Transplant Immunology 57 (2019) 101242

Contents lists available at ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

Negative impact of recipient SPRED2 deficiency on transplanted lung in a mouse model

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ARTICLE INFO

Keywords:
Lung transplantation
Ischemia reperfusion injury
Extracellular signal-regulated kinase
Sprouty-related EVH1 (enabled/vasodilator-stimulated phosphoprotein homology 1)-domain-containing protein (SPRED)

ABSTRACT

Ischemia-reperfusion injury (IRI) after lung transplantation mainly contributes to the development of primary graft dysfunction. The Sprouty-related EVH1-domain-containing (SPRED) protein family inhibits the mitogen activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK) pathway. Our study was aimed at examining the role of SPRED2 in IRI in mice that received orthotopic lung transplantation. Syngeneic mouse lung transplantation was performed in wild-type C57BL/6 J (WT) mice and Spred2 knockout (Spred2 − / −) mice on the C57BL/6 J background from the WT donor. Four hours after reperfusion, blood gas analysis was performed, and lung grafts were sacrificed and analyzed. By using arterial oxygen tension measurements and histological evaluation using Lung Injury Score, we revealed more severe IRI in the grafts transplanted to *Spred2*$^{-/-}$ recipients, which manifested as exacerbated airway epithelial cell damage, interstitial edema with hemorrhage and neutrophil infiltration. Intragraft ERK1/2 activation and expression levels of proinflammatory cytokines and chemokines in *Spred2*$^{-/-}$ recipients were higher than those in WT recipients. SPRED2 plays an important role in protecting the lungs from IRI in lung transplantation recipients. We suggest that focused treatments suppressing the activity of the MAPK/ERK pathway in transplantation recipients could be the potential therapeutic option for the prevention of lung IRI.

1. Introduction

Primary graft dysfunction of the lung induced by ischemic reperfusion injury (IRI) after transplantation is not only a risk factor of perioperative mortality but may also promote acute rejection or chronic allograft dysfunction [1–7]. Because the need for transplantable organs is increasing, the number of cases of acceptance of organs donated after cardiac death is also on the rise. However, the graft is more likely to result in severe IRI because of the longer warm ischemia time after cardiac arrest. From the clinical standpoint, prevention of IRI is important for lung transplantation. In particular, precise algorithms of clinical manipulations in the recipient are needed, depending on the clinical strategy. The development of IRI involves multiple mechanisms that involve many signaling pathways. A direct relationship between the activity of the mitogen-activated protein kinase (MAPK) pathway and IRI has been demonstrated previously [8,9].

Sprouty-related, EVH1 domain 2 protein (SPRED2), described for the first time in 2001, is one of the inhibitors of extracellular signal-regulated kinase (ERK)1/2, which is a major signaling element of the MAPK pathway [10]. External stress stimuli to cells promote phosphorylation of various domains of several cellular receptors, causing activation of rat sarcoma virus oncogene (Ras), v-raf-leukemia viral oncogene 1 (Raf1), and MAPK/ERK kinase (MEK), which, in turn, activates ERK1/2 [7,8]. Although ERK translocates to the nucleus, where it induces expression of genes related to cell growth, differentiation, and migration, SPRED2, which is widely expressed in various tissues, including the lung, inhibits the MAPK/ERK pathway by binding to Ras and inhibiting phosphorylation of Raf [10–15].

The importance of SPRED2 in counteracting IRI was revealed in our previous study, where we applied hilar clamping technique to *Spred2*$^{-/}$

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https://doi.org/10.1016/j.trim.2019.101242
Received 16 April 2019; Received in revised form 10 August 2019; Accepted 21 August 2019
Available online 22 August 2019
0966-3274/ © 2019 Published by Elsevier B.V.

$^{-}$ mice [16]. We found that IRI of the lungs in *Spred2*$^{-/-}$ mice was significantly more severe than that in wild-type (WT) mice. A positive relationship between the extent of MAPK activation and lung IRI was revealed. However, the selective impact on IRI of MAPK/ERK activation in the recipient has not been clarified. We hypothesized that MAPK activation in the recipient origins could independently affect lung function in transplanted normal donors. In this study, we utilized a murine lung transplantation model to examine whether there was a relationship between the magnitude of IRI and selected recipient factors, and to explore possible clinical approaches to the treatment of IRI that could be applied specifically to the recipient.

