Inhibition of Src ameliorates the progression of pulmonary arterial hypertension in experimental mouse model

（肺動脈性肺高血圧症モデルマウスにおいて、Srcの抑制は肺高血圧症の進行を改善する）

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ABSTRACT

Backgrounds: Pulmonary arterial hypertension (PAH) is characterized by progressive remodeling of pulmonary arterioles, resulting in right heart failure. Although currently available therapeutic agents have improved clinical outcomes of PAH patients, the efficacy remains unsatisfactory. Src, originally identified as oncogene, participates in numerous signaling pathways regulating cell survival and proliferation. However, its role in the pathogenesis of PAH is largely unknown.

Methods and Results: We treated wild-type mice with monocrotaline (MCT, 600 mg/kg) or vehicle (control) once a week for eight weeks to induce PAH, and Src-specific inhibitor PP1 (4 mg/kg) was injected three times per week concurrently with MCT. Right ventricular systolic pressure was significantly higher in MCT-treated mice than in control (60.1±8.1 vs. 20.6±1.1 mmHg, $p<0.0001$), and PP1 attenuated the increase to 32.8±12.7 mmHg ($p=0.0005$). The fibrotic area in the lung was greater in MCT-treated mice than in control (14.7±0.6 vs. 5.6±2.0%, $p<0.0001$), and PP1 attenuated the increase to 8.9%±2.6% ($p=0.02$). In human pulmonary artery smooth muscle cells, endothelin increased mRNA expression of interleukin-6, and pretreatment with PP1 inhibited its increase.

Conclusions: Src inhibition may attenuate the progression of PAH induced by MCT through inhibiting inflammatory cytokine production. Our results may provide a novel clinical implication for PAH treatment.

Key words: Pulmonary arterial hypertension, Src, endothelin, pulmonary artery smooth muscle cells, interleukin-6

INTRODUCTION

Pulmonary hypertension is a progressive and life-threatening disease caused by various systemic, cardiovascular, and pulmonary diseases [1]. Pulmonary arterial hypertension (PAH), classified as group 1 of pulmonary hypertension, is characterized by progressive remodeling of pulmonary arterioles (50-100 μm in size) including prolonged vasoconstriction and increased proliferation of vascular smooth muscle cells (VSMCs), generation and accumulation of extracellular matrix, local expression of inflammatory cytokines, and endothelial injury, resulting in increased pulmonary artery resistance and right heart failure [2]. Although advancements of therapeutic agents such as endothelin receptor antagonists, phosphodiesterase 5 inhibitors, and prostacyclin have improved clinical outcome, their effects remain unsatisfactory [3, 4]. Besides, despite the accumulation of extensive research over the past decade, the mechanism of PAH remains to be elucidated [5].

Src, a non-receptor protein-tyrosine kinase, has been intensively investigated due to its association with oncogenesis. Src participates in numerous signaling pathways regulating cell survival, proliferation, and gene expression [6]. Although previous studies have demonstrated the possible involvement of Src and Src-family kinases in the pathogenesis of PAH [7, 8], these has been still poorly understood. In the present study, we examined the effect of Src inhibitor on the development of PAH to test whether Src inhibition could have a protective effect for PAH progression using monocrotaline (MCT)-induced PAH mouse model.

METHODS

Animals and study protocols

We used 8-week-old male wild type (WT, C57BL/6) mice in this study. We treated WT mice with subcutaneous injection of MCT (600 mg/kg, Sigma, St. Louis, MO, USA) or vehicle (control) once a week for eight weeks to induce PAH. Src-specific inhibitor PP1 [9] (Sigma, 4mg/kg) or vehicle was injected peritoneally three times per week for eight weeks concurrently with MCT. Systolic blood pressure (SBP) and pulse rate were measured at baseline and eight weeks of the study period. After recording of right ventricular (RV) pressure, the mice were sacrificed, and the lung was rapidly extracted.

All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH) and approved by the Institutional Animal Care and Use Committee of Hirosaki University Graduate School of Medicine, Japan.

Blood pressure and pulse rate measurements

WT mice were maintained in a warm chamber set at 37°C for 5 min before measurements. SBP and pulse rate were measured by the tail-cuff method using BP-98A

(Softron, Tokyo, Japan). After discarding the highest and lowest readings, at least 10 readings were averaged.

RV pressure measurement

Mice were anesthetized with intraperitoneal injection of a mixture of 0.75 mg/kg of medetomidine, 4 mg/kg of midazolam, and 5 mg/kg of butorphanol tartrate. 1.4 F Millar catheter (AD instruments, Dunedin, New Zealand) was inserted from right cervical vein into right ventricle, and RV pressure was analyzed using the Labchart (AD Instruments) and averaged from 10 sequential cycles.

