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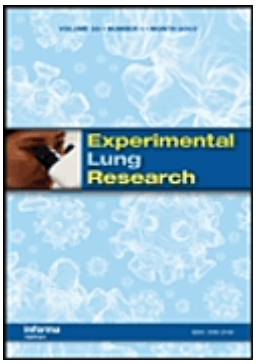


# The novel inhibitor PRI-724 for Wnt/β-catenin/CBP signaling ameliorates bleomycin-induced pulmonary fibrosis in mice

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# The novel inhibitor PRI-724 for Wnt/β-catenin/CBP signaling ameliorates bleomycin-induced pulmonary fibrosis in mice

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**ABSTRACT**

Purpose/Aim of the Study: Wnt/β-catenin signaling was reported to be activated in pulmonary fibrosis, and was focused on as a target for antifibrotic therapy. However, the mechanism how the inhibition of Wnt/β-catenin signaling ameliorate pulmonary fibrosis has not been fully elucidated. The purpose of this study is to explore the target cells of Wnt/β-catenin inhibition in pulmonary fibrosis and to examine the antifibrotic effect of the novel inhibitor PRI-724 specifically disrupting the interaction of β-catenin and CBP.

Materials and Methods: The effect of C-82, an active metabolite of PRI-724, on the expression of TGF-β1 and α-smooth muscle actin (SMA) was examined on fibroblasts and macrophages. We also examined the effects of PRI-724 in mouse model of bleomycin-induced pulmonary fibrosis.

Results: The activation and increased accumulation of β-catenin in the canonical pathway were detected in lung fibroblasts as well as macrophages stimulated by Wnt3a using Western blotting. Treatment with C-82 reduced CBP protein and increased p300 protein binding to β-catenin in the nucleus of lung fibroblasts. In addition, C-82 inhibited the expression of SMA in lung fibroblasts treated with TGF-β, indicating the inhibition of

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myofibroblast differentiation. In the fibrotic lungs induced by bleomycin, β-catenin was stained strongly in macrophages, but the staining of β-catenin in alveolar epithelial cells and fibroblasts was weak. The administration of PRI-724 ameliorated pulmonary fibrosis induced by bleomycin in mice when administered with a late, but not an early, treatment schedule. Analysis of bronchoalveolar fluid (BALF) showed a decreased number of alveolar macrophages. In addition, the level of TGF-β1 in BALF was decreased in mice treated with PRI-724. C-82 also inhibited the production of TGF-β1 by alveolar macrophages.

Conclusions: These results suggest that the β-catenin/CBP inhibitor PRI-724 is a potent antifibrotic agent that acts by modulating the activity of macrophages in the lungs.

**Key Words**: Wnt/β-catenin/CBP signaling, Idiopathic pulmonary fibrosis, Alveolar macrophage, PRI-724.

## INTRODUCTION



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Idiopathic pulmonary fibrosis (IPF) is a chronic devastating lung disease, characterized by the proliferation of fibroblasts and deposition of extracellular matrix in the lungs (1, 2). The prognosis associated with IPF is poor, and the median survival time after diagnosis is approximately 2 to 4 years (1). Although there are some antifibrotic agents available for the treatment of IPF (3), the efficacy is not yet sufficient for patients because the advantage is limited in terms of ameliorating the decline in the lung function, but not the survival benefit or recovery of injured lungs. Thus, the development of novel drugs with superior effects is strongly expected.

The pathogenesis of IPF is based on the repeated injury of alveolar epithelial cells (AECs) and subsequent activation of mesenchymal cells with the formation of fibroblastic and myofibroblastic foci (2, 4). The Wnt/β-catenin pathway is well-known as essential signaling during lung development and homeostasis (5). Activated Wnt/β-catenin signaling is associated with fibrosis in the liver, heart, kidney, skin, and other organs including the lung (5, 6). In the lungs of IPF patients, Wnt/β-catenin signaling is activated aberrantly in AECs and mesenchymal cells (fibroblasts/myofibroblasts) (7-9). Experimentally, the signaling is known to be induced in a murine model of lung fibrosis



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with the intratracheal administration of bleomycin (10-12).

However, the role of Wnt/β-catenin signaling in pulmonary fibrosis is complicated due to the pleiotropic action in many types of lung cells. Increased β-catenin in AECs is related to the hyperplasia and regeneration in IPF (7, 9, 11). It was reported that the Wnt/β-catenin pathway plays a role in the survival, migration, and proliferation of AECs (9-12). The specific deletion of β-catenin in AECs resulted in the enhanced apoptosis of AECs, leading to an increased number of fibroblasts and delayed recovery from epithelial injury in mice (12). However, the activation of Wnt/β-catenin signaling was also reported to be related to epithelial mesenchymal transition (EMT), indicating, on the contrary, the pro-fibrotic activity of Wnt/β-catenin (11). In addition, lung fibroblast cell lines are activated via Wnt/β-catenin signaling in migration, proliferation, and collagen production (8, 9, 11, 13). These results suggest that the inhibition and/or modulation of Wnt/β-catenin signaling may be applicable as a therapeutic strategy for pulmonary fibrosis. In fact, several reports demonstrated that the inhibition of Wnt/β-catenin signaling with inhibitors (14-18) or inhibitory protein, such as Dickkopf-1 (18), ameliorated pulmonary fibrosis in a bleomycin model.



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However, more recently, macrophages were also reported to be novel cells that are involved in the regulation of pulmonary fibrosis via the Wnt/β-catenin signaling pathway (19). In the present study, we performed cell-based analysis of the distribution and activation of β-catenin in fibrotic lungs, and demonstrated the antifibrotic effects and mode of action of the second-generation inhibitor PRI-724 specific for CBP/β-catenin, which has been used in clinical trials to treat patients with malignancy, in a bleomycin-induced pulmonary fibrosis model in mice.

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## METHOD

### Reagents.

PRI-724 and the active metabolite C-82 were from PRISM BioLab, Co., Ltd (Kanagawa, Japan). Recombinant Mouse Wnt3a Protein (R&D Systems, Minneapolis, MN, USA), recombinant TGF-β1 protein (R&D Systems), and Proteinase K (Sigma-Aldrich, Saint Louis, MO, USA) were also purchased.

### Animals and Treatments.

Eight-week-old C57BL/6 male mice were purchased from Charles River Japan, Inc. (Yokohama, Japan). Mice were maintained in the animal facility of the University of Tokushima under specific pathogen-free conditions according to the guidelines of our university (20). The present study was approved by the Institutional Animal Care and Use Committee of Tokushima University (Permission Number: 14099). Bleomycin sulfate (mixture) (Tokyo Chemical Industry Co., Tokyo, Japan) was dissolved in sterile saline and injected via oropharyngeal aspiration at a dose of 1.25 mg/kg of bleomycin in a total volume of 50 μL of saline. During the injection process, mice were anesthetized with



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2.5% isoflurane delivered in a box. Mice were suspended vertically on a stand for oropharyngeal aspiration. Osmotic minipumps (Alzet Osmotic Pumps, DURECT Corporation, CA, USA) containing PBS or 1 or 10 mg/kg/day of PRI-724 were implanted subcutaneously.

**Cell Lines.**

Murine lung fibroblasts were generated from the lungs of C57BL/6 mice as reported previously (20, 21). These fibroblasts were used at 5 to 10 passages. The human lung fibroblast cell line MRC5 and macrophage cell line RAW257.6 were obtained from DS PHARMA BIOMEDICAL (Osaka, Japan) and the American Type Culture Collection, ATCC (Manassas, VA, USA), respectively. These cells were cultured with RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) (GIBCO BRL, Rockville, MD, USA).

**Bronchoalveolar Lavage.**

Bronchoalveolar Lavage (BAL) was performed with saline (1 mL) using a soft cannula



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on mice treated with bleomycin (20). BAL fluid (BALF) was collected on days 28 and 35 after bleomycin injection, and centrifuged at 1,000 rpm for 10 minutes. The supernatant was used for ELISA to measure the TGF-β1 level. After counting the cell number in the BALF, cells were cytospun onto glass slides and stained with Diff-Quick (Baxter, Miami, FL, USA) for cell classification.

**Alveolar Macrophage Isolation.**

BAL was conducted for mice treated by bleomycin 7 days after administration. The cells were plated in wells in 1.0% FBS RPMI1640 medium. One hour later, the wells were washed with PBS three times and the adherent cells were used as alveolar macrophages (AMs). AMs ($1 \times 10^6$ /mL) were cultured on the well in 1.0% FBS RPMI1640 and the supernatants were collected after 24 hours-treatment with 1.0 μM of C-82 or DMSO. Cells were collected for real-time PCR to examine the mRNA level.

**Hydroxyproline Assay.**

Quantitative examination of the collagen content was performed using the



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hydroxyproline assay kit (BioVison, Milpitas, CA, USA) as previously described (22). The left lobes or total lungs were used for this assay to allow comparison.

