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Acute Amelioration of Inflammatory Activity Caused by Endothelin-2 Deficiency during Acute Lung Injury

AHMAD MUSTHAFA[1], GUSTY RIZKY TEGUH RYANTO[2], RATOE SURAYA[3], TATSUYA NAGANO[3], YOKO SUZUKI[2], TETSUYA HARA[2], KEN-ICHI HIRATA[1], and NORIAKI EMOTO[1, 2, *]

[1]*Division of Cardiovascular Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe, Japan;*

[2]*Laboratory of Clinical Pharmaceutical Science, Kobe Pharmaceutical University, Kobe, Japan;*

[3]*Division of Respiratory Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe, Japan*

**Corresponding author*

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In acute lung injury (ALI), a severe insult induces a hyperinflammatory state in the lungs. The mortality rate of severe ALI remains high, and novel mechanistic insights are required to improve therapeutic strategies. Endothelin-2 (Edn2), the least studied isoform of endothelin, is involved in lung physiology and development and can be affected by various factors. One of them is inflammation, and another isoform of endothelin, endothelin-1 (Edn1), affects lung inflammatory responses. Considering the importance of Edn2 in the lungs and how Edn2 works through the same receptors as Edn1, we postulated that Edn2 may affect inflammatory responses that are central to ALI pathophysiology. In this study, we performed 24 hours intratracheal lipopolysaccharide (LPS) instillation or PBS control as an *in vivo* ALI model in eight-week-old conditional *Edn2* knockout mice (*Edn2*-iKO), with *Edn2*-floxed mice as controls. Bronchoalveolar lavage (BAL) fluid and tissue were collected after exsanguination and analyzed for its cellular, molecular, functional, and histological inflammatory phenotypes. We found that *Edn2*-iKO mice displayed a reduced pro-neutrophilic inflammatory phenotype even after acute LPS treatment, shown by the reduction in the overall protein concentration and neutrophil count in bronchoalveolar lavage fluids. Further investigation revealed a reduction in mRNA interferon gamma (*IFNγ*) level of *Edn2*-iKO lungs and suppression of its downstream signaling, including phosphorylated level of STAT1 and IL-1β secretion, leading to reduced NFκB activation. To conclude, Edn2 deletion suppressed acute lung inflammation by reducing neutrophil-mediated IFNγ/STAT1/IL-1β/NFκB signaling cascade. Targeting Edn2 signaling may be beneficial for the development of novel treatment options for ALI.

Acute lung injury (ALI) is characterized by acute inflammation of the lungs owing to various triggers, causing increased vascular permeability, progressing to acute respiratory distress syndrome and lead to respiratory failure without immediate medical attention. The increase in vascular permeability is attributed to dysregulated inflammatory reactions in the alveolar bud, including excessive recruitment of neutrophils to trigger the release of inflammatory mediators and activate their activity cascade, including cytokines, oxidants, and chemokines, e.g., interleukin (IL)-1, IL-6, interferon gamma (IFNγ), and tumor necrosis factor alpha (TNFα) [1, 2]. As acute inflammation is important in ALI, understanding the mechanisms that regulate this process is essential to treat this condition.

Endothelin-2 (Edn2) is an isoform of well-known endothelin-1 (Edn1). Despite sharing similar amino acid sequences and binding the same receptors as Edn1, the molecular mechanism of Edn2 signaling remains elusive [3, 4]. During development, Edn1, Edn3, endothelin receptor A (Ednra), and endothelin receptor B (Ednrb) are vital to neural crest cells migration. Disruption of Edn1/Ednra axis impairs migration of vagal neural crest cells [5, 6], whereas disruption of Edn3/Ednrb axis impairs migration of trunk neural crest cells [7]. Deletion of *Edn2*, however, results in histological abnormalities of the lung after birth, whereas conditional deletion of *Edn2* in adulthood results in similar but less severe abnormalities, suggesting an important role of Edn2 in development and maintenance of lung structure [8]. Notably, *Edn2* in the lung is expressed mainly in epithelial and endothelial cells that are deeply involved in lung development, homeostasis, and physiology [9].

Phone: +81-78-441-7536 Fax: +81-78-441-7536 E-mail: emoto@kobepharma-u.ac.jp

The physiology of development and maintenance of the lung has been extensively reported [10, 11]. While numerous pathways are involved in ensuring the normalcy of these processes, some have been implied to adversely affect the lungs. Inflammation during the early neonatal period impairs lung development [12] leading to bronchopulmonary dysplasia [13, 14]. Induction of inflammation in adulthood via bronchial instillation of lipopolysaccharide (LPS), a disease model of ALI, damages alveolar structure [15]. Interestingly, the endothelin family of peptides, namely Edn1, has long been known to be involved in proinflammatory responses among several organs including the lungs [16, 17]. Edn1 has been linked with ALI and cytokines/chemokines production [18]. Considering how Edn1 and Edn2 signal through similar receptors and how Edn2 is important in lung physiology, Edn2 may also affect inflammation in the lungs. Therefore, we investigated the link of acute inflammation and ALI with Edn2 to uncover how Edn2 regulates inflammatory reactions in the lung.