Histological analysis

The lung was fixed in 10% formalin, embedded in paraffin, and stained with α-smooth muscle actin (SMA) or Masson-Trichrome (Soshiki Kagaku Kenkyujo, Co., Ltd.). Stained sections were visualized using a BZ-X710 fluorescence microscope (Keyence, Osaka, Japan). Pulmonary artery remodeling was assessed by measuring lumen area and vascular wall area normalized by total vascular area of the pulmonary arterioles with diameter of 20-100 μm. The total vascular area was calculated by outlining the outer smooth muscle cell layer of the vascular media, and the lumen area was calculated by outlining the inner boundary of the vascular intimal layer using image J software. Vascular wall area was calculated by

subtracting the lumen area from the total vascular area. Fibrotic area of the lung was analyzed using the BZ-X analyzer (Keyence). The captured images were imported into the software, and the fibrotic area in whole lung section was automatically extracted from the images and calculated.

Quantitative polymerase chain reaction

Human pulmonary artery smooth muscle cells (PASMCs) purchased from Lonza (Basel, Switzerland) were kept in 10% fetal bovine serum and 1% penicillin and streptomycin at 37°C in a humidified environment with 5% CO_2. PASMCs at passage 3 were plated on six-well dishes at a confluence of 70-80%. After overnight serum starvation, PASMCs were pretreated with 10 μM Src inhibitor PP1 (Sigma) or vehicle for 30 min. Then, PASMCs were stimulated with 100 nM endothelin (Sigma) or vehicle for 6 hours. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantitative polymerase chain reaction (PCR) was performed using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Specific probes were obtained from Applied Biosystems to detect interleukin (IL)-6 (Mm0046190_m1) and glyceraldehyde-3-phosphatasedehydrogenase (GAPDH) (Mm99999915_g1). The RNA expression of IL-6 was normalized by GAPDH.

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical significance was determined using two-way analysis of variance with Tukey's post hoc test using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA), and p-value less than 0.05 was determined to be significant.

RESULTS

Hemodynamic effects of Src inhibition

MCT has been widely used to induce PAH and right heart failure in rodents [7, 10, 11]. To examine whether Src inhibitor PP1 ameliorates the progression of PAH, we treated WT mice with MCT concurrently with or without PP1 and performed hemodynamic analysis. There were no significant differences in SBP or pulse rate at baseline or 8 weeks of the study period among the four groups (Table). RV systolic pressure was significantly higher in MCT-treated mice than in control mice (60.1±8.1 vs. 20.6±1.1 mmHg; $p<0.0001$), and PP1 attenuated the increase to 32.8±12.7 mmHg ($p=0.0005$) (Figure 1).

Src inhibition for vascular remodeling and fibrosis in the lung

To analyze the effect of Src inhibition on pulmonary artery remodeling, we measured lumen area and vascular wall area of the pulmonary arterioles. Representative sections of the pulmonary arterioles stained with αSMA are shown in Figure 2A. We measured a mean of 32.4±10.3 lumen area and vascular wall area per animal. The ratio of lumen area to total vascular area was significantly smaller in MCT-treated mice than in control (0.36±0.02 vs 0.54±0.06, p=0.004), whereas no difference was found between control and PP1 (0.42±0.07) in MCT-treated mice (p=0.66). The ratio of vascular wall area to total vascular area was significantly greater in MCT-treated mice than in control (0.64±0.02 vs 0.46±0.06, p=0.004), whereas no difference was found between control and PP1 (0.58±0.07) in MCT-treated mice (p=0.66) (Figure 2B). Progressive remodeling of pulmonary arterioles is one of the main features of PAH [2], and perivascular fibrosis in the lung was reported to be increased in MCT-treated mice [11]. Representative sections of the lungs stained with Masson's trichrome in the present study are shown in Figure 3A. The fibrotic area, especially in perivascular area, was greater in MCT-treated mice than in control mice, and treatment with PP1 attenuated perivascular fibrosis in MCT-treated mice. The quantification of fibrotic area in the lung was shown in Figure 3B. The area of fibrosis in the lung was greater in MCT-treated mice than in control (14.7±0.6 vs. 5.6±2.0%; p<0.0001), and PP1 attenuated the area to 8.9±2.6% in MCT-treated mice (p=0.02) (Figure 3B).

Src inhibition for IL-6 expression in PASMCs

Endothelin is considered as an essential molecule for the development of PAH, and inhibition of endothelin receptor signaling exerts a protective effect on the progression of PAH [12]. To investigate the underlying mechanism for the protective effects of Src inhibitor on the development of PAH, we measured mRNA expression levels of inflammatory cytokine IL-6 in response to endothelin with or without PP1 pretreatment in human PASMCs. IL-6 mRNA expression was increased by endothelin compared to control (2.1±0.5 vs 1.0±0.2, p=0.009), and pretreatment with PP1 significantly inhibited its increase (1.0±0.3, p=0.005) (Figure 4).

DISCUSSION

In the present study, we evaluated the effect of Src inhibitor PP1 on the development of PAH and demonstrated that 1) PP1 ameliorated the increase of RV systolic pressure and lung fibrosis in MCT-induced PAH mouse model, 2) PP1 attenuated the increase of IL-6 mRNA expression induced by endothelin in human PASMCs. Our results demonstrate the protective effects of Src inhibition on the progression of PAH and may provide new therapeutic perspectives of Src-inhibitor for the treatment of PAH.