**Western Blot Analysis.**

Serum-starved lung fibroblast B6 cells were harvested in RPMI1640 with 0.1% FBS and 1% penicillin-streptomycin. Thirty micrograms of protein per sample was loaded onto 8% tris-glycine SDS polyacrylamide gels (Invitrogen) for electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes (0.2 μM, ATTO). Membranes were then incubated with Blocking One (Nakalai Tesque Inc, Japan) for 1 hour at room temperature and then incubated with the appropriate primary antibody overnight. Secondary antibodies and an ECL kit (GE) were applied for generating chemiluminescent signals. Primary antibodies used for Western analysis included: anti-α-SMA (Sigma-Aldrich, St. Louis, MO, USA), anti-non-phospho (Active) β-catenin antibody (Ser33/37/Thr41) (D13A1) (Cell Signaling Technology, Danvers, MA, USA), anti-phospho-β-Catenin (Y654) antibody (St John's Laboratory, London, United Kingdom), anti-GAPDH (6C5) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-

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LAMIN A/C (Cell Signaling Technology, Danvers, MA, USA), anti-p300 (C-20) (Santa Cruz Biotechnology), and anti-CBP (A-22) (Santa Cruz Biotechnology) antibodies. Normal rabbit IgG (Millipore) and Protein G-Sepharose 4 Fast flow (GE Healthcare, Buckinghamshire, UK) were used. Densitometric analysis was performed using National Institutes of Health (NIH) Image J software.

**Cytoplasmic and Nuclear Protein Extraction.**

Proteins from cytoplasmic and nuclear compartments were separated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). Briefly, fibroblast and macrophage cells were harvested with trypsin-EDTA and then washed twice with PBS. Then, cells were centrifuged at 500 × g for 5 minutes and supernatants were removed. Ice-cold CER-I and CER-II solutions were added according to the manufacturer's instructions to separate the cytoplasmic from the nuclear compartment proteins. Western blot for GAPDH and LAMIN A/C were used to ensure no contamination in each part of the extracts.

**Histopathology.**



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The right lungs were fixed in 10% buffered formalin and embedded in paraffin. Sections (3-4 μm) were stained with hematoxylin and eosin. Azan staining was also performed.

**TGF-β1 ELISA.**

The concentrations of TGF-β1 in BALF and the culture supernatant of macrophages were determined using the TGF-β1 ELISA kit according to the manufacturer's instructions (R&D Systems). To determine the amount of total TGF-β1 in the conditioned media, samples were pretreated with 1 M HCl for 15 min at room temperature prior to neutralization 1 M NaOH, as suggested by the manufacturer.

**Immunofluorescence Staining.**

Paraffin-embedded lung-section slides were de-paraffinized, rehydrated, and subjected to antigen retrieval using proteinase K (20 μg/mL) for 25 minutes in 37°C before immunostaining (23). Non-specific binding was blocked using a solution of 5% BSA with 0.3% Triton X-100 in TBS. The lung sections were incubated with primary antibodies to phospho-Y654-β-catenin, nonphospho-β-catenin, CD68, pro-SPC, α-SMA, and Sca-1 in



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1% BSA with 0.3% Triton X-100 in TBS. The sections were then washed and incubated with Alexa Fluor-594 goat anti-rabbit IgG secondary antibody, and Alexa Fluor-488 anti-rat IgG secondary antibody and nuclei were counterstained with DAPI. Fluorescence analysis was performed using an Olympus inverted fluorescence microscope. Primary antibodies used included: anti-Active-β-catenin (anti-ABC) (Millipore, Massachusetts, USA), anti-Sca-1/Ly6A/E (D7) (FITC) (Abcam, Cambridge, MA, USA), anti-pro-SPC (Abcam), anti-CD68 (Abdserotec, Oxford, UK), and anti-α-SMA (1A4) (Biotin) (Abcam) antibodies. Other reagents such as Streptavidin, Alexa Fluor 488 conjugate (Thermo Fisher Scientific, Waltham, MA, USA) and APEX Alexa Fluor 488 antibody labeling kit (Thermo Fisher Scientific) were also used.

**Quantitative Real-time PCR Analysis.**

Total mRNA was extracted from cells using TRIzol reagent and cDNA was synthesized from mRNA using the Prime Script RT-PCR synthesis kit (High Capacity RNA-to-cDNA kit, Applied Biosystems) following the manufacturer's instructions. RT-PCR was performed using the standard protocol. Primers detecting TGF-β1 and GAPDH were



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used:

TGF-β1

For: CAACAATTCCTGGCGTTACCTTGG

Rev: GAAAGCCCTGTATTCCGTCTCCTT

GAPDH

For: CAACTACATGGTCTACATGTTC

Rev: CGCCAGTAGACTCCACGAC

**Statistical Analysis.**

Statistical analysis was performed with GraphPad Prism Ver. 5.01 (GraphPad Software Inc.). Dates are expressed as the mean ± standard error (SE). Differences in measured variables between experimental and control groups were assessed using Student's t-test. Values of $P < 0.05$ were considered significant.

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## RESULTS

### Activation of β-catenin in lung fibroblasts and macrophages.

We first demonstrated the activation of β-catenin in lung fibroblasts and macrophages with the primary culture of lung fibroblasts called B6 cells and macrophage cell line RAW257.6. The activation of β-catenin was evaluated by the nuclear accumulation with the anti-active (nonphospho) form of β-catenin dephosphorylated on Ser37 or Thr41 and phospho (p)-Y654-β-catenin antibodies by Western blotting. In lung fibroblast B6 cells, an increased accumulation of nonphospho-β-catenin was detected in the nucleus and the cytoplasm one hour after Wnt3a stimulation (Figure 1A, Supplementary Figure 1). Similarly, in macrophage RAW257.6 cells, the accumulation of both nonphospho- and pY654-β-catenin was demonstrated 12 hours after Wnt3a stimulation (Figure 1B, Supplementary Figure 2, 3). These results suggest that the activation of β-catenin was observed not only in fibroblasts in addition to alveolar epithelial cells, but also in macrophages.

### C-82, an active metabolite of PRI-724, blocks the binding of β-catenin to cAMP-



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**response element-binding protein (CREB)-binding protein (CBP) and inhibits the differentiation of lung fibroblasts to myofibroblasts induced by TGF-β1.**

ICG-001, a compound similar to PRI-724, was reported to specifically disrupt the interaction between β-catenin and CBP, not p300, in the nucleus (24). We therefore analyzed the activity of C-82 to block the binding of β-catenin and CBP in lung fibroblast B6 cells. B6 cells were stimulated with Wnt3a protein in the presence or absence of 1 or 2 μM C-82. The cell lysate was harvested 12 hours after Wnt3a stimulation, and was immunoprecipitated with anti-CBP or p300 antibody. The whole cell lysate and immunoprecipitated samples were electrophoresed and plotted with anti-nonphospho-β-catenin antibody. Treatment with C-82 clearly reduced the binding of β-catenin and CBP (Figure 1C). On the contrary, the binding of p300 to β-catenin was increased after C-82 treatment. Next, we examined the biological effect of C-82 on the myofibroblast differentiation of lung fibroblasts. Wnt/β-catenin signaling was reported to play a critical role in the TGF-β signaling pathway (25, 26). C-82 dose-dependently reduced the expression of α-smooth muscle actin (SMA) in MRC5, a human lung fibroblast cell line, treated with TGF-β1 (Figure1D, Supplementary Figure 4), indicating the inhibition of

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myofibroblast differentiation.

**PRI-724 ameliorates bleomycin-induced lung fibrosis with a late, but not an early, treatment schedule.**

We induced lung fibrosis in mice using the intratracheal administration of bleomycin. Administration with PRI-724 alone did not induce any adverse events including weight loss (data not shown) or fibrotic changes determined by the hydroxyproline content of the lungs (Figure 2A), suggesting the absence of toxic effects of PRI-724. Next, antifibrotic effects of PRI-724 were examined with an early treatment schedule (Figure 2B). In this setting, bleomycin sulfate (1.25 mg/kg) was injected via the oropharyngeal aspiration on day 0, and PRI-724 (1 or 10 mg/kg per day) or PBS was administered on day 3 subcutaneously using an osmotic pump. Three weeks after the bleomycin injection, we sacrificed mice, and measured the collagen content. As shown in Figure 2B, there were no significant differences in hydroxyproline levels among groups treated with bleomycin. We next examined a late treatment schedule using PRI-724. Under this experimental condition, the administration of PRI-724 was started on day 21, three weeks after the

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bleomycin injection. After PRI-724 treatment for two weeks, the mice were sacrificed five weeks after the bleomycin injection to examine the fibrotic changes in the lungs. The hydroxyproline content in the PRI-724 group was significantly decreased compared with that in the PBS group (393.4 ± 21.2 vs. 325.3 ± 17.3 μg/lung, $P < 0.05$) (Figure 2C). As shown in Figure 3, the histological examination also showed that the fibrotic changes around the peri-bronchiolar lesion in the lungs were reduced in the PRI-724 group.

