

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Figure 5. *In vitro* neutrophil migration imaging and migration analysis. (A-B) Pattern of Neutrophil migration induced by fMLP stimulation only. (C-F) Track length and velocity were significantly increased in EphA2 deficient neutrophils. *, p value < 0.05; **, p value < 0.01

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5. Bleomycin decreased the migration index value both in wild-type and EphA2 deficient mice *in vivo*.

Intravital imaging was performed to compare neutrophil migration between wild-type and EphA2 deficient mice in naïve or acute lung inflammation state induced by bleomycin. Intravital imaging results showed real-time blood flow and cell migration (Figure 5A).

After mice surgery, imaging was proceeded for 30 minutes and cell migration for 10 minutes was analyzed using Imaris software.

As a result, the number of neutrophils recruited to lung tissue induced by bleomycin was various in every individual mice but slightly increased after bleomycin injection (Figure 5B). Track length, displacement and velocity decreased in bleomycin-treated mice groups. Significant difference in neutrophil migration index between wild-type and EphA2 deficient mice was not observed (Figure 5C-F).

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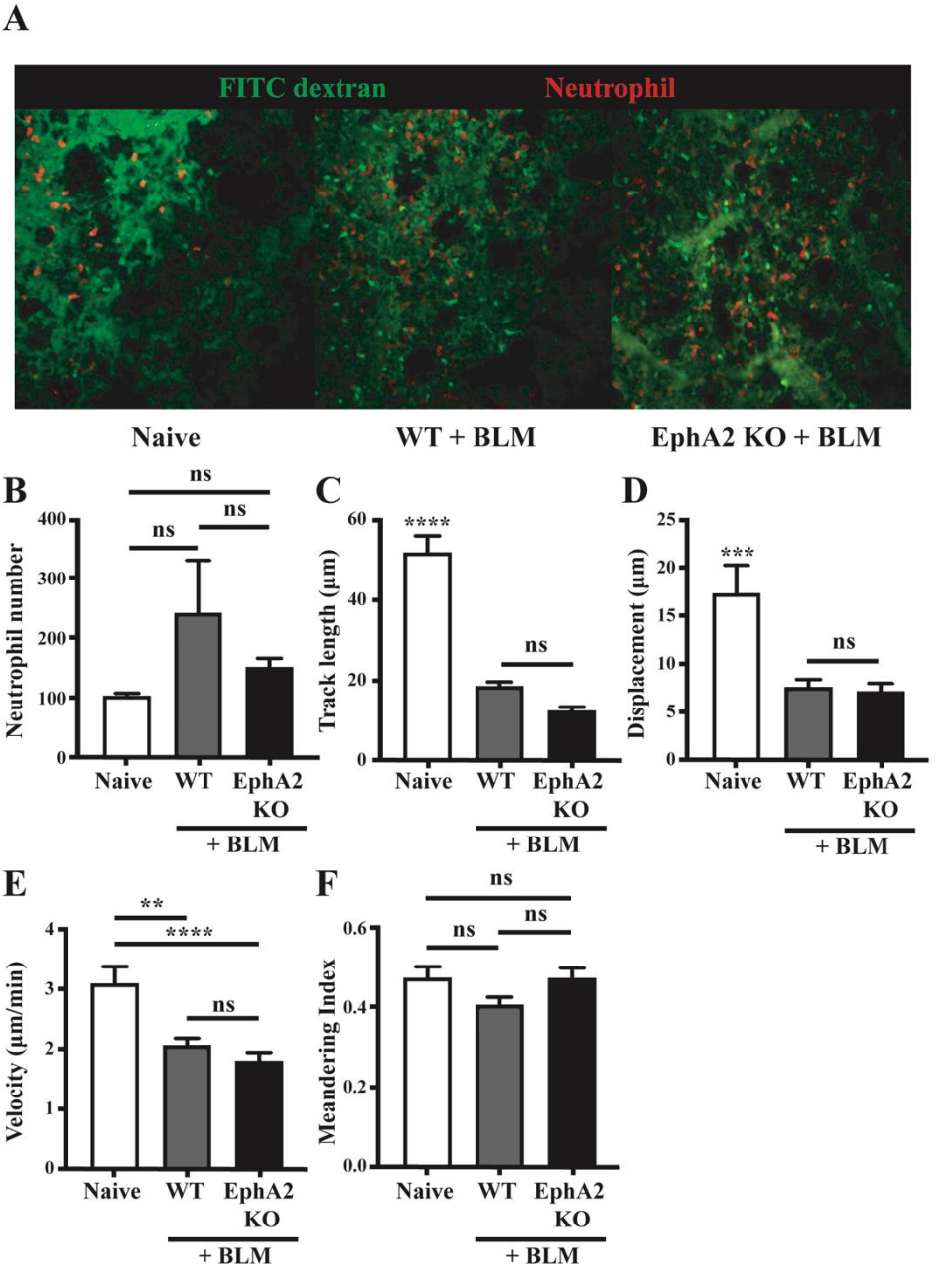