Involvement of Src in the pathogenesis of PAH

Protein tyrosine kinases catalyze the transfer of the phosphate of adenosine triphosphate to tyrosine residues on protein substrates, resulting in enhancement of enzymatic activity and recruitment of downstream signaling molecules [13]. Src is a non-receptor cytoplasmic tyrosine kinase activated by plasma membrane receptors including G-protein coupled receptors, receptor tyrosine kinases, cytokine receptors, and integrins [6]. Src is ubiquitously expressed in the diverse tissues and an essential molecule of multiple pathophysiological pathways such as the Ras/mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase/Akt pathways, which play key roles in cell proliferation, migration, angiogenesis, and cell survival [13]. In the past decade, the involvement of Src and Src family kinases in pulmonary vascular remodeling has been suggested [7, 8]. In fact, the expression of platelet-derived growth factor (PDGF), belonged to Src family kinases, in the lung was enhanced in hypoxia-induced PAH model, and PDGF and PDGF receptor expressions were enhanced in the lung tissue obtained from human PAH patients [14, 15]. More strikingly, Src was markedly activated in PASMCs isolated from PAH patients, and PP1 inhibited proliferation of PASMCs through hypoxia-inducible factor-1α and Akt/mTOR pathway [8]. Additionally, PP1 significantly ameliorated pulmonary artery remodeling and decreased RV systolic pressure in PAH mouse model [8]. indicating that Src is involved in the pathogenesis of PAH. In the present study, treatment with Src inhibitor PP1 significantly

ameliorated the increase of RV systolic pressure and lung fibrosis in MCT-induced PAH mouse model, and our results are consistent with the findings of the previous studies.

The endothelin/Src/IL-6 signaling axis in PASMCs

Regarding to the role of inflammatory cytokines in the pathogenesis of PAH, PAH patients exhibit higher circulating levels and pulmonary expression of various inflammatory cytokines such as IL-1β, IL-6, and monocyte chemoattractant protein-1 compared to healthy controls [16, 17]. Besides, serum levels of IL-1, IL-6, and TNF-α levels were associated with an increased risk of death in PAH patients [18]. IL-6 is a multifunctional inflammatory cytokine associated with a number of diseases including PAH. Higher IL-6 level is correlated with RV dysfunction, higher pulmonary arterial pressure, and worse clinical outcome in PAH patients [19, 20]. In experimental model, IL-6 expression in the lung was increased in MCT-induced PAH mouse model [10], and lung-specific IL-6-overexpressing transgenic mice exhibited elevated RV systolic pressure accompanied with increased muscularization of the pulmonary artery [21]. In contrast, RV systolic pressure and media thickness of pulmonary artery were decreased in IL-6 knockout mice or by IL-6 blockade [22, 23]. These previous findings indicate the importance of IL-6 on the progression of PAH [24]. Endothelin, originally identified as a strong vasoconstrictor [25], is one of the key molecules in the pathogenesis of PAH [12], and endothelin receptor antagonists have become an essential agent for the treatment of PAH in

current clinical practice [1]. Previous report showed that endothelin induced IL-6 secretion from VSMCs in a dose-dependent manner [26] . In our study, endothelin led to an enhancement of IL-6 mRNA expression, and pretreatment with Src inhibitor PP1 attenuated the enhancement in PASMCs. As noted above, Src mediates intracellular signaling such as MAPK in response to G-protein coupled receptor activation including the endothelin receptor [27]. In fact, endothelin rapidly induced the phosphorylation of MAPKs, and treatment with Src inhibitor reduced the increased phosphorylation in VSMCs [27]. Besides, Src inhibitor or Src knockdown inhibited endothelin-induced cell growth and protein synthesis in VSMCs and in mesangial cells [28, 29]. Our findings suggest the possible involvement of endothelin/Src/IL-6 signaling axis as one of the mechanisms of in the development of PAH and the possibility of Src inhibition for PAH treatment. A small number of experiments was major limitations of this study, therefore, further studies are clearly required to examine the possible involvement of endothelin/Src/IL-6 signaling axis in the development of PAH.

CONCLUSIONS

Src inhibition may attenuate the progression of PAH via inhibiting inflammatory cytokine production. Our results provide possible involvement of the endothelin/Src/IL-6 signaling axis in the development of PAH and may provide a new important clinical implication for PAH treatment.

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REFERENCES

1. Galiè N, Humbert M, Vachiery JL, Gibbs S, Lang I, Torbicki A, Simonneau G, et al. 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). Eur Heart J. 2016;37:67-119.
2. Rabinovitch M. Molecular pathogenesis of pulmonary arterial hypertension. J Clin Invest. 2008;118:2372-9.
3. Kozu K, Sugimura K, Ito M, Hirata KI, Node K, Miyamoto T, Ueno S, et al. Current status of long-term prognosis among all subtypes of pulmonary hypertension in Japan. Int J Cardiol. 2020;300:228-35.
4. Ogawa A, Satoh T, Tamura Y, Fukuda K, Matsubara H. Survival of Japanese patients with idiopathic/heritable pulmonary arterial hypertension. Am J Cardiol. 2017;119:1479-84.
5. Johnson S, Sommer N, Cox-Flaherty K, Weissmann N, Ventetuolo CE, Maron BA. Pulmonary hypertension: a contemporary review. Am J Respir Crit Care Med. 2023;208:528-48.
6. Guarino M. Src signaling in cancer invasion. J Cell Physiol. 2010;223:14-26.