**Immunofluorescence analysis of active β-catenin in various cells in the fibrotic lungs of mice.**

We analyzed the expression of active β-catenin in various cells of the lungs treated with bleomycin for three weeks in mice with double immunofluorescence staining. To detect active β-catenin, antibodies for nonphospho- and pY654-β-catenin were used. As shown in Figure 4A and 4B, alveolar macrophages detected by anti-CD68 antibody were strongly stained with both anti-nonphospho- and pY654-β-catenin antibodies. In most macrophages, their membranes were clearly stained, and the nucleus was also stained in some cells (Figure 4A and 4B, Supplementary Figure 5A, 5B), indicating the activation



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of β-catenin. Regarding AECs, nonphospho-β-catenin was detected by double-staining with pro-SPC, but the level of staining was less than that seen in macrophages (Figure 4C, Supplementary Figure 5C). The nuclear localization of nonphospho-β-catenin was also observed in some AECs (Figure 4C, Supplementary Figure 5C). The myofibroblasts were stained with anti-α-SMA antibody. As shown in Figure 4D, positive staining with anti-pY654-β-catenin antibody was detected in myofibroblasts, but the level was weak compared with that in macrophages. It was difficult to determine the nuclear localization of pY654-β-catenin in fibroblast-like cells. In addition, we found other cells, which were positive for Sca-1, which strongly stained for pY654-β-catenin in the fibrotic lungs, and some cells also showed nuclear localization (Figure 4E).

**Effects of PRI-724 in BAL cells in the fibrotic lungs of mice.**

To examine the effects of PRI-724 on the number and classification of BAL cells, mice were sacrificed on day 28, one week after starting PRI-724 administration with a late treatment schedule, and BAL was performed. As shown in Figure 5A, the total cell number showed a decreasing tendency in the group receiving PRI-724 treatment,



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although the difference was not significant. However, the number of macrophages was significantly reduced in mice treated with PRI-724 compared with the PBS group (8.3 ± 0.89 vs. 6.8 ± 1.09 × $10^4$/mL, respectively, $P < 0.05$) (Figure 5A).

**PRI-724 reduces the production of TGF-β1 in bleomycin-induced lung fibrosis in mice**

Next, we examined the level of active TGF-β1 in BAL fluid one (day 28) and two (day 35) weeks after the administration of PRI-724, as assessed by ELISA. The level of active TGF-β1 in BAL fluid in the PRI-724 groups was decreased significantly on day 28, one week after the PRI-724 treatment (Figure 5B). However, there was no significant difference in the TGF-β1 level on day 35, two weeks after the PRI-724 treatment, although a decreasing tendency was observed (Figure 5B). We also analyzed the expression level of TGF-β1 in BAL fluid collected from mice treated with an early treatment schedule as shown in Figure 2B. However, there was no significant difference in the TGF-β1 level on both day 7 and 14 (Supplementary Figure 6). These data support that PRI-724 ameliorates bleomycin-induced lung fibrosis with the late, but not the early,

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treatment schedule.

We finally examined the effects of C-82 on the production of TGF-β1 by AMs. Murine AMs were harvested from the BAL fluids one week after bleomycin injection. After 24 hours of treatment with C-82 or solvent in 1.0% FBS RPMI1640 medium, the level of TGF-β1 was measured in the supernatant treated with acid activation by ELISA, and the mRNA level of cells by RT-PCR. As shown in Figure 6B, the level of latent TGF-β1 protein was decreased in the C-82 group (Figure 6A), and the level of TGF-β1 mRNA was also suppressed in that group (Figure 6B).

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## DISCUSSION

In the present study, we examined the activated β-catenin signaling in various types of cells in the lungs treated with bleomycin by immunofluorescence staining, and found that AMs were stained most strongly, particularly in the nucleus, among lung cells including AECs and mesenchymal cells like fibroblasts. In addition, we demonstrated the antifibrotic effects of a novel inhibitor PRI-724, specifically disrupting the interaction of β-catenin and CBP, on pulmonary fibrosis induced by bleomycin in mice partly via inhibiting the production of TGF-β1 by AMs.

The Wnt/β-catenin signaling pathway was established to be highly activated in hyperplastic AECs and bronchiolar epithelial cells in IPF as well as in experimental pulmonary fibrosis model mice (7-11). However, as the specific deletion of β-catenin in AECs showed the deterioration of lung fibrosis and delayed recovery from lung injury, β-catenin signaling in AECs may serve rather to maintain the survival and promote regeneration in the fibrotic lungs (12), although profibrotic mediators such as WNT1-inducible signaling protein-1 was simultaneously induced (11). On the other hand, blockade of the Wnt/β-catenin signaling pathway using inhibitors or inhibitory protein



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Dickkopf-1 (DKK-1) suppressed pulmonary fibrosis in mice (14-18). These results suggest that major target cells for Wnt/β-catenin inhibition to inhibit pulmonary fibrosis are cells other than AECs, such as fibroblasts or myeloid cells that have migrated into the lungs. The fibroblasts could be possible targets because lung fibroblasts were reported to proliferate via activated β-catenin signaling including canonical and TGF-β-mediated pathways, and localized β-catenin staining of the nuclei of fibroblasts was observed (8, 9, 13, 26). In addition, Sennello et al recently reported that the activation of β-catenin signaling in lung myeloid cells such as macrophages, neutrophils, B lymphocytes, and T lymphocytes, except eosinophils, in the Axin2-β-galactosidase reporter mouse (19). They also mentioned that the majority of β-catenin-activated cells appear morphologically to be of immune cell origin, although there are some nonimmune cell types in the distal lungs, such as AECs or fibroblasts (19). These observations are consistent with our results of immunofluorescence staining of the fibrotic lungs, in which strong signals of activated β-catenin were frequently observed in CD68-positive macrophages, but less in AECs or fibroblasts. In addition, we also observed an activated β-catenin signal in Sca-1-positive progenitor-like cells. Although these cells are CD45-negative and co-stained for TGF-β1



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(data not shown), the role in pulmonary fibrosis should be explored in a further study.

Next, we investigated the antifibrotic effects of PRI-724, a novel second-generation-specific inhibitor for the interaction between β-catenin and CBP (27). The first-generation β-catenin/CBP inhibitor ICG-001 has been demonstrated to show antifibrotic effects on various organs with fibrosis, including the lungs, skin, and liver (14, 28, 29). PRI-724 is a novel inhibitor generated for clinical use (27). Notable points regarding the antifibrotic effects of PRI-724 were the effective phase and mode of action in pulmonary fibrosis. The antifibrotic effects were observed when treatment with PRI-724 was started on day 21 in the progressive and/or resolution phase, although the initiation of PRI-724 from day 3 in the inflammatory phase did not lead to any antifibrotic effects. These results may indicate the therapeutic effects of PRI-724 because the effects in an early phase in a bleomycin model were mediated predominantly by anti-inflammatory and not antifibrotic effects. In addition, early treatment may induce dual bidirectional effects, profibrotic and antifibrotic, which are mediated by AECs and fibroblasts in response to epithelial injury. The mechanistic study identified a reduced number of AMs on day 28 one week after PRI-724 administration. These results are consistent with the previous data in which the

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number of monocyte-derived Siglec-F$^{low}$ recruited AMs was decreased in bleomycin-treated mice lacking lipoprotein receptor-related protein 5 (Lrp5), which is a Wnt coreceptor and mediates a positive signal in the Wnt/β-catenin pathway (19). These data suggest that β-catenin signaling promotes the differentiation of recruited monocyte-AM populations that contribute to the fibrotic phenotype (19). A more recent study reported the involvement of the Wnt/β-catenin signal in the proliferation of macrophages (30). In addition to our immunofluorescence study, the Wnt/β-catenin signal may be strongly associated with activities of subpopulations of macrophages, particularly AMs. We also identified a reduced level of TGF-β1 in BALF on days 28, one week after PRI-724 initiation. This is also compatible with the results from Lrp$^{-/-}$ mice, with a reduced level of TGF-β production by alveolar leukocytes reported in the later, not early, phase of pulmonary fibrosis (17). Furthermore, we noted the suppressive effects of C-82, an active metabolite of PRI-724, on TGF-β1 production of AMs *in vitro*. However, it was demonstrated that the loss of Lrp5 and/or 6 does not have an impact on the TGF-β-dependent up-regulation of TGF-β (17). There is a report that β-catenin is not involved in Smad3-dependent transcriptional activities in the macrophage cell J774 (31). Thus, the



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possibility that the reduction of TGF-β1 expression is mediated by inhibition of the Wnt/β-catenin signal was supported by other reports (14-17), and another signaling pathway interacting with CBP may be involved in the suppressive activity of PRI-724/C-82 on AMs.

These results suggest that the novel β-catenin/CBP inhibitor PRI-724 is a potent antifibrotic agent that acts through modulating the activity of AMs in the lungs, although a further mechanistic study is required to explore the mode of action.

**Acknowledgements**

The authors thank Tomoko Oka and Megumi Kume for technical assistance. We also thank the members of the Nishioka lab for their technical advice and fruitful discussions.

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**Declaration of interest statement**

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## FIGURE LEGENDS

**Figure 1. Activation of β-catenin in lung fibroblasts and macrophages and effects of C-82 on the binding of β-catenin with CBP and TGF-β-induced differentiation of lung fibroblasts.** A: Lung fibroblast B6 cells were stimulated with Wnt3a (50 ng/mL) for 1 hour. The accumulation of β-catenin in the nuclei was examined with anti-nonphospho (active) -β-catenin antibody. B: The accumulation of β-catenin in the nuclei in the macrophage cell line RAW257.6 was examined after treatment with Wnt3a for 12 hours with anti-nonphospho (active)-β-catenin antibody. C: The effects of C-82 on the interaction between β-catenin and CBP were examined with the nuclear extracts of lung fibroblast B6 cells stimulated with Wnt3a for 90 min. D: Effects of C-82 on the expression of α-SMA in MRC5, a human lung fibroblast cell line. MRC5 cells were cultured with TGF-β1 (5 ng/mL) in the presence or absence of various concentrations of C-82 for 48 hours. The expression of α-SMA was examined with Western blotting. Data are representative of two separate experiments. The relative expression of each band was calculated by densitometric analysis. Data were analyzed using a Student's t-test and

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displayed as means ± SE. *$P < 0.05$ versus the control group in Figure 1A and 1B, and versus the TGF-β only group in Figure 1D.