MATERIALS AND METHODS

Animal studies

The Ethics Review Committee of Animal Experiments at Kobe Pharmaceutical University (Approval Number: 2023-057) approved the protocols for animal experiments conducted in this study. No human subjects were involved in this study. Tamoxifen-inducible non-specific *Edn2* knockout (*Edn2*-iKO) mice were generated by crossing *Edn2*$^{flox/flox}$ (*Edn2*-floxed) mice with mice harboring tamoxifen-inducible Cre mutation (R26CreR). *Edn2*-floxed mice were generated as previously described [19]. R26CreR mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) [B6;129-*Gt(ROSA)26Sor*$^{tm1(cre/ERT)Nat}$/J]. The animals were housed in a facility with controlled temperature, humidity and a 12-h dark-light cycle. Standard chow and water were provided *ad libitum*. Mice aged 8–10 weeks were used for the experiments. Recombination was induced by intraperitoneal injection of tamoxifen at 75 mg/kg body weight/day for five consecutive days.

Mouse model of ALI

Mice were anesthetized by intraperitoneal injection of tribromoethanol (TBE) (250 mg/kg). The trachea was exposed and mice were positioned at an angle of approximately 60°. Mice received 75 µl of either sterile phosphate-buffered saline (PBS) or 1 mg/ml LPS extracted from *Escherichia coli* O111:B4 (Sigma-Aldrich; St. Louis, MO, USA), injected through the trachea using a 26-G needle. Mice were sacrificed after 24 h of instillation.

Pulmonary function test

Mice anesthetized with intraperitoneal injection of TBE were tracheostomized. Mice were subsequently connected to a FlexiVent system (SCIREQ; Montreal, Canada) and paralyzed using pancuronium bromide. Static Compliance (Cst), Tissue Damping (G), Tissue Elasticity (H), Tissue Hysteresis (G/H), Respiratory System Compliance (Crs), Respiratory System Elastance (Ers), and Respiratory System Resistance (Rrs) were measured according to the manufacturer's instructions.

Bronchoalveolar lavage (BAL) collection and analysis

BAL fluid was collected and analyzed as previously described [20]. Briefly, following the pulmonary function test, mice were euthanized by exsanguination. Bronchoalveolar fluid was collected by three serial instillations of 500 µl sterile PBS. BAL fluid was centrifuged at 1,500 rpm for 10 min at 4°C. The supernatant was separated, treated with protease and phosphatase inhibitors, and used for cytokine analysis. Protein concentration in BAL fluid was determined using Bio-Rad protein assay (Bio-Rad; Hercules, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)

ELISA for IL-6 (BioLegend; San Diego, CA, USA) and IL-1β (Abcam; Cambridge, United Kingdom) was performed on BAL fluid following the manufacturer's instructions.

Histology

Following exsanguination, PBS was perfused into the right ventricle to clear the red blood cells in the lungs. A 22-G angiocatheter was inserted and tied into the trachea, through which 4% formaldehyde was instilled at a pressure of 25 cmH_2O. Lungs were then carefully removed and submerged in 4% formaldehyde for 24 h at 4°C. Lungs were then dehydrated using graded ethanol and Hemo-De (Falma; Tokyo, Japan) and embedded in paraffin. Lung sections cut at 3 µm thickness were HE-stained and captured using All-in-One fluorescence microscope (BZ-X800; Keyence; Osaka, Japan) at 400x magnification. Lung injury score was measured as previously described [21].

Immunofluorescence

After deparaffinization and rehydration, epitope was retrieved by incubating sections in citrate-based buffer (pH 6) at sub-boiling temperature for 10 min. Sections were subsequently cooled and blocked with 5% donkey serum in PBS containing 0.1% Triton X-100 (PBS-T). Sections were then incubated overnight at 4°C using primary antibodies against NIMP-R14 (Santa Cruz Biotechnology; Dallas, Texas, USA). The slides were subsequently washed with PBS-T and incubated with Alexa Fluor 594-conjugated (1:500; Invitrogen; Waltham, MA, USA) secondary antibody at room temperature (25°C) for 1 h. The slides were incubated with Hoechst 33342 in PBS (1:2500; Invitrogen; Waltham, MA, USA) for 5 min at 25°C and mounted with 70% glycerol. Images were captured using All-in-One Fluorescence microscope (BZ-X800; Keyence; Osaka, Japan) at 400x magnification.

RNA extraction and real-time polymerase chain reaction (PCR)

RNA extraction and real-time PCR were performed as previously described [22]. Lung tissues were homogenized in RNAiso Plus (Takara; Kusatsu, Shiga, Japan), and RNA was extracted and cleaned using an RNA clean-up kit (Macharey-Nagel; Düren, Germany). Next, cDNA at concentration of 1000 ng/µl was prepared using a PrimeScript RT-PCR Kit (Takara; Kusatsu, Shiga, Japan) according to the manufacturer's instructions. Real-time PCR was conducted using FastStart SYBRgreen (Takara; Kusatsu, Shiga, Japan) with the primers listed in Table I.