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Figure 6. Intravital imaging analysis. (A) Intravital imaging results showed real-time blood flow and cell migration. (B) The number of neutrophils recruited to lung tissue induced by bleomycin was various in every individual mice but slightly increased after bleomycin injection. (C-F) Track length, displacement and velocity were decreased in bleomycin-treated mice groups but, Significant difference in neutrophil migration index between wild-type and EphA2 deficient mice was not observed. **, p value < 0.01; ****, p value < 0.0001

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IV. DISCUSSION

Inflammation is series of processes including recognition of infection or tissue damage, secretion of cytokine and chemokines, and recruitment of immune cells into infection site[36]. Several adhesion and junctional molecules regulate immune cell transmigration through modulating endothelial integrity[37]. Eph/ephrin protein, related to neural, vascular development and epithelial homeostasis, is also known for regulating inflammation, and its expression level is increased by pro-inflammatory stimulators[38-40]. EphA2 receptor and ephrinA1 were expressed on lung endothelium and their expression levels were increased after bleomycin injection, which induces acute lung inflammation. Activated EphA2 receptor is concerned with increase of monolayer permeability, migration of immune cells from blood to interstitial tissue, and regulation of chemokine production[11,15].

Previous research have shown the relationship between EphA2 receptor and neutrophil influx using bronchoalveolar lavage fluid (BALF) and hematoxylin & eosin staining. However, to observe migration and retention in acute lung inflammation induced by bleomycin, *in vitro* migration assay and intravital cell migration are necessary.

Therefore, we examined the relationship between EphA2 receptor and neutrophil migration. Wild-type and EphA2 deficient mice were injected with bleomycin, inducing acute lung inflammation. 3 hours after injection, recruitment of neutrophils was increased both in wild-type and EphA2 deficient mice compared to naïve state, but there was no significant difference between wild-type and EphA2 deficient mice. Neutrophils isolated from wild-type and EphA2

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deficient mice bone marrow were used for *in vitro* migration assay, and significant differences in migration velocity and track length were observed between wild-type and EphA2 deficient neutrophils. After bleomycin injection to wild-type and EphA2 deficient mice, intravital imaging was performed to observe blood flow and neutrophil migration in real-time. The number of recruited neutrophils to lung tissue varied but slightly increased after bleomycin injection. Both wild-type and EphA2 deficient mice that were injected with bleomycin showed downregulated migration index in track length, displacement, and velocity, which can be interpreted as bleomycin induced rolling, adhesion and crawling of neutrophils.

In this study, we discovered that EphA2 receptor expressed on neutrophils can modulate neutrophil migration in a ligand-independent manner *in vitro* but not *in vivo*, due to the complexity of *in vivo* systems and the diversity of factors that can modulate neutrophil migration simultaneously.