**Figure 2. PRI-724 reduces pulmonary fibrosis with a late treatment schedule in a bleomycin-induced pulmonary fibrosis model.** A: C57BL/6 mice were treated with the subcutaneous injection of PBS or PRI-724 (1 or 10 mg/kg per day) with osmotic minipumps from days 3 to 21, and left lobe was harvested for analysis of the collagen content by the hydroxyproline assay on day 21. B: C57BL/6 mice were treated with the intratracheal administration of saline or bleomycin on day 0. In bleomycin groups, mice were injected subcutaneously with osmotic minipumps containing PBS or PRI-724 (1 or 10 mg/kg/day) from days 3 to 21, and the left lobe was harvested for analysis of the collagen content by hydroxyproline assay on day 21. C: C57BL/6 mice were treated with the intratracheal administration of saline or bleomycin on day 0. In bleomycin groups, mice were injected subcutaneously with osmotic minipumps containing PBS or PRI-724 (10 mg/kg/day) from days 21 to 35, and whole lungs were harvested for analysis of the collagen content by the hydroxyproline assay on day 35. Data are presented as the mean

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± SE. (A) n=4; (B) n=6-9; (C) n=7-9, $P < 0.05$ (Student's t-test)

**Figure 3. Histological evaluation of the anti-fibrotic effects of PRI-724 on mice with bleomycin-induced lung fibrosis.** C57BL/6 mice were treated with the intratracheal administration of saline or bleomycin on day 0. In bleomycin groups, mice were injected subcutaneously with osmotic minipumps containing PBS or PRI-724 (10 mg/kg/day) from days 21 to 35, and the right lobe was harvested for histological examination on day 35. Hematoxylin and eosin (H&E) (A, C, and E) and azan mallory (B, D, and F) (scale bar = 100 μm) stainings were performed. (A, B) Saline. (C, D) BLM and PBS. (E, F) BLM and PRI-724 (10 mg/kg).

**Figure 4. Immunofluorescence images of the β-catenin-positive cells' nuclei, membranes, and cytoplasm in murine lungs treated with bleomycin.**

Immunofluorescent analyses were performed in the murine lungs on day 21 after the bleomycin (1.5 mg/kg) injection. β-Catenin was stained with anti-phospho-Y654-β-catenin (A, D, E) or nonphospho (active)-β-catenin (B, C) antibodies. Macrophages were



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also stained with anti-CD68 antibody (A, B). Alveolar epithelial cells were stained with anti-pro-SPC-antibody (C). Lung myofibroblasts were stained with anti-α-smooth muscle actin antibody (D). Sca-1-positive cells were analyzed (E). Arrows indicate the nuclear staining of each β-catenin. Scale bars, 10 μm.

**Figure 5. Analysis of bronchoalveolar lavage fluid.** BAL was performed on day 28 or 35 after one or two weeks of treatment with PRI-724. A: Cell classification of BAL cells on day 28 (n=6: BLM/PBS, n=5: BLM/PRI-724). B: Level of TGF-β1 in BAL fluids on days 28 and 35. Active TGF-β1 was measured with ELISA (n=6: saline/PBS/day 28, n=14: BLM/PBS/day28, n=12: BLM/PRI-724/day 28, n=6: BLM/PBS/day 35, n=6: BLM/PRI-724/day 35). Data are the mean ± SE. $P < 0.05$ using Student's t-test.

**Figure 6. C-82 inhibits the expression of TGF-β1 on alveolar macrophages.** The AMs were collected from mice 1 week after treatment with bleomycin with the BAL procedure. The AMs were treated with saline or C-82 (1 μM) in 1.0% FBS RPMI1640 medium for 24 hours, and the supernatants were collected. The latent TGF-β1 levels were measured



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using ELISA (n=4 in each group) (A). Real-time PCR was performed using RNA extracted from the AMs after being treated with DMSO or C-82 for 24 hours (n=4 in each group) (B). Data are the mean ± SE. $P < 0.05$ using Student's t-test.

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Fig.1

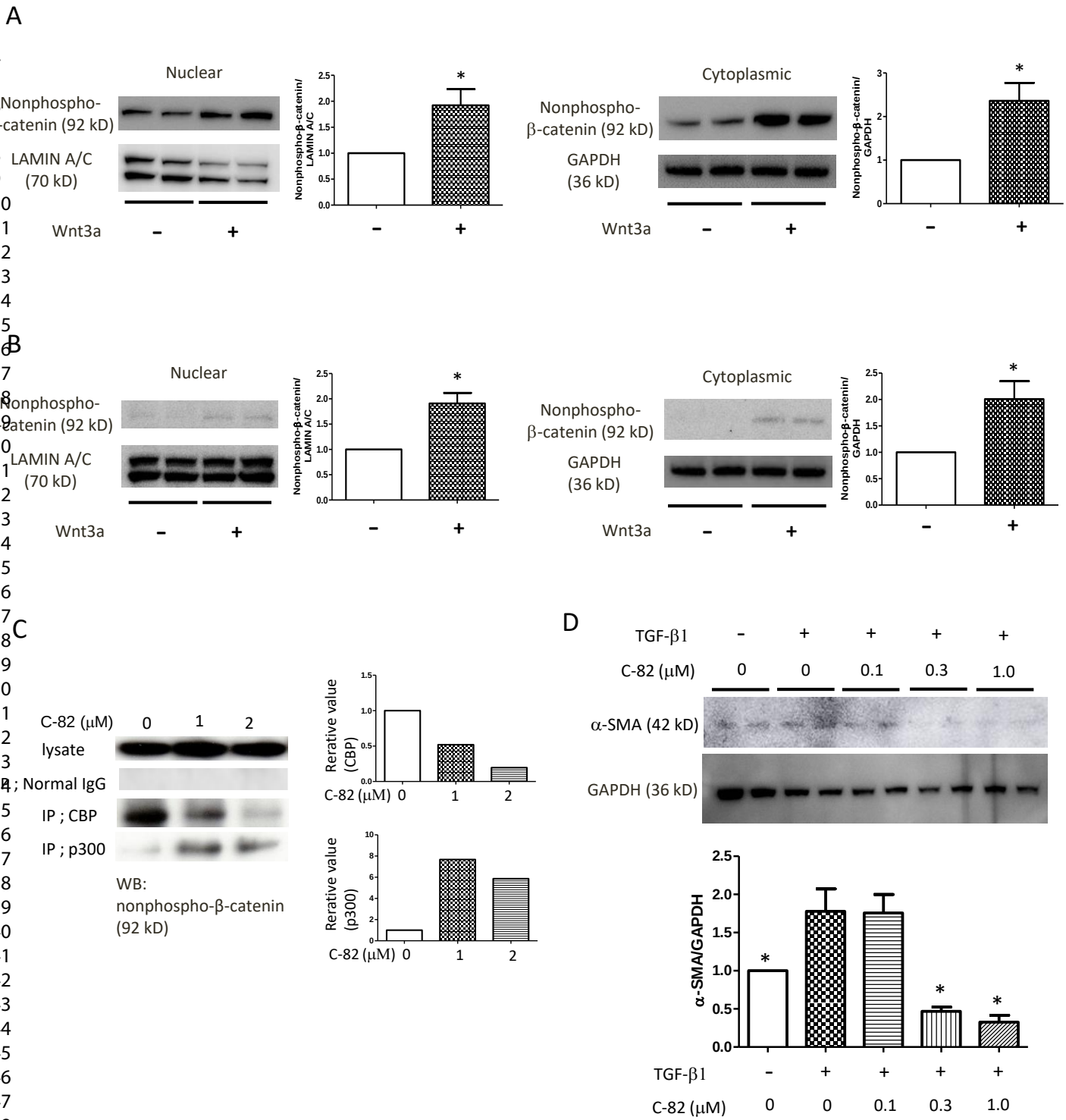

A
Nuclear
Nonphospho-β-catenin (92 kD)
LAMIN A/C (70 kD)
Wnt3a
Nonphospho-β-catenin/ LAMIN A/C
Cytoplasmic
GAPDH (36 kD)
Nonphospho-β-catenin/ GAPDH
B
C
C-82 (μM) 0 1 2
lysate
IP ; Normal IgG
IP ; CBP
IP ; p300
WB: nonphospho-β-catenin (92 kD)
Rerative value (CBP)
Rerative value (p300)
D
TGF-β1
C-82 (μM) 0 0 0.1 0.3 1.0
α-SMA (42 kD)
GAPDH (36 kD)
α-SMA/GAPDH