2. Materials and methods

2.1. Animals

WT C57BL/6J mice (8–12 weeks old) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). *Spred2*$^{-/-}$ mice (8–12 weeks old) backcrossed onto the C57BL/6 J background [13,17] were bred and maintained at the Department of Animal Resources of the Okayama University (Okayama, Japan). Our experimental protocol was approved by the Animal Care Committee at Okayama University. All experimental mice received humane care in accordance with the "Principles of Laboratory Animal Care" of the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals," prepared by the National Academy of Science and published by the National Institutes of Health (NIH).

2.2. Murine lung transplantation

Orthotopic transplantation of a single (left) lung was performed by using the previously described technique [18]. Two experimental groups were employed with distinct "donor → recipient" combinations: WT → WT and WT → *Spred2*$^{-/-}$ (n = 5 in both combinations). Donor mice were anesthetized by intraperitoneal injection of ketamine (0.1 mg/g bodyweight) and xylazine (0.01 mg/g bodyweight), cannulated via tracheotomy, and provided ventilation in the form of room air at a tidal volume of 0.5 mL and a respiratory rate of 120 breaths/min. Median sternotomy was performed and heparinized by an intravenous injection of 100 IU/bolus heparin. The lungs were flushed with extracellular phosphate-buffered lung preservation solution (EP-TU solution) at 4 °C, harvested, prepared for vessel anastomosis, and stored first at 4 °C for 120 min and then at room temperature for 30 min. Recipient mice were anesthetized by the same protocol as donor animals, intubated, provided with ventilation using the same protocol as in the donors, and maintained under general anesthesia with 0.5% sevoflurane admixed in 100% oxygen. The left thoracotomy was performed, and each vessel of the donor lung was anastomosed by the cuff technique. After reperfusion, the chest was closed, and the recipient was kept under anesthesia for 4 h until sacrifice. After aspiration of the arterial blood, the grafts were harvested, divided into four parts, and stored until analysis. Each of these four parts was used for hematoxylin-eosin staining, flow cytometry, Western blotting, and RT-PCR.

2.3. Blood gas analysis

To evaluate graft function, arterial blood gas analysis was performed. The mouse was anesthetized and provided with ventilation in the same way as the donor operation. Median sternotomy was performed, right hilum was clamped with a clip for 10 min, and arterial blood was taken from the ascending aorta. Partial pressure of oxygen (PaO_2) in arterial blood was measured by a Rapid Lab 348 apparatus (Siemens Healthcare Diagnostics, Tokyo, Japan).

2.4. Histological evaluation

To evaluate the severity of graft injury after ischemic reperfusion, one of the sections of lung graft tissue was fixed in 10% formalin and stained with hematoxylin-eosin. All sections were evaluated by two pathologists blinded to the assignment and given points under the criteria described previously as the Lung Injury Score (LIS): (1) neutrophil infiltration or aggregation; (2) airway epithelial cell damage; (3) interstitial edema; (4) hyaline membrane formation; and (5) hemorrhage [19]. Each item was evaluated as normal, minimal, mild, moderate, or severe change (score 0–4) and the values were added.

2.5. Flow cytometry

Lung graft tissue was used for flow cytometry analysis following a previously described protocol [20]. In order to evaluate infiltration of neutrophils, samples were stained with fluorochrome-labeled anti-CD45, anti-CD11b, and anti-Gr-1 antibodies (BD Bio sciences, Franklin Lakes, NJ), and flow cytometry analysis was performed by using BD Accuri™ C6 (BD Bio sciences, Franklin Lakes, NJ).

2.6. Western blotting

Lung graft tissues stored at −80 °C were used for Western blotting. A detailed protocol of this procedure has been described previously [21]. The primary antibodies were anti-p44/42 MAPK (Cell Signaling Technology, Beverly, MA, USA) to evaluate total ERK1/2 (t-ERK) level, anti-phosphorylated p44/42 MAPK (Cell Signaling Technology) to assess ERK1/2 phosphorylation (p-ERK), and anti-actin (Cell Signaling Technology) to be used as control. Goat anti-mouse or anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as secondary antibodies. The membranes were analyzed using an enhanced chemiluminescence Advanced Western Blotting Detection Kit (GE Healthcare, Piscataway, NJ, USA).