For further study, downstream signaling study of EphA2 receptor expressed on neutrophils must be performed to verify *in vitro* migration assay results. Also, intravital imaging can be performed using EphA2 deficient neutrophils transferred to wild-type mice to exclude effects of EphA2 receptor expressed on endothelium[41]. Previous studies have shown that activation of EphA2 receptor on dendritic cells increases dendritic cell adhesion using adhesion assays; therefore, adhesion assays can be used to examine the relationship between EphA2 receptor and neutrophil adhesion[18]. Lung inflammation is initiated with neutrophil recruitment, so EphA2 receptor can be a target for regulating neutrophil migration and inflammation process.

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V. CONCLUSION

In this study, we examined the effect of EphA2 receptor on neutrophil migration. *In vitro* migration assay and intravital imaging were mainly used for study. 5 mg/kg and overdose of bleomycin induced severe expansion of alveolar septa and recruitment of immune cells in lung tissue. 3 hours after bleomycin injection, recruitment of neutrophils was significantly increased compared to naïve state, but there were not significant differences in population of infiltrated neutrophils between wild-type and EphA2 deficient mice. *In vitro* migration assay showed that EphA2 deficient neutrophils have more active migration pattern than wild-type neutrophil. Bleomycin induced decrease of migration index compared to naïve state, but there were no significant differences in migration index between wild-type and EphA2 deficient mice.

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REFERENCES

1. Landen NX, Li D, Stahle M. Transition from inflammation to proliferation: a critical step during wound healing. Cell Mol Life Sci 2016;73:3861-85.
2. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124:783-801.
3. Beutler B, Jiang Z, Georgel P, Crozat K, Croker B, Rutschmann S, et al. Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large. Annu Rev Immunol 2006;24:353-89.
4. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell 2010;140:805-20.
5. Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol 2009;1:a001651.
6. de Oliveira S, Rosowski EE, Huttenlocher A. Neutrophil migration in infection and wound repair: going forward in reverse. Nat Rev Immunol 2016;16:378-91.
7. Yeung L, Hickey MJ, Wright MD. The Many and Varied Roles of Tetraspanins in Immune Cell Recruitment and Migration. Front Immunol 2018;9:1644.
8. Chae YK, Choi WM, Bae WH, Anker J, Davis AA, Agte S, et al. Overexpression of adhesion molecules and barrier molecules is associated with differential infiltration of immune cells in non-small cell lung cancer. Sci Rep 2018;8:1023.

연세대학교
YONSEI UNIVERSITY

9. Kinashi T, Katagiri K. Regulation of immune cell adhesion and migration by regulator of adhesion and cell polarization enriched in lymphoid tissues. Immunology 2005;116:164-71.

10. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol 2007;7:678-89.

11. Cercone MA, Schroeder W, Schomberg S, Carpenter TC. EphA2 receptor mediates increased vascular permeability in lung injury due to viral infection and hypoxia. Am J Physiol Lung Cell Mol Physiol 2009;297:L856-63.

12. Ivanov AI, Romanovsky AA. Putative dual role of ephrin-Eph receptor interactions in inflammation. IUBMB Life 2006;58:389-94.

13. Larson J, Schomberg S, Schroeder W, Carpenter TC. Endothelial EphA receptor stimulation increases lung vascular permeability. Am J Physiol Lung Cell Mol Physiol 2008;295:L431-9.

14. Hong JY, Shin MH, Chung KS, Kim EY, Jung JY, Kang YA, et al. EphA2 Receptor Signaling Mediates Inflammatory Responses in Lipopolysaccharide-Induced Lung Injury. Tuberc Respir Dis (Seoul) 2015;78:218-26.

15. Carpenter TC, Schroeder W, Stenmark KR, Schmidt EP. Eph-A2 promotes permeability and inflammatory responses to bleomycin-induced lung injury. Am J Respir Cell Mol Biol 2012;46:40-7.

16. Tanaka M, Kamata R, Sakai R. EphA2 phosphorylates the cytoplasmic tail of Claudin-4 and mediates paracellular permeability. J Biol Chem

연세대학교
YONSEI UNIVERSITY

2005;280:42375-82.

17. Aasheim HC, Munthe E, Funderud S, Smeland EB, Beiske K, Logtenberg T. A splice variant of human ephrin-A4 encodes a soluble molecule that is secreted by activated human B lymphocytes. Blood 2000;95:221-30.