Fig.2

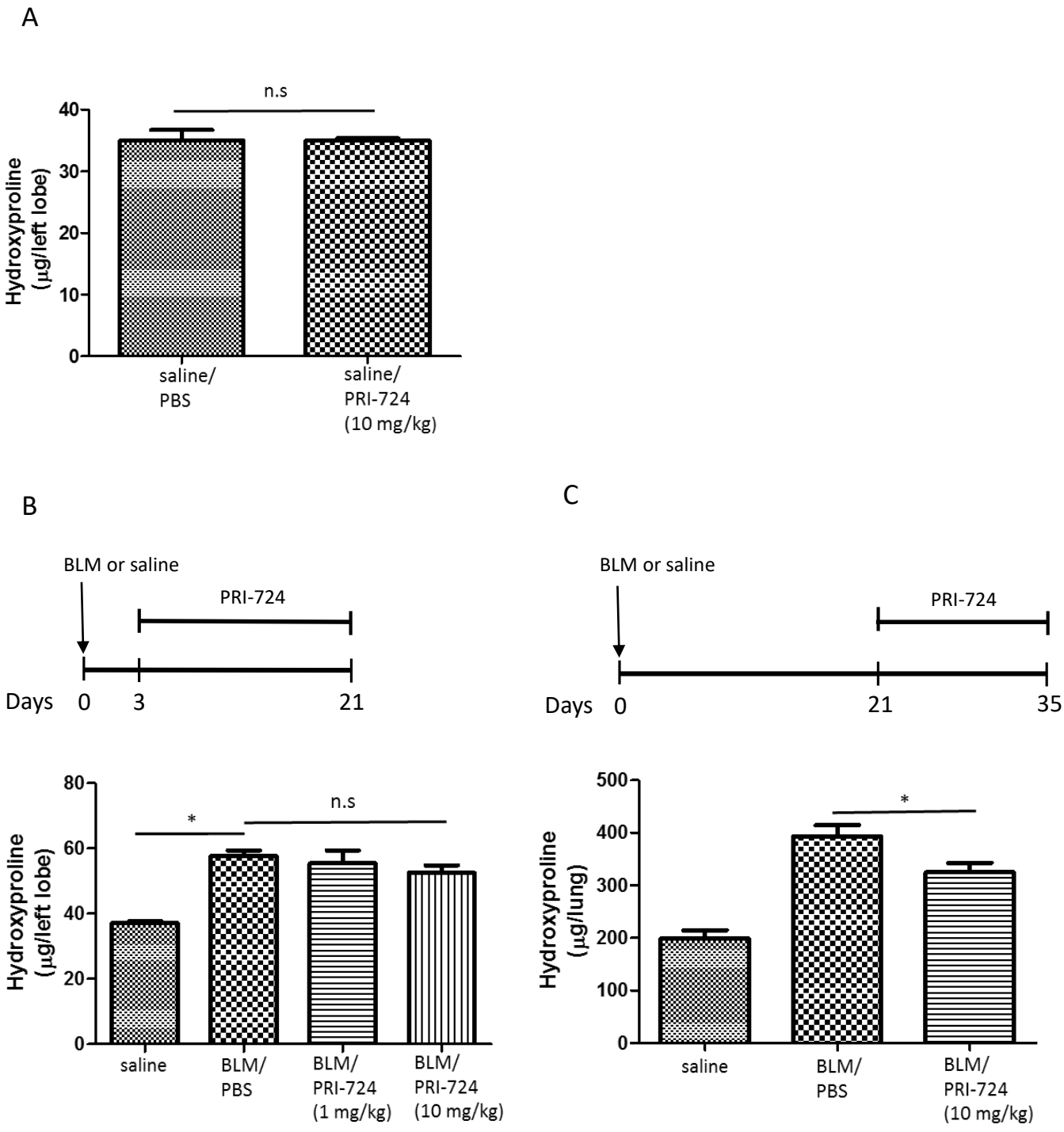

A
n.s
Hydroxyproline (μg/left lobe)
40
30
20
10
0
saline/ PBS
saline/ PRI-724 (10 mg/kg)
B
BLM or saline
PRI-724
Days 0 3 21
Hydroxyproline (μg/left lobe)
80
60
40
20
0
*
n.s
saline
BLM/ PBS
BLM/ PRI-724 (1 mg/kg)
BLM/ PRI-724 (10 mg/kg)
C
BLM or saline
PRI-724
Days 0 21 35
Hydroxyproline (μg/lung)
500
400
300
200
100
0
*
saline
BLM/ PBS
BLM/ PRI-724 (10 mg/kg)