Table I. Sequence of primers used for real time PCR

Organism	Name		Sequence 5'→ 3'
mouse	Edn2	F	TCTGCCACCTGGACATCATC
		R	GAGCACTCACAACGCTTTGG
mouse	EdnrA	F	TATCCTGCACCATTTTCATCGTGGG
		R	ATAAGGTCTCCAAGGGCCAGGCT
mouse	EdnrB	F	CATGCGCAATGGTCCCAATA
		R	GCTCCAAATGGCCAGTCCTC
mouse	Edn1	F	TGTATCTATCAGCAGCTGGTGGAA
		R	AAAAATGCCTTGATGCTATTGC
mouse	TNFα	F	TGGGAGTAGACAAGGTACAACCC
		R	CATCTTCTCAAAATTCGAGTGACAA
mouse	IL-1α	F	CACATCAGCTGCTTATCCAGAGCTG
		R	GGTACATACAGACTGTCAGCACTTCC
mouse	IL-6	F	CCCCAATTTCCAATGCTCTCC
		R	CGCACTAGGTTTGCCGAGTA
mouse	IFNγ	F	AGCGCCAAGCATTCAATGAG
		R	AATCTCTTCCCCACCCCGAA
mouse	ICAM1	F	TGTCAGCCACCATGCCTTAG
		R	CAGCTTGCACGACCCTTCTA
mouse	VCAM1	F	CTGGGAAGCTGGAACGAAGT
		R	GCCAAACACTTGACCGTGAC
mouse	iNOS	F	AGGTGCACACAGGCTACTCC
		R	GCCACCAGCTTCTTCAATGT
mouse	CXCL5	F	CAGTGCCCTACGGTGGAAG
		R	TAGCTTTCTTTTTGTCACTGCCC
mouse	CCL3	F	CCTACAGCCGGAAGATTCCACG
		R	ATCTGCCGGTTTCTCTTAGTCAGG
mouse	CXCL2	F	ACCAACCACCAGGCTACAGG
		R	GTTCTTGAAGTCAACCCTTGGCAG
mouse	CathepsinG	F	CTCATTGCTTGGGAAGCTCCA
		R	TAATCAGGATGGCGGATGGC
mouse	IL-18	F	TCCAACTGCAGACTGGCACA
		R	CTGATGCTGGAGGTTGCAGA
mouse	CXCR2	F	AGCAAACACCTCTACTACCCTCTA
		R	GGGCTGCATCAATTCAAATACCA
mouse	18s	F	GTAACCCGTTGAACCCCATT
		R	CCATCCAATCGGTAGTAGCG

Protein extraction and immunoblotting

Protein extraction and immunoblotting were performed as previously described [22]. Briefly, lung tissues were minced, immersed in lysis buffer containing phosphatase and protease inhibitors (Sigma Aldrich; St. Louis, MA, USA), and their concentrations were calculated using Bio-Rad protein assay (Bio-Rad; Hercules, CA, USA) and adjusted using the modified Bradford method. Protein samples were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and transferred onto nitrocellulose membranes (Bio-Rad; Hercules, CA, USA). Membranes were incubated using primary antibodies against phosphorylated signal transducer and activator of transcription 1 (STAT1) (Cell Signaling Technology; Danvers, MA, USA), STAT1, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)-p65 (Cell Signaling Technology; Danvers, MA, USA), phospho-NFκB-p65 (Cell Signaling Technology; Danvers, MA, USA), and β-actin (Cell Signaling Technology; Danvers, MA, USA) diluted at 1:1000 ratio, followed by incubation with anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:3000; Cell Signaling Technology; Danvers, MA, USA). The antibodies were diluted in Can Get Signal (Toyobo; Osaka, Japan). Protein bands were detected using Amersham ECL Select Western Blotting Detection Reagent (Cytiva; Marlborough, MA, USA).

Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Differences between two groups were analyzed using a two-tailed Student's *t*-test. Differences among three or more groups were analyzed using two-way analysis of variance (ANOVA) with Tukey's post-hoc test. A p-value < 0.05 was considered significant. Outliers were removed using the ROUT method with Q = 0.1%. All statistical analyses were performed using GraphPad Prism v.9 (GraphPad Software Inc.).