18. de Saint-Vis B, Bouchet C, Gautier G, Valladeau J, Caux C, Garrone P. Human dendritic cells express neuronal Eph receptor tyrosine kinases: role of EphA2 in regulating adhesion to fibronectin. Blood 2003;102:4431-40.

19. Sakamoto A, Sugamoto Y, Tokunaga Y, Yoshimuta T, Hayashi K, Konno T, et al. Expression profiling of the ephrin (EFN) and Eph receptor (EPH) family of genes in atherosclerosis-related human cells. J Int Med Res 2011;39:522-7.

20. Funk SD, Orr AW. Ephs and ephrins resurface in inflammation, immunity, and atherosclerosis. Pharmacol Res 2013;67:42-52.

21. Pfaff D, Heroult M, Riedel M, Reiss Y, Kirmse R, Ludwig T, et al. Involvement of endothelial ephrin-B2 in adhesion and transmigration of EphB-receptor-expressing monocytes. J Cell Sci 2008;121:3842-50.

22. Zhou Y, Yamada N, Tanaka T, Hori T, Yokoyama S, Hayakawa Y, et al. Crucial roles of RSK in cell motility by catalysing serine phosphorylation of EphA2. Nat Commun 2015;6:7679.

23. Walker-Daniels J, Hess AR, Hendrix MJ, Kinch MS. Differential regulation of EphA2 in normal and malignant cells. Am J Pathol 2003;162:1037-42.

연세대학교
YONSEI UNIVERSITY

24. Li RX, Chen ZH, Chen ZK. The role of EPH receptors in cancer-related epithelial-mesenchymal transition. Chin J Cancer 2014;33:231-40.

25. Adamson IY, Bowden DH. The pathogenesis of bleomycin-induced pulmonary fibrosis in mice. Am J Pathol 1974;77:185-97.

26. Chaudhary NI, Schnapp A, Park JE. Pharmacologic differentiation of inflammation and fibrosis in the rat bleomycin model. Am J Respir Crit Care Med 2006;173:769-76.

27. Crystal RG, Bitterman PB, Mossman B, Schwarz MI, Sheppard D, Almasy L, et al. Future research directions in idiopathic pulmonary fibrosis: summary of a National Heart, Lung, and Blood Institute working group. Am J Respir Crit Care Med 2002;166:236-46.

28. Shi K, Jiang J, Ma T, Xie J, Duan L, Chen R, et al. Pathogenesis pathways of idiopathic pulmonary fibrosis in bleomycin-induced lung injury model in mice. Respir Physiol Neurobiol 2014;190:113-7.

29. Cheong SH, Lee JH, Lee KM, Cho KR, Yang YI, Seo JY, et al. The effects of hemodilution on acute inflammatory responses in a bleomycin-induced lung injury model. Exp Lung Res 2009;35:841-57.

30. Bakowitz M, Bruns B, McCunn M. Acute lung injury and the acute respiratory distress syndrome in the injured patient. Scand J Trauma Resusc Emerg Med 2012;20:54.

31. Johnson ER, Matthay MA. Acute lung injury: epidemiology, pathogenesis, and treatment. J Aerosol Med Pulm Drug Deliv 2010;23:243-52.

32. Nauseef WM. Biological roles for the NOX family NADPH oxidases. J

연세대학교
YONSEI UNIVERSITY

Biol Chem 2008;283:16961-5.

33. Yum HK, Arcaroli J, Kupfner J, Shenkar R, Penninger JM, Sasaki T, et al. Involvement of phosphoinositide 3-kinases in neutrophil activation and the development of acute lung injury. J Immunol 2001;167:6601-8.

34. Swidergall M, Solis NV, Wang Z, Phan QT, Marshall ME, Lionakis MS, et al. EphA2 Is a Neutrophil Receptor for Candida albicans that Stimulates Antifungal Activity during Oropharyngeal Infection. Cell Rep 2019;28:423-33 e5.