7. Pullamsetti SS, Berghausen EM, Dabral S, Tretyn A, Butrous E, Savai R, Butrous G, et al. Role of Src tyrosine kinases in experimental pulmonary hypertension. Arterioscler Thromb Vasc Biol. 2012;32:1354-65.

8. Liu P, Gu Y, Luo J, Ye P, Zheng Y, Yu W, Chen S. Inhibition of Src activation reverses pulmonary vascular remodeling in experimental pulmonary arterial hypertension via Akt/mTOR/HIF-1 signaling pathway. Exp Cell Res. 2019;380:36-46.

9. Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA, et al. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J Biol Chem. 1996;271:695-701.

10. Yamazato Y, Ferreira AJ, Hong KH, Sriramula S, Francis J, Yamazato M, Yuan L, et al. Prevention of pulmonary hypertension by Angiotensin-converting enzyme 2 gene transfer. Hypertension. 2009;54:365-71.

11. Qin L, D'Alessandro-Gabazza CN, Aoki S, Gil-Bernabe P, Yano Y, Takagi T, Boveda-Ruiz D, et al. Pulmonary hypertension is ameliorated in mice deficient in thrombin-activatable fibrinolysis inhibitor. J Thromb Haemost. 2010;8:808-16.

12. Hoeper MM, McLaughlin VV, Dalaan AM, Satoh T, Galie N. Treatment of pulmonary hypertension. Lancet Respir Med. 2016;4:323-36.

13. Yeatman TJ. A renaissance for SRC. Nat Rev Cancer. 2004;4:470-80.

14. Katayose D, Ohe M, Yamauchi K, Ogata M, Shirato K, Fujita H, Shibahara S, et al. Increased expression of PDGF A- and B-chain genes in rat lungs with hypoxic pulmonary hypertension. Am J Physiol. 1993;264:L100-6.

15. Perros F, Montani D, Dorfmüller P, Durand-Gasselin I, Tcherakian C, Le Pavec J, Mazmanian M, et al. Platelet-derived growth factor expression and function in idiopathic pulmonary arterial hypertension. Am J Respir Crit Cre Med. 2008;178:81-8.

16. Humbert M, Monti G, Brenot F, Sitbon O, Portier A, Grangeot-Keros L, Duroux P, et al. Increased interleukin-1 and interleukin-6 serum concentrations in severe primary pulmonary hypertension. Am J Respir Crit Care Med. 1995;151:1628-31.

17. Itoh T, Nagaya N, Ishibashi-Ueda H, Kyotani S, Oya H, Sakamaki F, Kimura H, et al. Increased plasma monocyte chemoattractant protein-1 level in idiopathic pulmonary arterial hypertension. Respirology. 2006;11:158-63.

18. Soon E, Holmes AM, Treacy CM, Doughty NJ, Southgate L, Machado RD, Trembath RC, et al. Elevated levels of inflammatory cytokines predict survival in idiopathic and familial pulmonary arterial hypertension. Circulation. 2010;122:920-7.

19. Prins KW, Archer SL, Pritzker M, Rose L, Weir EK, Sharma A, Thenappan T. Interleukin-6 is independently associated with right ventricular function in pulmonary arterial hypertension. J Heart Lung Transplant. 2018;37:376-84.

20. Chen JY, Griffiths M, Yang J, Nies MK, Damico RL, Simpson CE, Vaidya RD, et al. Elevated interleukin-6 levels predict clinical worsening in pediatric pulmonary arterial hypertension. J Pediatr. 2020;223:164-9.e1.

21. Steiner MK, Syrkina OL, Kolliputi N, Mark EJ, Hales CA, Waxman AB. Interleukin-6 overexpression induces pulmonary hypertension. Circ Res. 2009;104:236-44.

22. Savale L, Tu L, Rideau D, Izziki M, Maitre B, Adnot S, Eddahibi S. Impact of interleukin-6 on hypoxia-induced pulmonary hypertension and lung inflammation in mice. Respir Res. 2009;10:6.

23. Hashimoto-Kataoka T, Hosen N, Sonobe T, Arita Y, Yasui T, Masaki T, Minami M, et al. Interleukin-6/interleukin-21 signaling axis is critical in the pathogenesis of pulmonary arterial hypertension. Proc Natl Acad Sci U S A. 2015;112:E2677-86.

24. Pullamsetti SS, Seeger W, Savai R. Classical IL-6 signaling: a promising therapeutic target for pulmonary arterial hypertension. J Clin Invest. 2018;128:1720-3.

25. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature. 1988;332:411-5.