RESULTS

Loss of Edn2 impairs neutrophil recruitment during acute lung inflammation

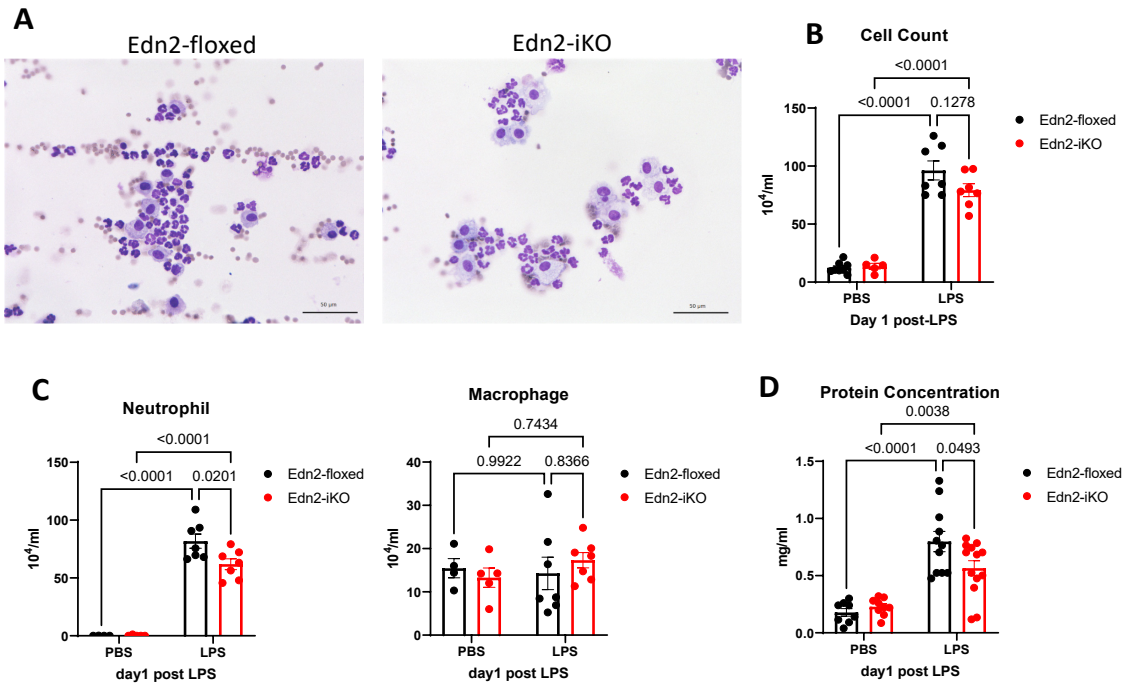

Fig. 1. Reduced neutrophil recruitment in Edn2-deficient lungs after LPS treatment. (A) Representative images of Giemsa-stained BAL fluid from *Edn2*-floxed and *Edn2*-iKO mice after 24 h of LPS or PBS treatment, (B) overall cell count, and (C) number of neutrophils and macrophages. (D) Protein concentration in BAL fluids of *Edn2*-floxed and *Edn2*-iKO mice 24 h after LPS or PBS treatment. n = 4–9 for all figures. Data are presented as mean ± standard error of mean (SEM). Two-way ANOVA with Tukey's multiple comparisons test was used for all figures. BAL, bronchoalveolar lavage; PBS, phosphate-buffered saline; LPS, Lipopolysaccharide.

As we aimed to find the link between Edn2 and lung inflammation, the effect of *Edn2* deletion during acute inflammation of the lungs was investigated first. We utilized an *in vivo* ALI disease model induced by intratracheal administration of LPS or PBS as a negative control, generated the model in *Edn2*-iKO and control *Edn2*-floxed

mice, and analyzed their phenotypes after 24 h. Surprisingly, the overall inflammatory response in the lungs was reduced in *Edn2*-iKO mice. While we confirmed the effectiveness of LPS-induced lung injury, the overall cell count in BAL fluid was not significantly different between *Edn2*-iKO and *Edn2*-floxed control mice after LPS treatment (Fig. 1A, B). However, a significantly lower number of neutrophils was observed in LPS-treated *Edn2*-iKO mice than in control *Edn2*-floxed mice (Fig. 1C), indicating reduced neutrophil recruitment. Furthermore, protein concentration in BAL fluid of LPS-treated *Edn2*-iKO mice was lower than that of *Edn2*-floxed mice (Fig. 1D).