Fig.3

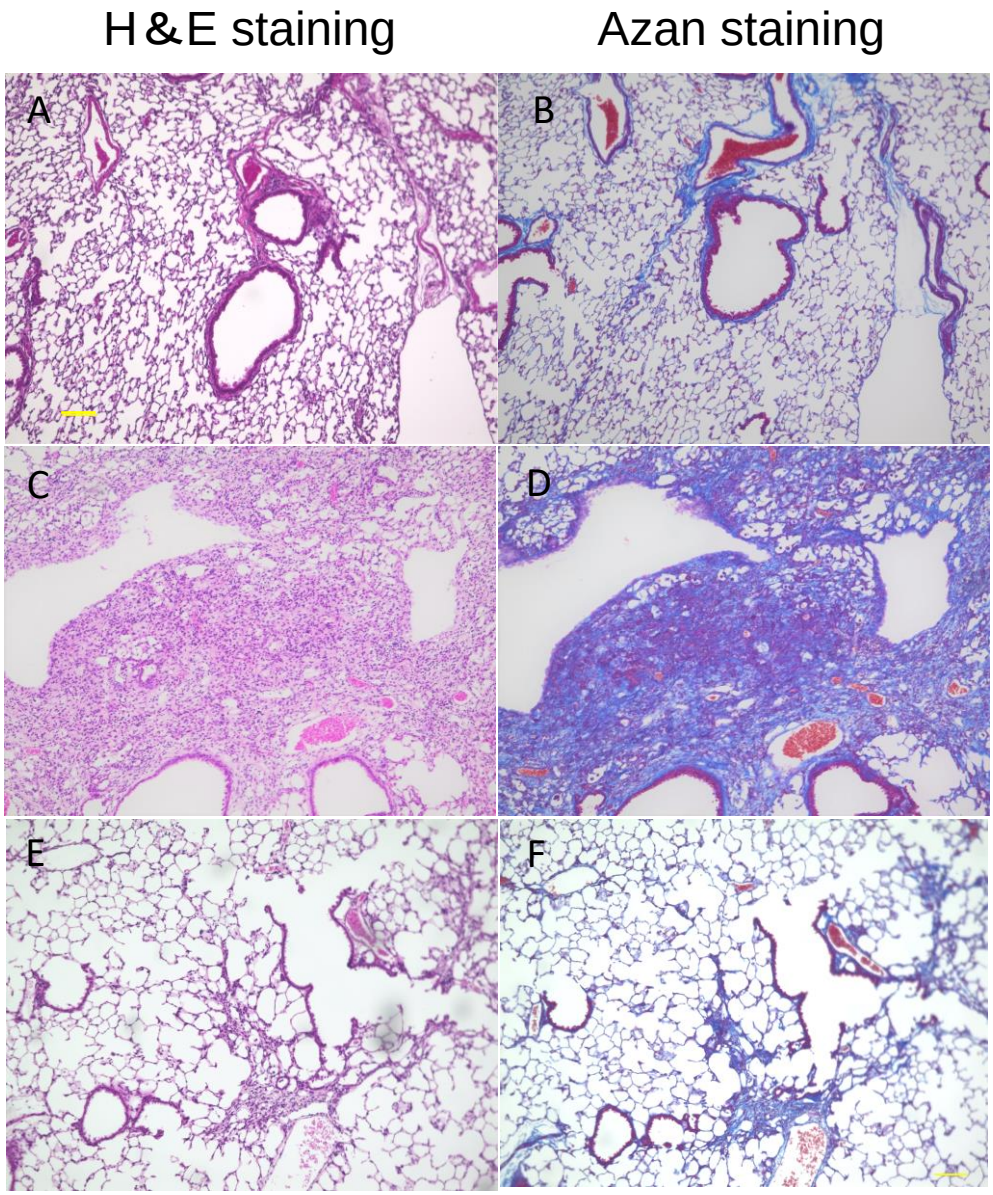

H&E staining
Azan staining
saline
BLM/
PBS
BLM/
PRI-724
(10 mg/kg)
A
B
C
D
E
F

Fig.4

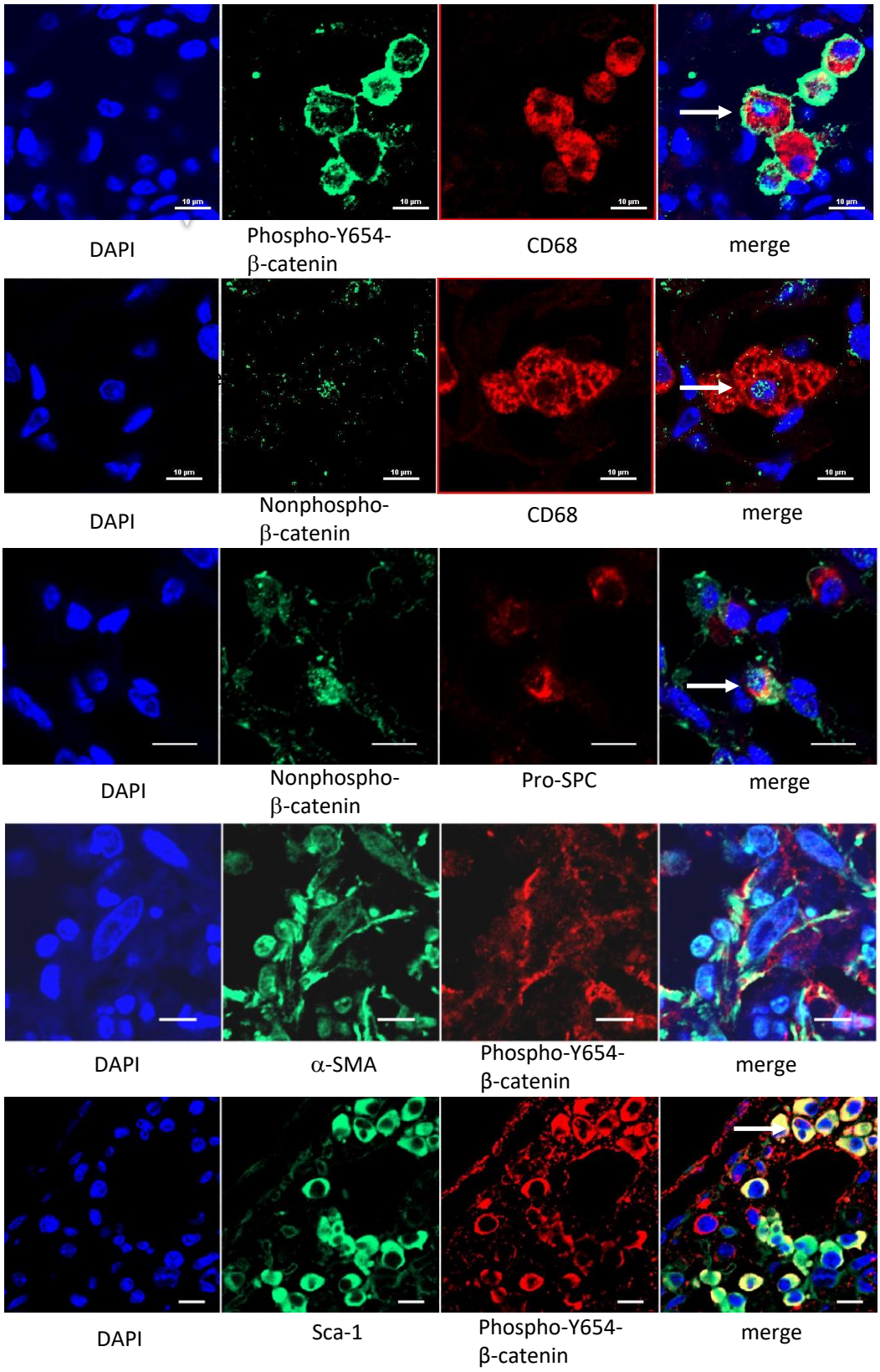

A
DAPI
Phospho-Y654-β-catenin
CD68
merge
B
DAPI
Nonphospho-β-catenin
CD68
merge
C
DAPI
Nonphospho-β-catenin
Pro-SPC
merge
D
DAPI
α-SMA
Phospho-Y654-β-catenin
merge
E
DAPI
Sca-1
Phospho-Y654-β-catenin
merge

Fig.5

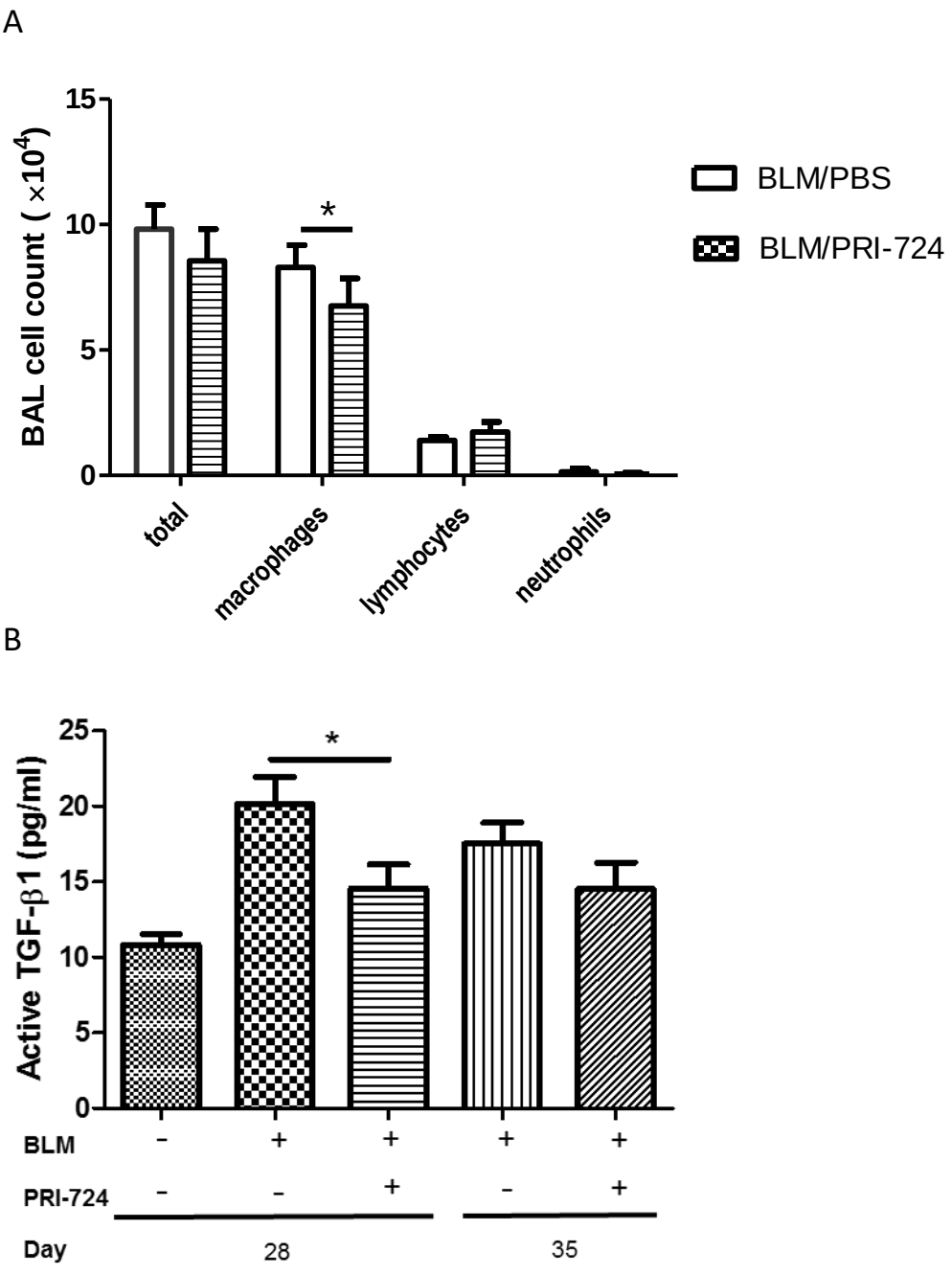
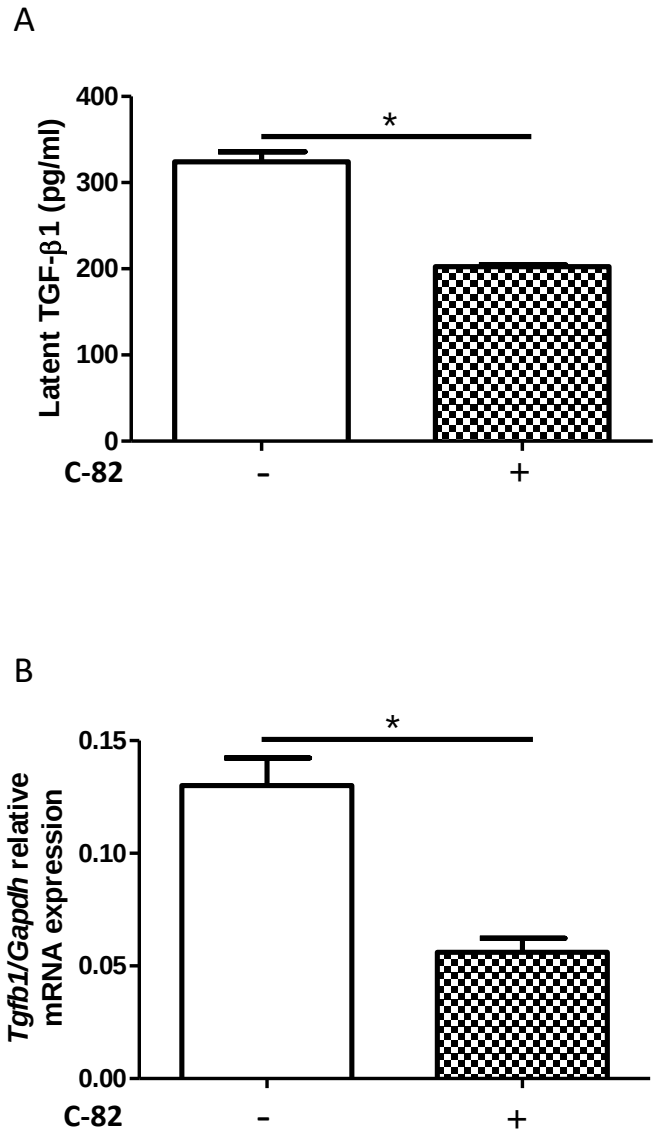