26. Browatzki M, Schmidt J, Kübler W, Kranzhöfer R. Endothelin-1 induces interleukin-6 release via activation of the transcription factor NF-kappaB in human vascular smooth muscle cells. Basic Res Cardiol. 2000;95:98-105.

27. Simo-Cheyou ER, Vardatsikos G, Srivastava AK. Src tyrosine kinase mediates endothelin-1-induced early growth response protein-1 expression via MAP kinase-dependent pathways in vascular smooth muscle cells. Int J Mol Med. 2016;38:1879-86.

28. Bouallegue A, Vardatsikos G, Srivastava AK. Role of insulin-like growth factor 1 receptor and c-Src in endothelin-1- and angiotensin II-induced PKB phosphorylation, and hypertrophic and proliferative responses in vascular smooth muscle cells. Can J Physiol Pharmacol. 2009;87:1009-18.

29. Mishra R, Wang Y, Simonson MS. Cell cycle signaling by endothelin-1 requires Src nonreceptor protein tyrosine kinase. Mol Pharmacol. 2005;67:2049-56.

FIGURE LEGENDS

Figure 1. Right ventricular (RV) systolic pressure in monocrotaline (MCT)- or vehicle-treated mice with or without treatment with Src inhibitor PP1.

Figure 2. (A) Representative images of the pulmonary arterioles stained with αSMA in monocrotaline (MCT)- or vehicle-treated mice with or without treatment with Src inhibitor PP1. (B) Analysis of pulmonary artery remodeling assessed by the ratios of lumen area or vascular wall area to total vascular area in MCT- or vehicle-treated mice with or without treatment with Src inhibitor PP1.

Figure 3. (A) Representative images of the lung stained with Masson-Trichrome in monocrotaline (MCT)- or vehicle-treated mice with or without treatment with Src inhibitor PP1. (B) Analysis of fibrotic area of the lung in MCT- or vehicle-treated mice with or without treatment with Src inhibitor PP1.

Figure 4. mRNA expression of interleukin (IL)-6 with or without pretreatment with Src inhibitor PP1 in human pulmonary artery smooth muscle cells treated with endothelin.

TABLE

Table. SBP and pulse rate at baseline and 8 weeks of the study period.

	SBP (mmHg)		Pulse rate (bpm)	
	Baseline	8 weeks	Baseline	8 weeks
Control	100±12	106±5	607±51	649±22
Control+PP1	102±4	106±6	561±37	584±69
MCT	104±5	98±5	577±70	592±32
MCT+PP1	110±8	106±4	593±59	565±83

Data were expressed as mean ± standard deviation. MCT, monoclotarine; SBP, systolic blood pressure.

RV systolic pressure

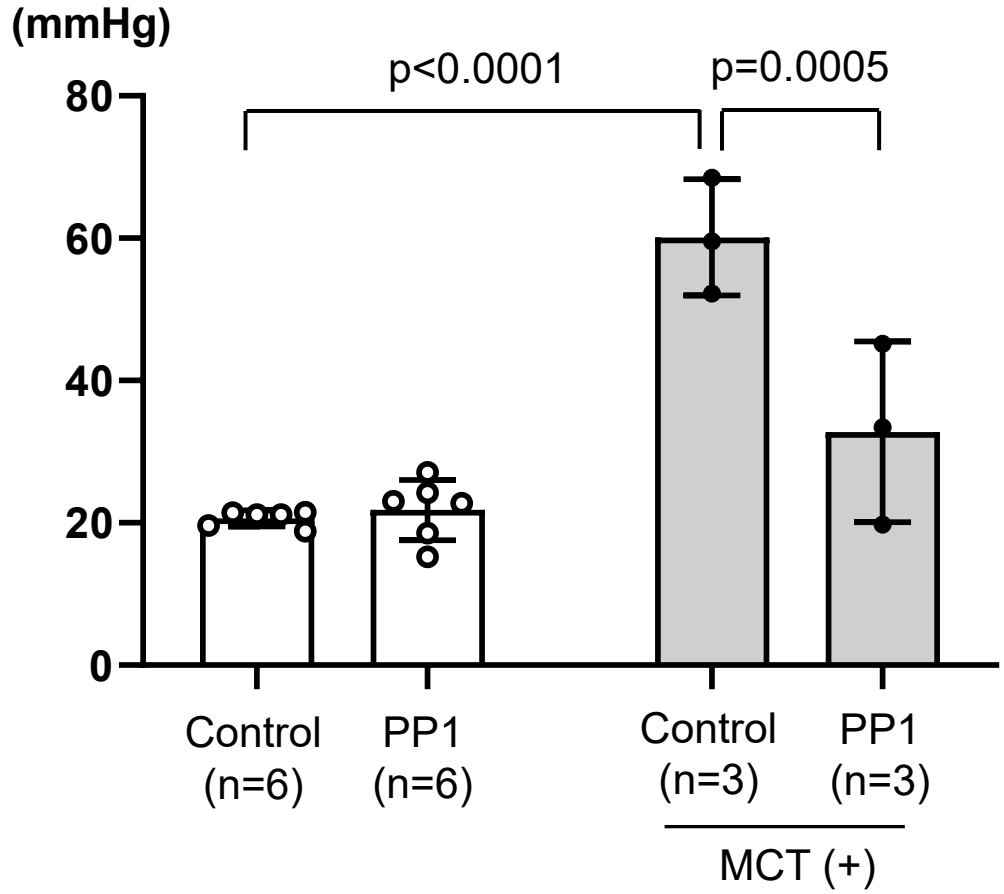
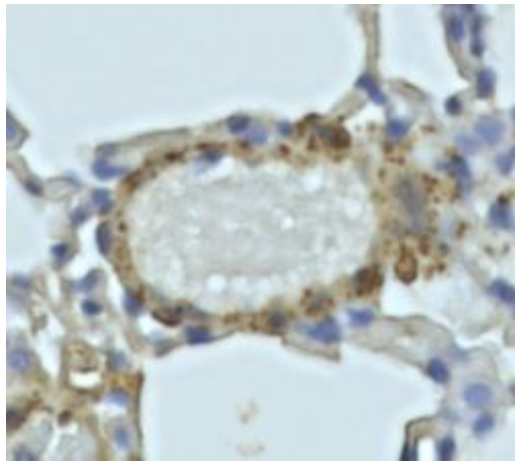
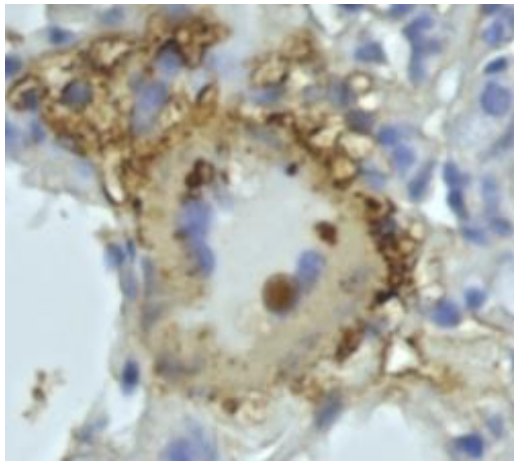