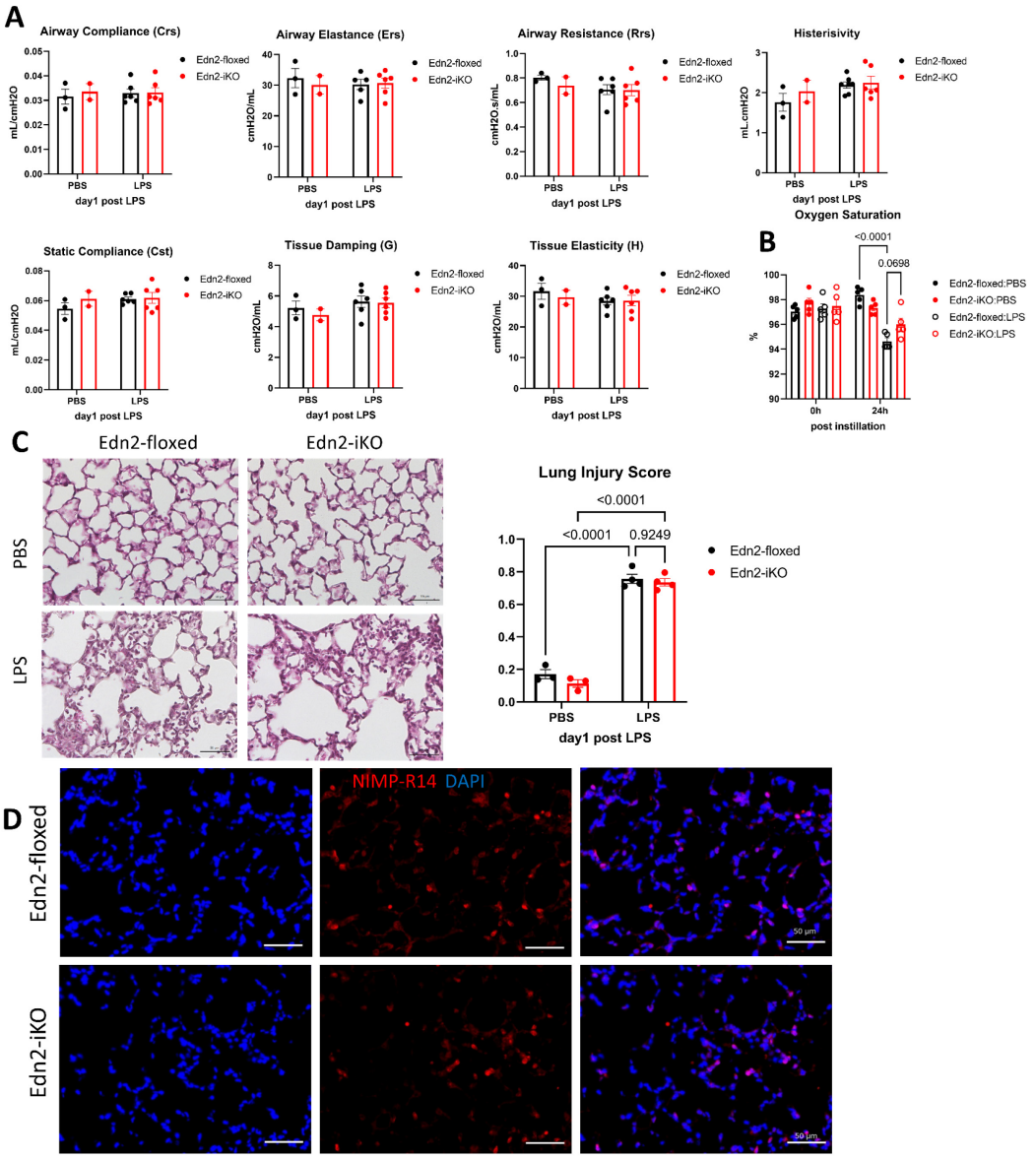

Fig. 2. Edn2 deletion did not acutely alter LPS-induced inflamed lung function and structure.
(A) Lung function parameters of *Edn2*-floxed and *Edn2*-iKO mice were analyzed after 24 h of LPS or PBS treatment. (B) Oxygen saturation levels of *Edn2*-floxed and *Edn2*-iKO after 24 h of LPS or PBS treatment. (C) Representative picture of Hematoxylin-Eosin-stained lung sections *Edn2*-floxed and *Edn2*-iKO after 24 h of LPS or PBS treatment and lung injury score quantification of the same sample. (D) Representative picture of NIMP-R14-immunostained lung sections *Edn2*-floxed and *Edn2*-iKO after 24 h of LPS treatment. n = 2–6 for figure A, n = 5–6 for figure B, and n = 3–4 for figure C&D. Data are presented as mean ± SEM. Two-way ANOVA with Tukey's multiple comparisons test was used for figure A&C. Repeated Measurement two-way ANOVA with Tukey's multiple comparisons test was used for Fig. B. PBS, phosphate-buffered saline; LPS, Lipopolysaccharide.

Both LPS treatment groups showed a worsening tendency of lung functions and peripheral oxygen saturation compared to its PBS counterparts (Fig. 2A, B). However, the suppression of neutrophilic inflammation via *Edn2* deletion could not significantly alter its functions. Similarly, while histological changes after LPS could be seen

in the lung of both mice, there was no significant difference in the lung injury score between *Edn2*-iKO and *Edn2*-floxed mice (Fig. 2C). As such, we concluded that Edn2-loss-derived inflammatory suppression alone is not enough to alter the LPS-induced changes in the lung structure and function.

Interfering with Edn2 expression reduces neutrophil recruitment to the lungs

Next, we sought to assess whether the reduction in neutrophil recruitment during acute lung inflammation has implications in cytokine production and its downstream pathways. Consistent with the general finding that deletion of *Edn2* reduces inflammation in the lungs, the expression of major inflammatory cytokines was downregulated in LPS-treated *Edn2*-iKO, although the difference was not statistically significant (Fig. 3A). In contrast, the mRNA expression of *IFNγ* was found to be blunted in LPS-treated *Edn2*-iKO mice compared to that in the LPS-treated control (Fig. 3A). However, several signaling cascades downstream of IFNγ and interferon stimulated genes were not similarly reduced (Fig. 3B, C).