35. Cho YJ, Yi CO, Jeon BT, Jeong YY, Kang GM, Lee JE, et al. Curcumin attenuates radiation-induced inflammation and fibrosis in rat lungs. Korean J Physiol Pharmacol 2013;17:267-74.

36. Newton K, Dixit VM. Signaling in innate immunity and inflammation. Cold Spring Harb Perspect Biol 2012;4.

37. Miao H, Wang B. Eph/ephrin signaling in epithelial development and homeostasis. Int J Biochem Cell Biol 2009;41:762-70.

38. Lisabeth EM, Falivelli G, Pasquale EB. Eph receptor signaling and ephrins. Cold Spring Harb Perspect Biol 2013;5.

39. Wu B, Rockel JS, Lagares D, Kapoor M. Ephrins and Eph Receptor Signaling in Tissue Repair and Fibrosis. Curr Rheumatol Rep 2019;21:23.

40. Zhang J, Hughes S. Role of the ephrin and Eph receptor tyrosine kinase families in angiogenesis and development of the cardiovascular system. J Pathol 2006;208:453-61.

41. Miyabe Y, Kim ND, Miyabe C, Luster AD. Studying Neutrophil Migration In Vivo Using Adoptive Cell Transfer. Methods Mol Biol

연세대학교
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2016;1407:179-94.

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ABSTRACT (IN KOREAN)

Bleomycin 유도 폐 손상에서
EphA2에 따른 선천 면역 세포 이동 분석

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박 은 지

혈관 내피 세포에서 발현하는 Eph 수용체와 ephrin은 혈관 투과성에 영향을 주어, 혈류나 면역 세포가 내피 세포의 사이에 있는 공간을 통과하여 세포간 조직으로 이동하는 것을 조절한다. 면역 세포에서 발현하는 Eph/ephrin 단백질은 리간드 의존적인 방식으로 면역 세포의 교통을 조절하며, 암세포에서 Eph 수용체는 리간드 비의존적인 방식으로 인테그린과 작용하여 세포 부착을 조절한다고 알려져 있다. EphA2 수용체 결손은 bleomycin에 의한 폐 손상을 감소시킨다고 알려져 있지만 EphA2 수용체가 세포의 혈구누출과 정체에 영향을 주는지는 알려지지 않았다.

본 연구에서는 EphA2 knock out (KO) 마우스와 대조군인

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C57BL/6J 마우스에 bleomycin으로 급성 염증 반응을 유도하고, 폐에 침윤된 호중구를 유세포 분석과 H&E 염색으로 확인하였다. EphA2 수용체에 의해 호중구의 이동 양상이 달라지는 지 확인하기 위해 골수에서 분리한 호중구를 이용하여 *in vitro* 세포 이동 분석을 진행하였다. 또한, 이광자 현미경을 이용한 생체 이미징을 진행하여 정상 상태와 염증 상태에서, EphA2 수용체 유무에 따른 호중구의 움직임을 분석하였다.

5mg/kg 이상의 bleomycin은 폐포 중격의 확장을 유도하였으며, bleomycin 투여 3시간 후, 폐에 침윤된 호중구의 수는 정상 상태일 때 보다 증가하였지만, EphA2 KO 마우스와 대조군간의 차이는 존재하지 않았다. *In vitro*에서 EphA2 결손 호중구는 대조군보다 높은 이동 길이와 속도를 보였고, *in vivo*에서 bleomycin은 세포 이동을 감소시켰지만, EphA2 KO과 대조군과의 차이는 존재하지 않았다.

따라서, 호중구에 존재하는 EphA2 수용체는 리간드 비의존적인 방법으로 세포 이동을 조절할 수 있지만, *in vivo*에서는 호중구의 이동을 조절하는 다양한 요소들이 복합적으로 작용하기 때문에 EphA2의 영향이 크지 않은 것으로 보인다. 염증은 호중구의 모집으로 시작하기 때문에, EphA2 수용체는 호중구의 이동과 면역 반응을 조절하는 타겟이 될 수 있다.

핵심되는 말: 호중구, 급성 폐 손상, ephA2, 세포 이동