2.7. Real-time reverse transcription-polymerase chain reaction technique

Lung graft tissue stored in RNAlater RNA stabilization reagent (Qiagen, Venlo, Netherlands) was used for real-time reverse transcription-polymerase chain reaction analysis. Total RNA was extracted from the stored tissue by using an RNeasy Mini Kit (Qiagen). A high capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA) was used for synthesizing first strand cDNA from the respective RNA, according to the manufacture's recommendations. Quantitative real-time reverse transcription-polymerase chain reaction was performed with primers for genes encoding TNF-α, IL-1β, CCL2, CXCL2, and GAPDH, which is encoded by a housekeeping gene, as control. The cDNA and each primer and probe set (purchased from Thermo Fisher Scientific) were injected into TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA), and mRNA expression levels were determined by an ABI StepOnePlus Real-Time PCR Instrument (Thermo Fisher Scientific) using the delta-delta-CT method.

2.8. Statistical analysis

All data were analyzed by using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). PaO_2 levels, the numbers of cells sorted by flow cytometry, and levels of proinflammatory molecules are expressed as the mean ± SEM. Statistical analysis was performed by using Mann-Whitney U test for LIS and Student's t-test for others. All statistical analyses were conducted with a significance level of $\alpha = 0.05$ ($P < .05$).

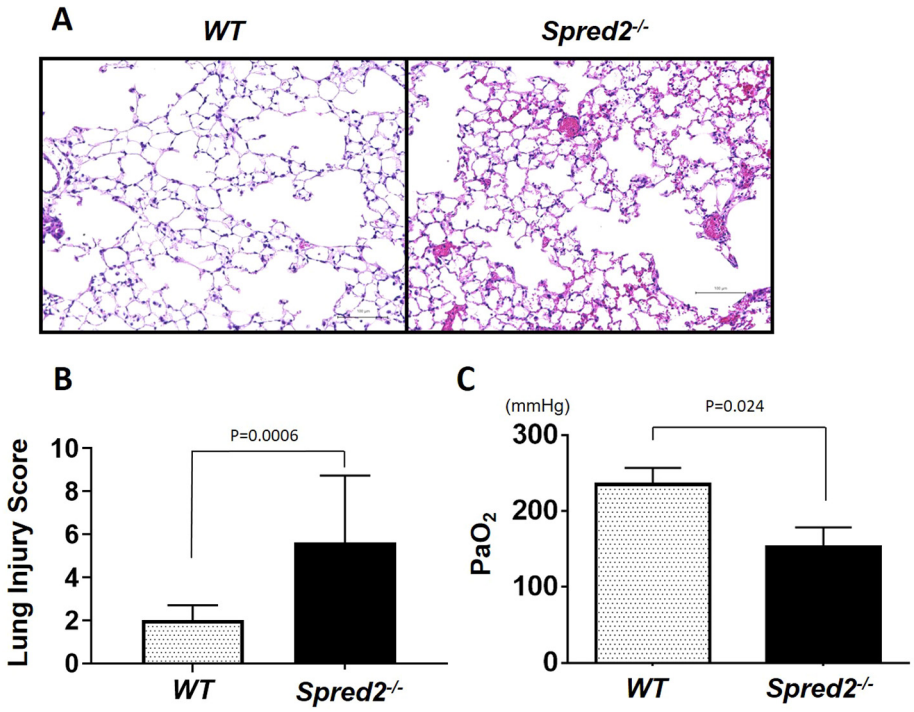

Fig. 1. Histological evaluation and pulmonary function of the graft after ischemia-reperfusion.
(A) Comparison of the hematoxylin and eosin-stained pulmonary parenchyma sections from WT and *Spred2*$^{-/-}$ mice 4 h after the reperfusion (40× magnification). The section from a *Spred2*$^{-/-}$ animal showed more severe inflammatory changes, such as interstitial edema, hemorrhagic congestion, septal thickening, and neutrophil infiltration, than the section from a WT mouse. (B) LIS in the WT and *Spred2*$^{-/-}$ groups. The score was significantly higher in *Spred2*$^{-/-}$ group than in the WT group ($P = 0.027$). (C) Pulmonary graft function 4 h after the reperfusion was evaluated by PaO_2 levels after ventilation with 100% oxygen. Arterial blood sample was obtained from ascending aorta after right hilar clamping in order to evaluate the graft function independently. PaO_2 level was significantly lower in *Spred2*$^{-/-}$ group than in WT group ($P = 0.0006$).