A
BAL cell count ( ×10^4)
15
10
5
0
*
total
macrophages
lymphocytes
neutrophils
BLM/PBS
BLM/PRI-724
B
Active TGF-β1 (pg/ml)
25
20
15
10
5
0
*
BLM - + + + +
PRI-724 - - + - +
Day 28 35

Fig.6


A
Latent TGF-β1 (pg/ml)
400
300
200
100
0
*
C-82
-
+
B
Tgfb1/Gapdh relative mRNA expression
0.15
0.10
0.05
0.00
*
C-82
-
+

# The novel inhibitor PRI-724 for Wnt/β-catenin/CBP signaling ameliorates bleomycin-induced pulmonary fibrosis in mice

Hiroyasu Okazaki[1], Seidai Sato[1], Kazuya Koyama[1], Shun Morizumi[1], Shuichi Abe[1], Momoyo Azuma[1], Yajuan Chen[1], Hisatsugu Goto[1], Yoshinori Aono[1, 2], Hirohisa Ogawa[3], Kozo Kagawa[1], Haruka Nishimura[1], Hiroshi Kawano[1], Yuko Toyoda[1], Hisanori Uehara[3], Hiroyuki Kouji[4,5], Yasuhiko Nishioka[1]

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## SUPPLEMENT DATA

## FIGURE LEGENDS

**Supplementary Figure 1. Expanded blots from Figure 1A**

**Supplementary Figure 2. Expanded blots from Figure 1B**

**Supplementary Figure 3. Activation of β-catenin in macrophages and effects of C-82 on the binding of β-catenin with CBP.**

The accumulation of β-catenin in the nuclei in the macrophage cell line RAW257.6 was examined after treatment with Wnt3a for 12 hours with pY654-β-catenin antibodies.

**Supplementary Figure 4. Expanded blots from Figure 1D**

**Supplementary Figure 5. Low magnification images of Figure 4.**

Immunofluorescence analyses were performed in the murine lungs on day 21 after the bleomycin (1.5 mg/kg) injection. β-Catenin was stained with anti-pY654-β-catenin (A) or nonphospho (active)-β-catenin (B, C) antibodies. Macrophages were stained with anti-CD68 antibody (A, B). Alveolar epithelial cells were stained with anti-pro-SPC-antibody (C). Arrows indicate the nuclear staining of each β-catenin. Scale bars, 100 μm.

**Supplementary Figure 6. The expression level of TGF-β in BAL fluid collected from mice treated with an early treatment schedule.**

C57BL/6 mice were treated with the intratracheal administration of saline or bleomycin on day 0. In bleomycin groups, mice were injected subcutaneously with osmotic minipumps containing PBS or PRI-724 (1 or 10 mg/kg/day) started from days 3. BAL was performed on day 7 or 14 with mice treated with an early treatment schedule. Total TGF-β1 was measured with ELISA (n=5 in each group). Data are the mean ± SE. $P < 0.05$ using Student's t-test.



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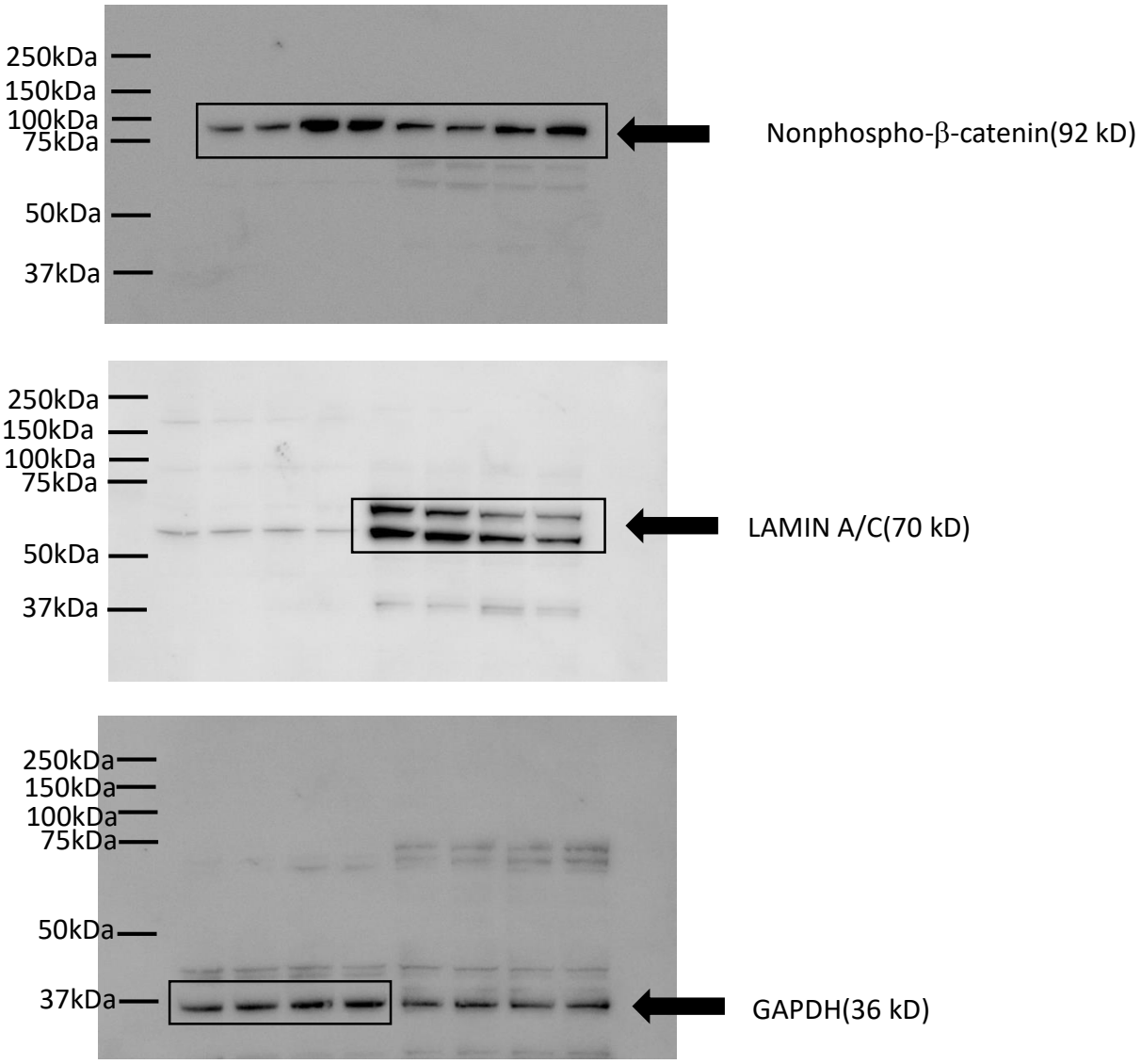
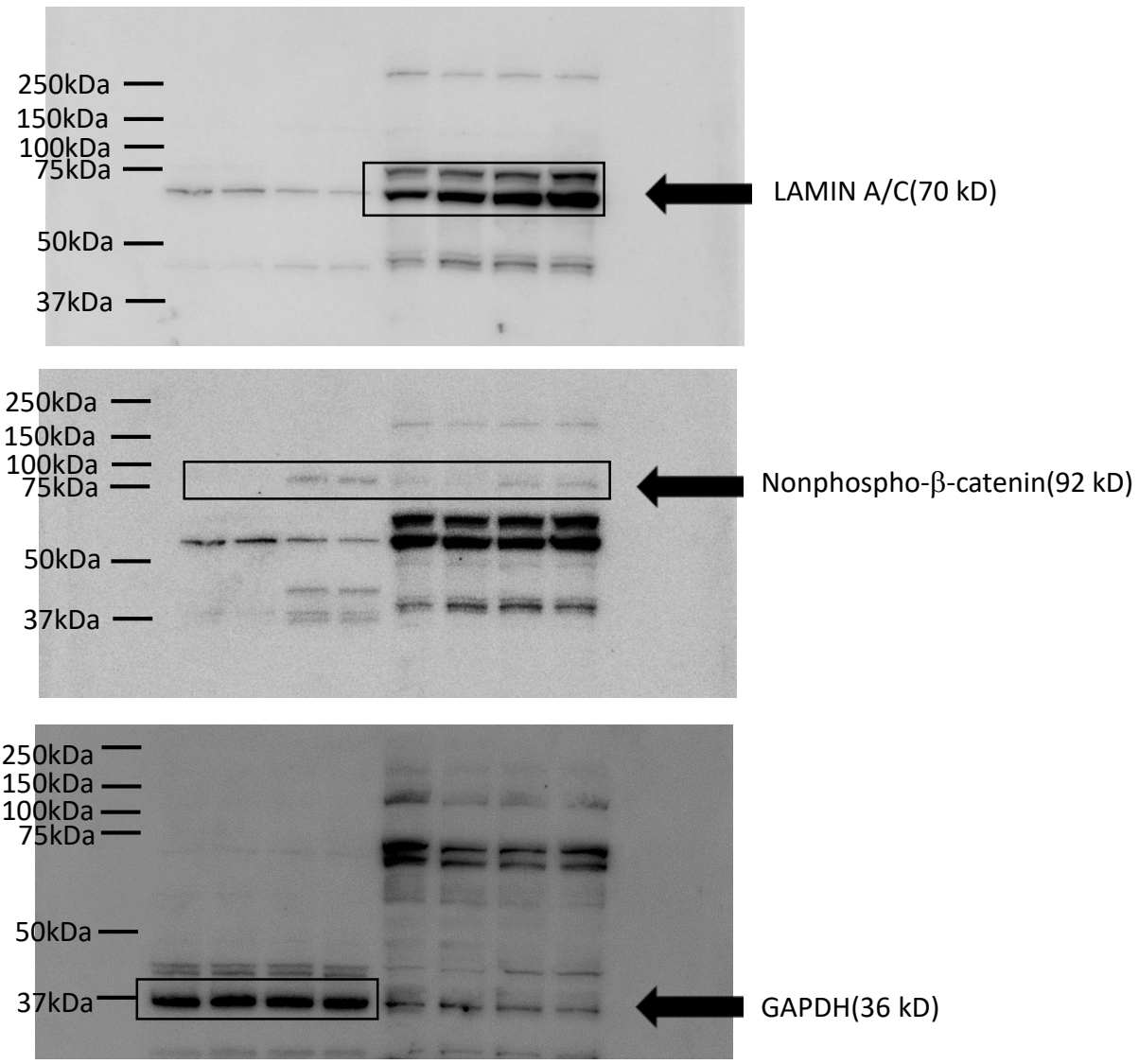
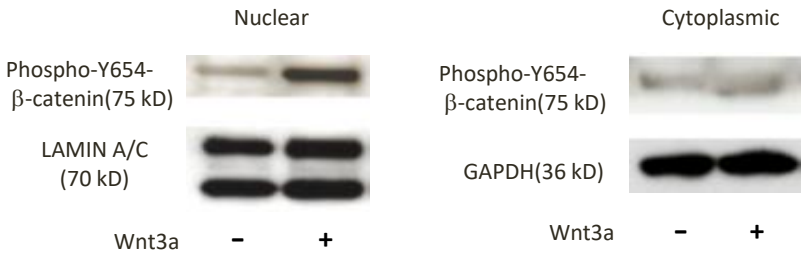
Supplementary Figure 1.