Figure 1

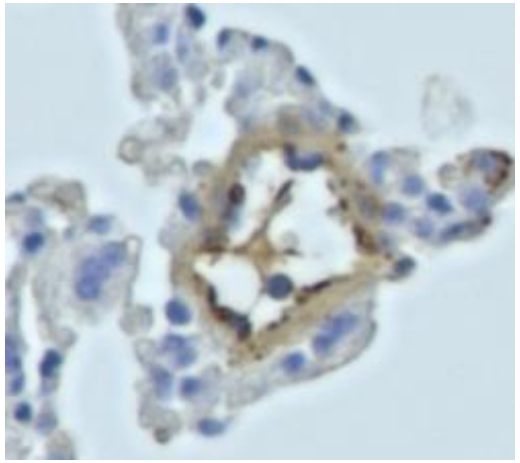
(A)



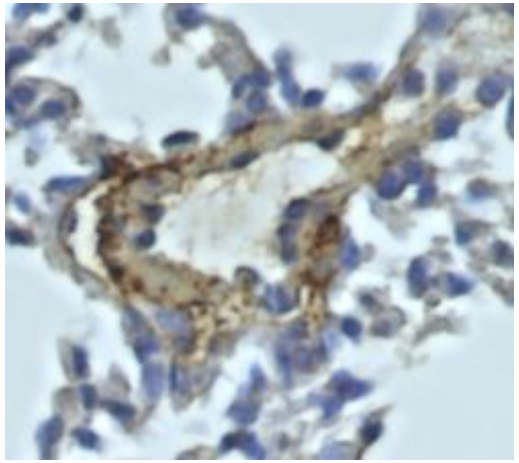
Control



MCT



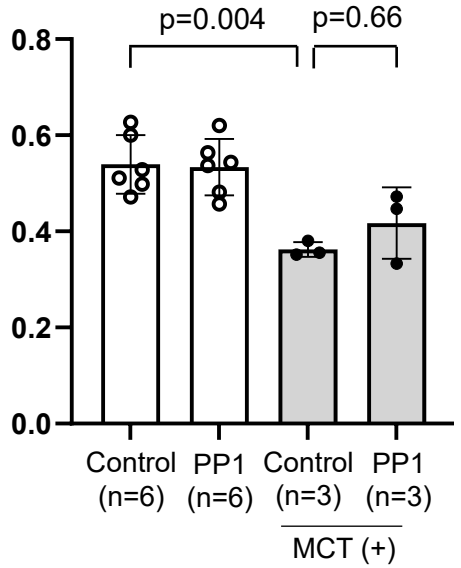
Control+PP1



MCT+PP1

50μm

(B) Lumen area / total vascular area



Vascular wall area / total vascular area

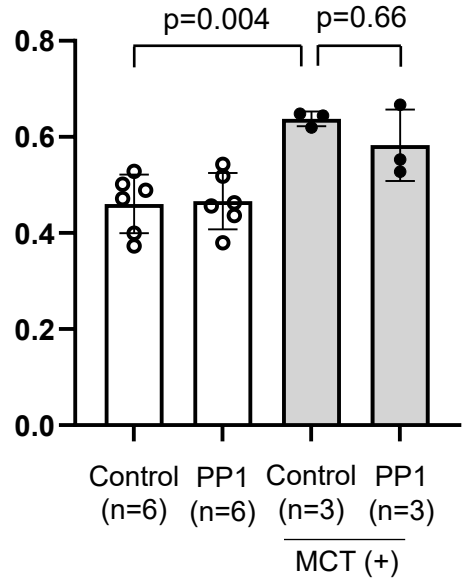
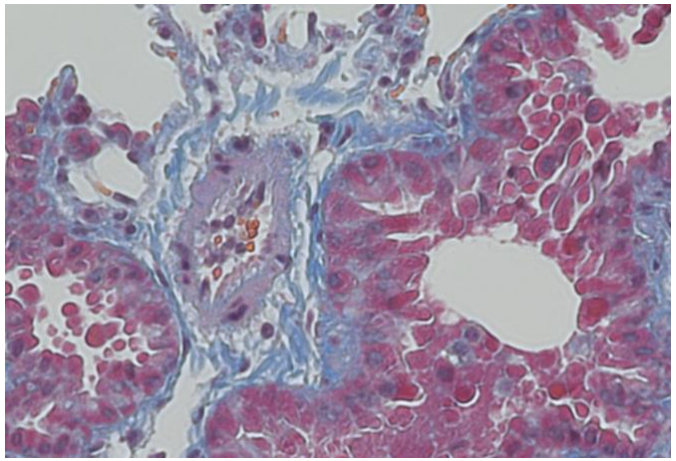
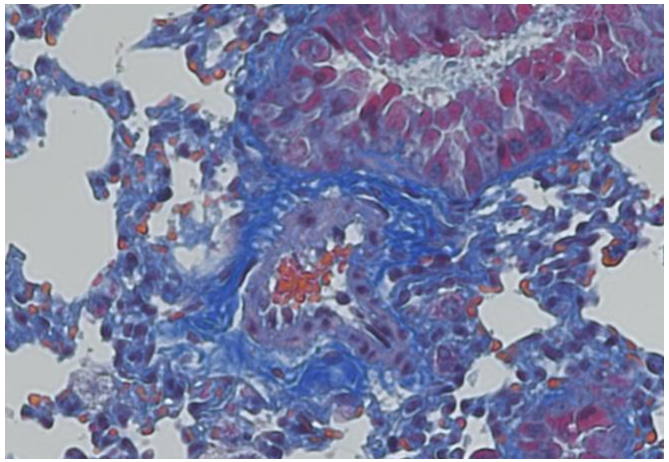


Figure 2

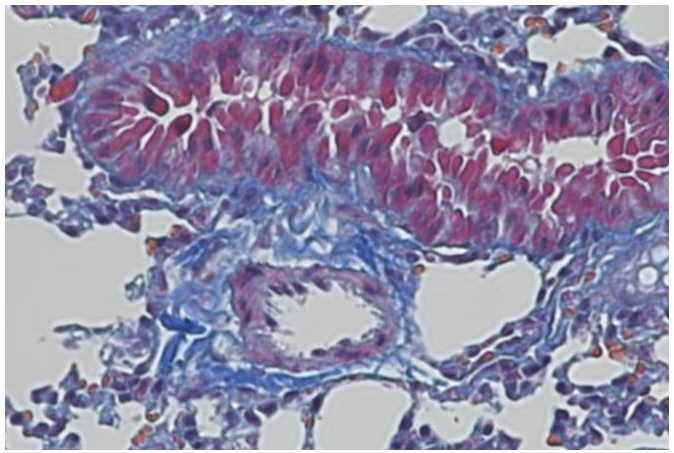
(A)