3. Results

3.1. Pulmonary graft function

Observations of hematoxylin-eosin stained sections of the transplanted lung revealed stronger proinflammatory changes, such as interstitial edema, hemorrhagic congestion, septal thickening, and neutrophils infiltration, in *Spred2*$^{-/-}$ mice compared to those in WT animals (Fig. 1A). LIS showed significant difference between the two groups (WT: 2.0 ± 0.63; *Spred2*$^{-/-}$: 5.6 ± 2.8; $P = .027$; Fig. 1B). PaO_2 level in the *Spred2*$^{-/-}$ group was significantly lower than that in the WT group (WT: 235.2 ± 9.7; *Spred2*$^{-/-}$: 152.5 ± 11.7 mmHg; $P = .0006$; Fig. 1C).

3.2. Number of infiltrated neutrophils in the transplanted lung graft

Gr-1$^+$, CD11b$^+$, and CD11c$^-$ characteristics were used as markers of neutrophils. Gr-1$^+$CD11b$^+$ cells and Gr-1$^+$CD11c$^-$ cells were sorted under CD45$^+$ gating (Fig. 2) by flow cytometry. Both cell subtypes were more abundant in *Spred2*$^{-/-}$ group than in WT group (Gr-1$^+$CD11b$^+$ cells: WT, 378.6 ± 57.7; *Spred2*$^{-/-}$, 856.5 ± 149.6; $P = .018$; Gr-1$^+$CD11c$^-$ cells: WT, 353.3 ± 46.5; *Spred2*$^{-/-}$, 857.4 ± 152.8; $P = .014$). These findings strongly suggested that more activated neutrophils infiltrated graft lungs in *Spred2*$^{-/-}$ than in WT recipients.

3.3. Intragraft expression status of proinflammatory molecules and ERK1/2

We examined mRNA expression levels of cytokines and chemokines involved in the regulation of neutrophil migration and infiltration in transplanted lung grafts. We found that mRNAs encoding CCL2, CXCL2, IL-1β, and TNF-α were expressed at a significantly higher level in the *Spred2*$^{-/-}$ group than in WT animals: CCL2, $P = .035$; CXCL2, $P = .012$; IL-1β, $P = .046$; TNF-α, $P = .028$. (Fig. 3A). In addition, to examine the activation of ERK1/2 of the MAPK pathway, Western blotting for p-ERK1/2 was performed. The level of p-ERK1/2 was higher in the *Spred2*$^{-/-}$ group than in WT mice, whereas t-ERK1/2 expression was equivalent in both groups (Fig. 3B).

4. Discussion

This study investigated the role of SPRED2, an inhibitor of the MAPK/ERK signaling pathway, in the development of lung IRI in recipient mice that underwent orthotopic lung transplantation. Normal donor lungs transplanted in the *Spred2*$^{-/-}$ recipient mice suffered from more severe IRI than those in WT recipient mice, which indicated that SPRED2 plays an important role by inhibiting ERK1/2 activation and attenuating IRI development in transplant recipients.

Several complex signaling pathways affect the emergence of lung IRI. Under ischemic conditions, vascular endothelial cells and alveolar macrophages in the lung tissue produce various pro-inflammatory molecules. Once reperfusion occurs, pro-inflammatory molecules stimulate migration and infiltration of the inflammatory cells, typified by the neutrophils, and induce further stress response and activation of signaling cascades, including the MAPK pathway. These physiological reactions lead to increased microvascular permeability, pulmonary vascular resistance, pulmonary edema, impaired oxygenation, and pulmonary hypertension. Collectively, these processes ultimately cause primary graft dysfunction after lung transplantation [2,8,9,22].