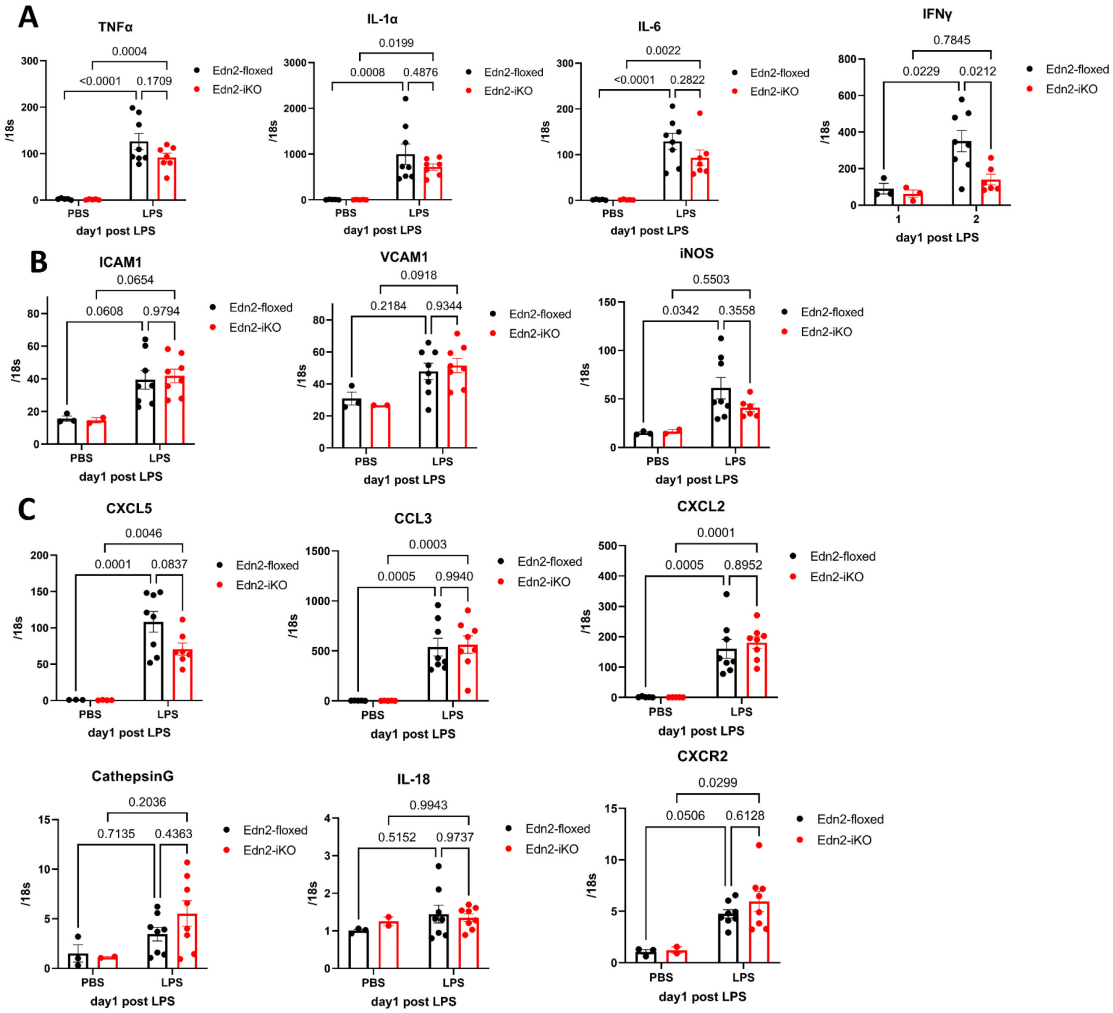

Fig. 3. Loss of Edn2 impairs upregulation of IFNγ following LPS-induced inflammation.
(A) mRNA expression of inflammatory markers in the lungs of *Edn2*-floxed and *Edn2*-iKO mice after 24 h of LPS or PBS treatment. (B) mRNA expression of IFNγ-related genes and (C) neutrophil recruitment-related genes in the lungs of *Edn2*-floxed and *Edn2*-iKO mice after 24 h of LPS or PBS treatment. n = 2–8 for all figures. Data are presented as mean ± SEM. Two-way ANOVA with Tukey's multiple comparisons test was used for statistical analysis. IFNγ, interferon gamma; PBS, phosphate-buffered saline; LPS, Lipopolysaccharide.

Further investigation into the IFNγ signaling pathway revealed a notable alteration in a specific molecular pathway leading to inflammation. A major downstream signaling molecule of IFNγ is the intracellular protein STAT1 that is phosphorylated through IFNγ [23]. In our study, lung lysates of LPS-treated *Edn2*-iKO mice had a dramatically reduced phosphorylated STAT1 level compared to that in the control mice, suggesting a potential reduction of IFNγ activity in the lungs (Fig. 4A). We then analyzed the production of inflammatory cytokines

directly involved with STAT1 activity [24] in BAL fluid and noticed a reduced level of IL-1β in LPS-treated *Edn2*-iKO mice, while no change in IL-6 levels was observed among the study groups (Fig. 4B).

We then investigated the level of NFκB, a transcription factor that can be activated by IL-1β and play a role in facilitating neutrophil recruitment and subsequent acute inflammatory responses during ALI [25, 26]. Phosphorylation of NFκB at the p65 site was significantly suppressed in *Edn2*-iKO mice even after LPS treatment (Fig. 4C). Taken together, our data indicated that *Edn2* deletion could affect the IFNγ/STAT1/IL-1β cascade of pathway during acute inflammation, which explains the reduced phenotypic severity in the lungs of *Edn2*-iKO mice.