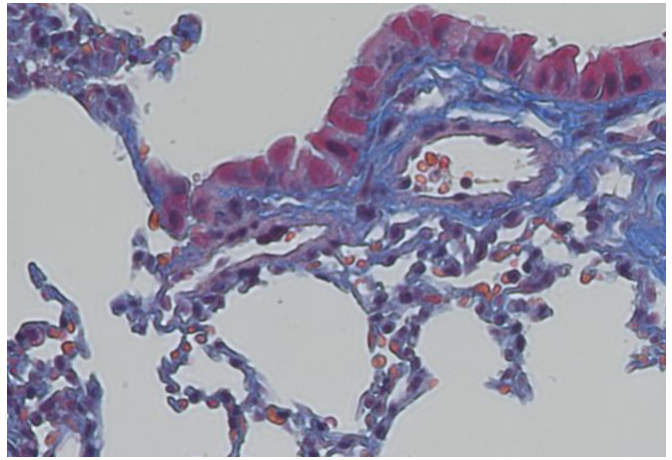
Control



MCT



Control+PP1



MCT+PP1

100μm

(B) The area of fibrosis in the lung

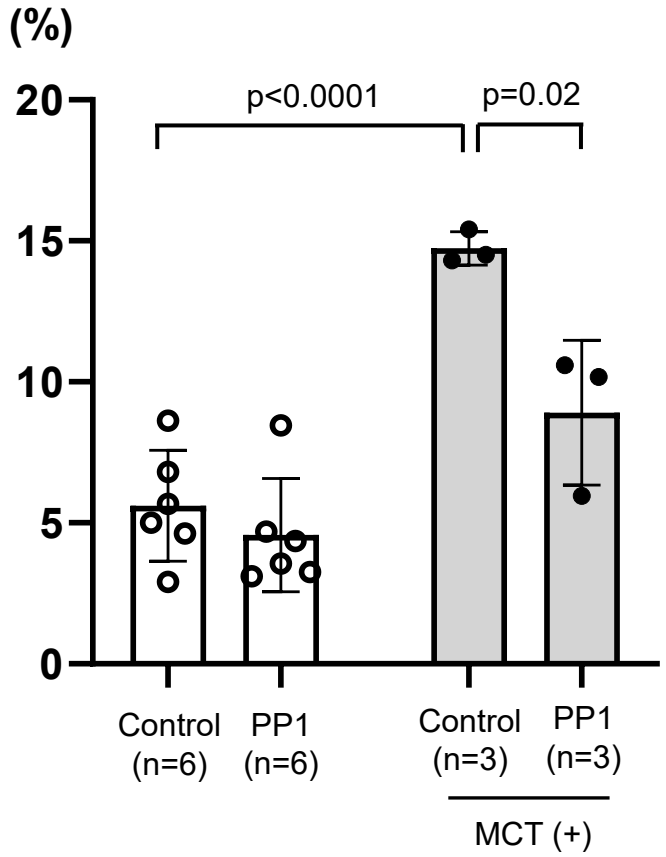


Figure 3

IL-6 / GAPDH

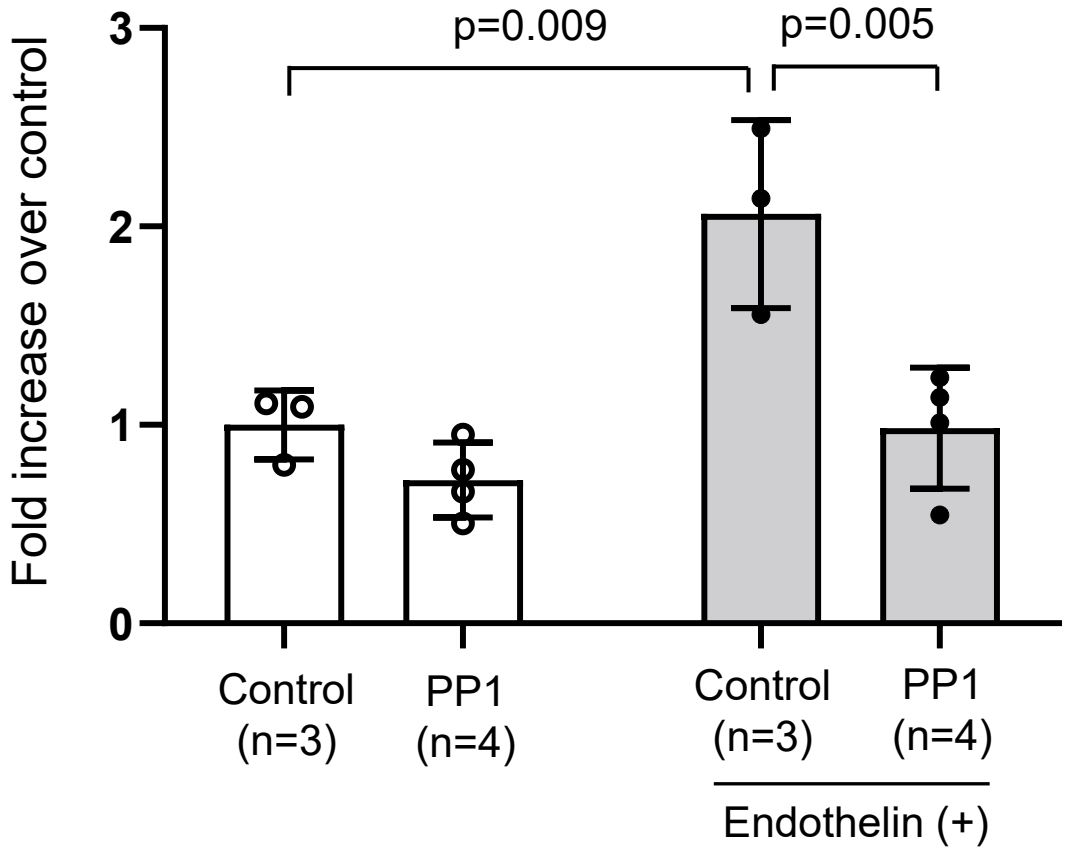


Figure 4