The reaction of MAPK pathways proceeds rapidly, from several minutes to just a few hours from the activation, resulting in the stimulation of ERK. Translocation of ERK into the nucleus promotes synthesis of several cytokines and chemokines that facilitate migration of inflammatory cells, including neutrophils, edema, and other proinflammatory manifestations [23–25]. These observations suggest that the severity of IRI seems to depend on donor factors, resulting in rapid activation of MAPK signaling pathways in the lung. Indeed, the majority of studies on IRI after transplantation have focused on the donor rather than the recipient's original factors. However, activated inflammatory cells or molecules of recipient origin play an important role in the promotion of additional inflammatory phenomena. Several studies reported that a number of recipient factors correlated with the extent of lung IRI [26,27]. Liu et al. reported that recipient monocyte migration caused by ischemia reperfusion triggered IRI in a mouse model of lung transplantation [26]. Additionally, other researchers demonstrated that the severity of inflammation in lung transplantation recipients influenced overall lung IRI in a clinical study [27]. They

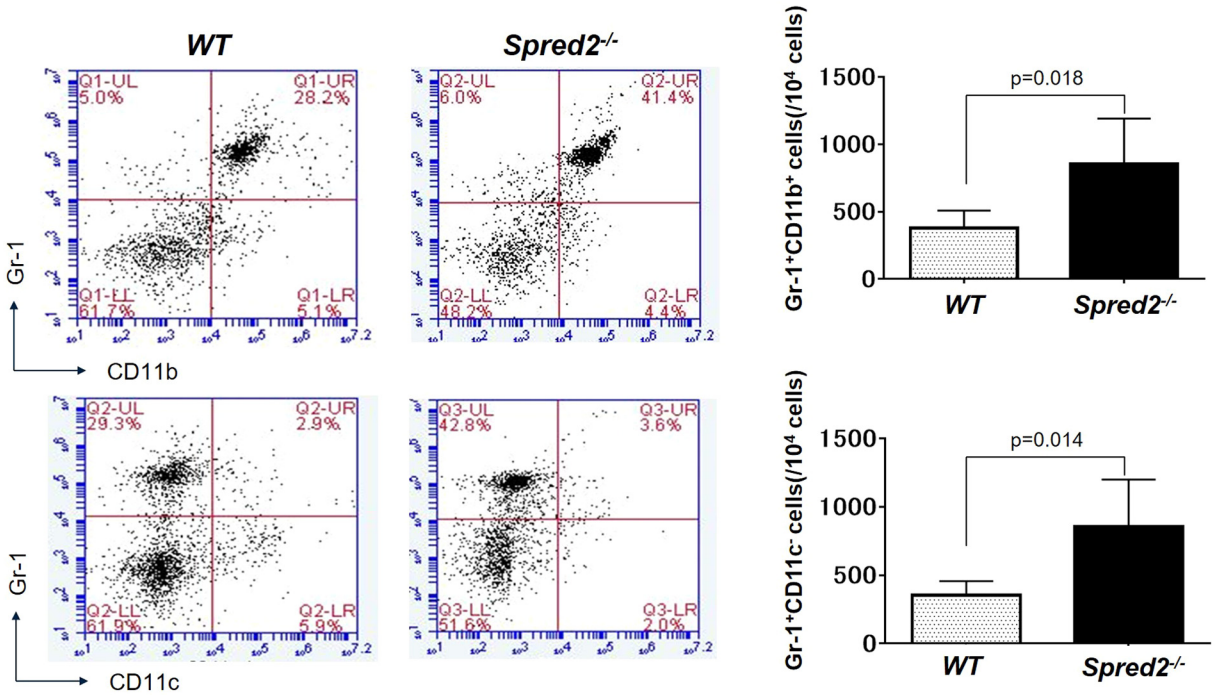

Fig. 2. Flow cytometry analysis of lung neutrophil infiltration.
Flow cytometry was performed to sort Gr-1$^+$/CD11b$^+$ and Gr-1$^+$/CD11c$^-$ cells under CD45$^+$ gating, as they were deemed to be neutrophils. Both types of cells were significantly more abundant in *Spred2*$^{-/-}$ lung grafts (*$P < 0.05$).

focused on several inflammatory cytokines and chemokines, including CCL2, in their experiment and showed that preoperative levels of those molecules in the recipient blood samples correlated with the extent of IRI development. From these findings, it follows that inflammatory cells and proinflammatory signaling pathway molecules of the recipient also have key roles in IRI development.