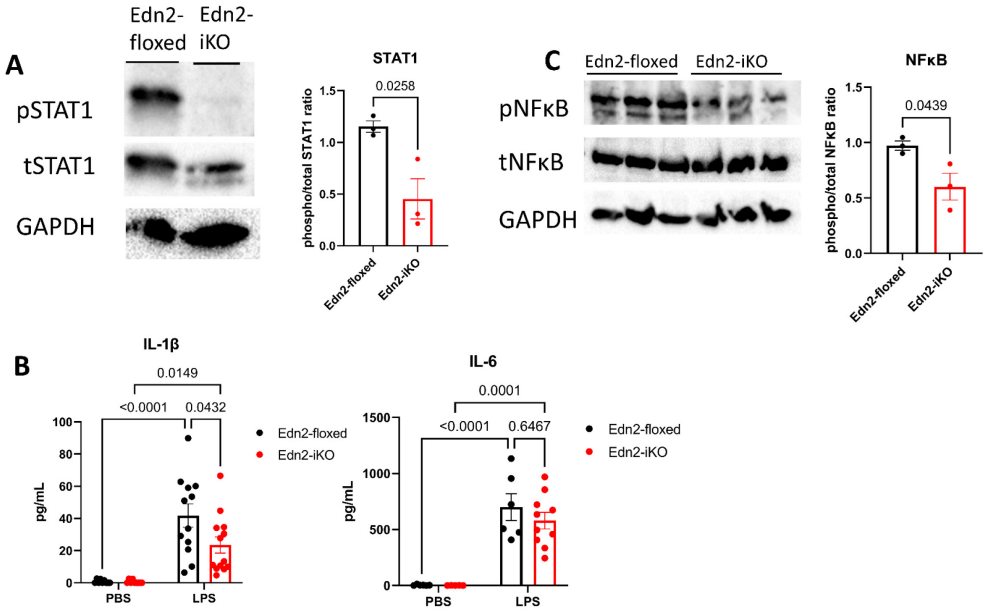

Fig. 4. Reduced activity of the STAT1-IL-1β-NFκB axis in *Edn2*-iKO mice during acute lung inflammation. (A) Representative immunoblot of phosphorylated STAT1, total STAT1, and GAPDH from lung lysates of *Edn2*-floxed and *Edn2*-iKO mice 24 h post-LPS treatment. Blotting images were cropped between 100 kDA and 75 kDA for phosphorylated STAT1 and total STAT1, and between 37 kDA and 25 kDA for GAPDH. (B) Circulating IL-1β and IL-6 levels in BAL fluids of *Edn2*-floxed and *Edn2*-iKO mice after 24 h of LPS or PBS treatment. (C) Representative immunoblot of phosphorylated NFκB, total NFκB, and GAPDH from lung lysates of *Edn2*-floxed and *Edn2*-iKO mice 24 h post-LPS treatment. Blotting images were cropped between 75 kDA and 50 kDA for phosphorylated NFκB and total NFκB, and between 37 kDA and 25 kDA for GAPDH. Data are presented as mean ± SEM. Unpaired *t*-test was used for data of A and C, and two-way ANOVA with Tukey's multiple comparisons test was used for data of B. STAT1, signal transducer and activator of transcription 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; BAL, bronchoalveolar lavage; PBS, phosphate-buffered saline; LPS, Lipopolysaccharide; NFκB, nuclear factor kappa B.

Compensatory alterations of endothelin family of peptides are not found in Edn2-iKO mice

Since Edn2 modulates its own expression by changes in the surrounding environment, we suspected that LPS might alter Edn2 expression. Instillation of LPS effectively reduced mRNA expression of *Edn2* in the *Edn2*-floxed lungs (Fig. 5A). Additionally, transcriptional modulation of proteins from the same family is common [27], possibly including the endothelin peptide family. Therefore, we verified whether this phenomenon occurs during lung inflammation. Notably, both Ednra and Ednrb, the two canonical receptors of endothelin peptides, were drastically downregulated, suggesting a downregulation of Edn2 signaling during inflammation (Fig. 5B). Edn1, the more widely studied isoform of Edn2, which is linked to various proinflammatory molecular changes, was surprisingly unaffected after LPS treatment, regardless of the presence of Edn2 in the lungs (Fig. 5B). Therefore, the phenotypic and molecular changes observed in *Edn2*-iKO mice were solely owing to the loss of Edn2 rather than a secondary effect of Edn1.

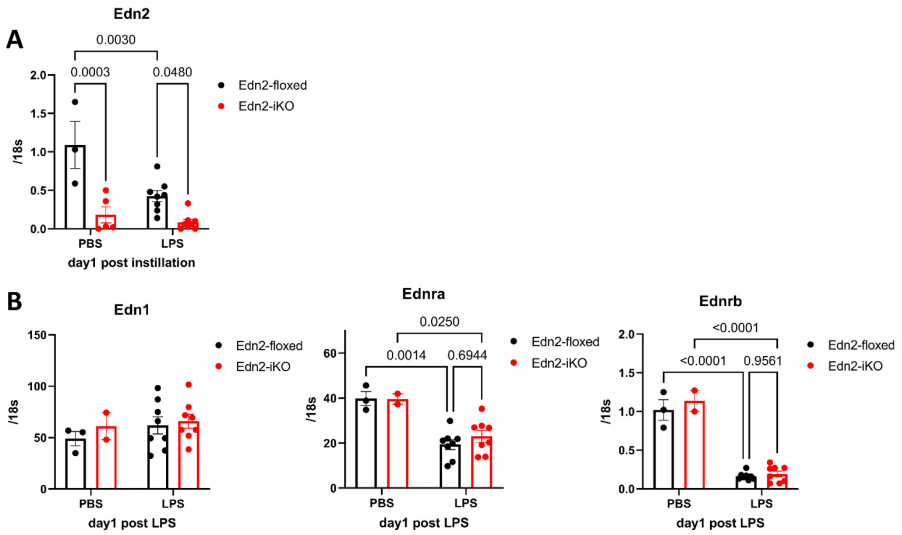

Fig. 5. LPS-induced inflammation downregulates endothelin signaling without apparent change in Edn1 level. (A) *Edn2* mRNA expression in *Edn2*-floxed lung tissue 24 h following PBS or LPS treatment. (B) mRNA expression of *Edn1*, *Ednra*, and *Ednrb* in lung tissue 24 h post-LPS treatment. n = 3–7 for A and 2–8 for B. Data are presented as mean ± SEM. Unpaired *t*-test was used for data of A, and two-way ANOVA with Tukey's multiple comparisons test was used for data of B. PBS, phosphate-buffered saline; LPS, Lipopolysaccharide; Edn1, endothelin-1; Ednra, endothelin receptor A; Ednrb, endothelin receptor B.