In this study, WT mice were utilized as normal recipients, whereas *Spred2*$^{-/-}$ mice mimicked recipients with the potential of developing an inflammatory response. The lung grafts, which were supposed to retain their function, caused severe IRI at 4 h after reperfusion when transplanted into recipients with the deletion of *Spred2*. Thus, recipients with the potential of developing inflammatory hyperactivation of the MAPK pathway can suffer from IRI irrespective of the lung graft condition. Moreover, our data suggest that anti-inflammatory treatment of the recipient in the perioperative period can prevent lung IRI. In our previous study of lung IRI, in which we utilized the hilar clamping technique, it was revealed that an ERK inhibitor ameliorated IRI that developed in *Spred2*$^{-/-}$ mice [16].

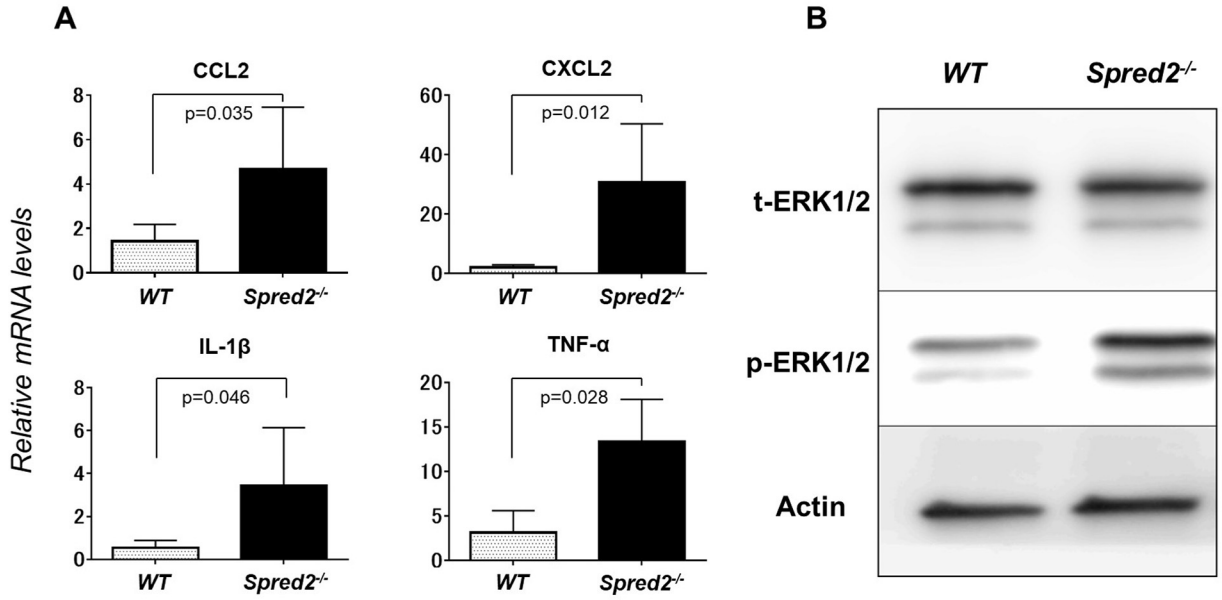

Fig. 3. Expression levels of ERK1/2 and mRNAs encoding proinflammatory molecules.
(A) Relative expression levels of mRNAs encoding proinflammatory cytokines and chemokines involved in neutrophil activation were examined by real time PCR. mRNAs encoding CCL2, CXCL2, IL-1β, and TNF-α were significantly more highly expressed in *Spred2*$^{-/-}$ group than in WT animals. (B) Total ERK1/2 (t-ERK) and phosphorylated ERK (p-ERK) expression levels were examined by Western blotting to evaluate the extent of MAPK pathway activation.

In conclusion, activation of the MAPK/ERK pathway by the deletion of SPRED2 in the recipient affects the severity of IRI after lung transplantation. Furthermore, anti-inflammatory treatment for the recipient may be the potential therapeutic option in cases of lung transplantation characterized by augmented inflammatory potential due to MAPK pathway hyperactivation.

Funding

This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS KAKENHI grant number: 16K19980 to S.O.).

Declaration of Competing Interest

Kohei Hashimoto and other co-authors have no conflict of interest.

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