DISCUSSION

ALI, a condition that can rapidly progress to respiratory failure and death without rapid and proper management, is a medical problem that demands effective and efficient therapeutic solutions to improve the survival and long-term prognosis of affected patients [1, 28]. Vital in its pathophysiology is the dysregulation of inflammatory responses in the alveolar bed, amplifying its destruction without proper control [2]. Inflammation, in both neonates and adults, can adversely affect the lungs [14]. Endothelin, mainly Edn1, plays a role in various pathological conditions related to dysregulated inflammation, such as pulmonary hypertension, chronic obstructive pulmonary diseases, and ALI. Moreover, proinflammatory cytokines, such as interleukins, interferons, and TNFα, are induced by Edn1 [16–18]. With Edn1 and Edn2 binding the same Ednra and Ednrb receptors, Edn2 might modulate inflammation, a fact that may serve as a basis for further studies in this topic.

Presently, we investigated the link between Edn2 and inflammation using an *in vivo* ALI model. Deletion of Edn2 was deleterious for lung development in neonate mice, as well as for lung structure maintenance in adult mice [8]. Therefore, we initially assumed that knocking out Edn2 will similarly worsen inflammation. Surprisingly, we found that without Edn2, LPS-induced inflammation generally improved, with a specific pathway affected by the loss of Edn2. There were reduced number of neutrophils in the BAL fluid of *Edn2*-iKO mice, indicating lower neutrophil recruitment. Downregulation of the IFNγ/STAT1/IL-1β axis with corresponding reduction in NFκB activity further suggest suppressed inflammation, indicating that lungs devoid of Edn2 exhibited lesser inflammatory responses. Each of these molecules is important in the progression of ALI [29–31], and that lungs devoid of Edn2 repressed the abovementioned inflammatory axis during LPS-induced ALI could provide a novel insight into the mechanism of ALI.

Tackling hyperresponsive proinflammatory signaling has been a therapeutic strategy for ALI and other pathological conditions [32]. Our results indicated that a clear improvement in acute inflammation could be achieved in *Edn2*-iKO mice, an important finding with vital clinical implications, in line with the current ALI treatment studies which majorly focus on repressing acute inflammatory reactions in the lungs [32]. Studies pointed out how repressing neutrophil activity and pro-inflammatory signaling could prove beneficial for reversing the damaged lung, both in pre-clinical and clinical settings [1]. As we showed in this study, Edn2-null lung could achieve the desired neutrophil- and subsequent inflammatory-repressing effect in the early phase of ALI. Thus, the potency of Edn2 loss in preventing overactive inflammation in the lungs could prove beneficial for the immediate treatment of ALI.

Considering that Edn2 acts through the same receptors as Edn1, and how Edn1 is also a pro-inflammatory molecule, endothelin receptor antagonists (ERA) could become a promising treatment option during ALI. Indeed, previous studies indicated that ERA administration could lead to anti-inflammatory effects in various organs [33,

34]. In addition, several ERAs (e.g., bosentan, macitentan, and ambrisentan) has been clinically approved as a treatment option for pulmonary arterial hypertension (PAH), while in other conditions (e.g., diabetic nephropathy and essential hypertension), ERA have successfully shown its efficacy [4, 35]. Although caution should appropriately be applied, the clinical utilization of ERA could also be expanded to ALI. For this, further studies analyzing molecular works of endothelin in lung inflammation are recommended.

We were curious to see whether any changes in endothelin signaling could indicate a regulatory mechanism within the family of peptides during inflammation. However, our results showed an overall suppression of Edn2 and its two receptors, Ednra and Ednrb, suggesting that Edn2 signaling may inhibit inflammation, and its deletion amplify inflammation. Notably, Edn1 expression remained unchanged regardless of Edn2 availability or LPS administration. As Edn1 shares the same receptor with Edn2 in the lungs, any difference found between *Edn2*-floxed and *Edn2*-iKO mice is exclusively owing to the knockout of *Edn2* rather than any compensatory change of Edn1. While involvement of Edn1 in inflammation has been known [16], this is the first study to link Edn2 to production and activities of inflammatory cytokines. We expect that this study will be an impetus for future research regarding Edn2 physiology.

This study has some limitations. LPS-treated *Edn2*-iKO mice harbored lower neutrophil infiltration, lesser IL-1β levels, and decreased NFκB level, which are all related. As we focused only in one timepoint during the acute phase, we cannot ascertain the chronological order of the findings. Furthermore, direct effect of Edn2 in the lungs and how it downregulates IFNγ are still unclear. Because lung Edn2 is expressed in many cells important in pathophysiology of ALI [9], removal of Edn2 from multiple lung cells might contribute to the phenotype observed in our mice. Determining whether each Edn2-expressing cell has different roles in the mechanism of ALI and whether cell-specific *Edn2* deletion could achieve the desired anti-inflammatory effect would be interesting. Lastly, it must be acknowledged that the long-term effects of Edn2 loss in the adult mice could cause lung structural abnormality, which was reported to appear 8 weeks after Edn2 knockout induction [8]. To minimize this factor, we decided to focus on the acute effects of Edn2 loss in the lung shortly after complete Edn2 knockout induction. As such, in this study, we specifically analyzed the inflammatory phenotype of Edn2-deficient lung 24 hours after LPS treatment. Nevertheless, it is important to study what the effect of long-term Edn2 loss in the lung would be, structurally and molecularly, in the future.

To conclude, our results suggested that the loss of Edn2 could ameliorate the proinflammatory effects of LPS by reducing the neutrophil-mediated IFNγ/STAT1/IL-1β/NFκB cascade of signaling. Targeting Edn2 signaling might be beneficial for treatment of ALI, and future studies delving further into the relationship of lung Edn2 and inflammation is warranted to successfully devise endothelin blockade-based strategies to combat ALI.

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