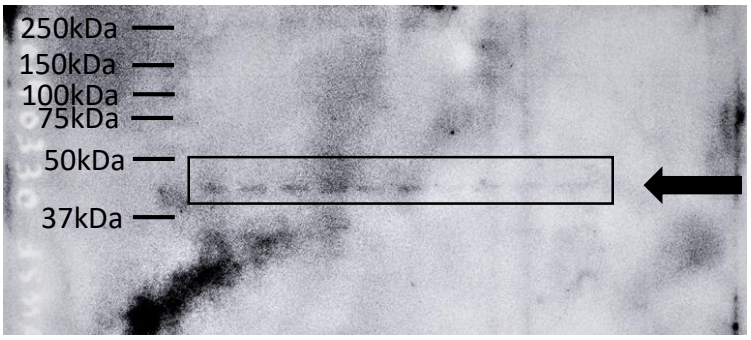


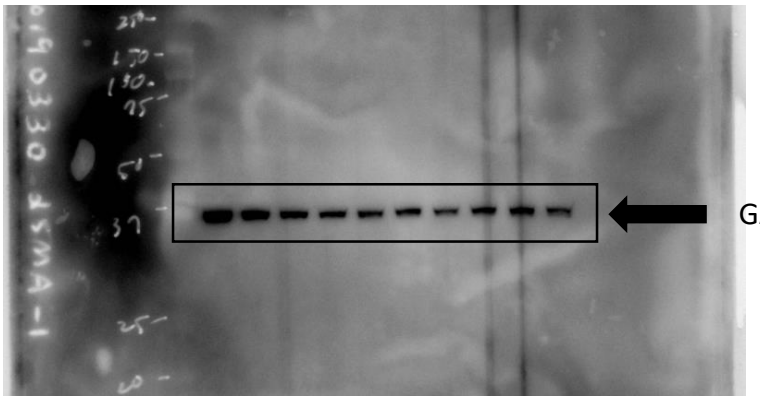
Supplementary Figure 2.



# Supplementary Figure 3.

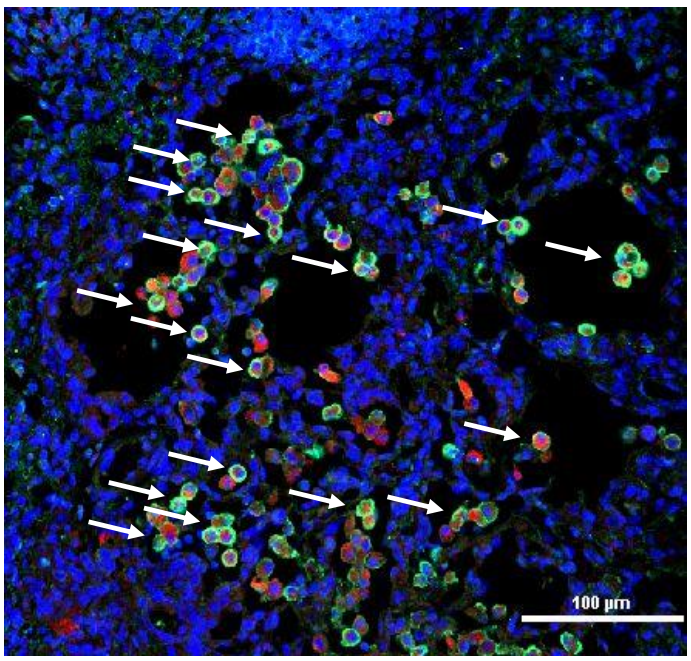



250kDa
150kDa
100kDa
75kDa
50kDa
37kDa
α-SMA(42 KD)


250kDa
150kDa
100kDa
75kDa
50kDa
37kDa
GAPDH(36 kD)

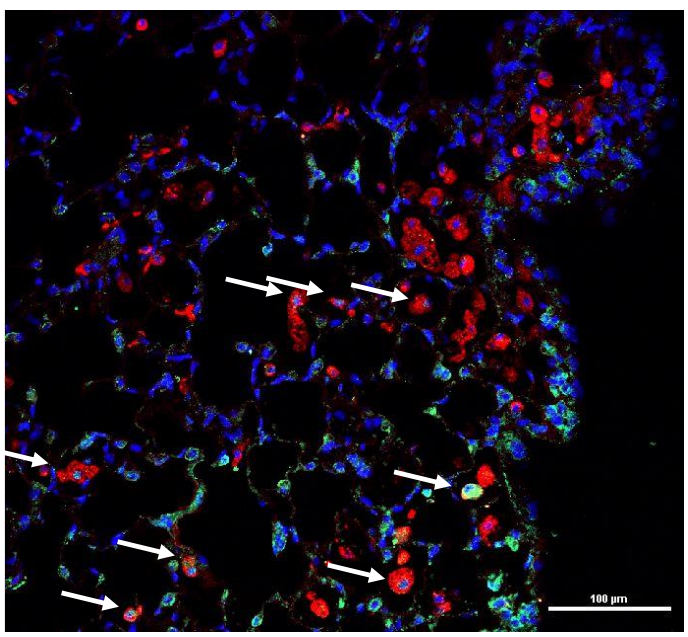
# Supplementary Figure 5.

**A**



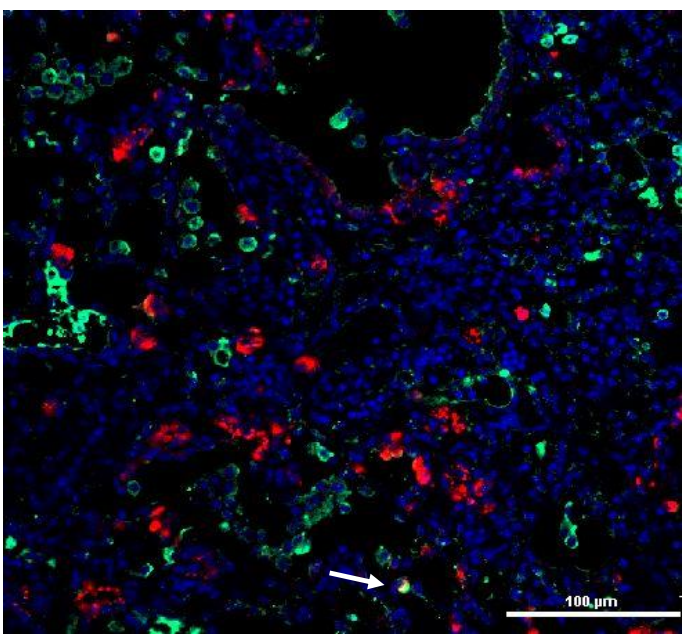

CD68 (red)
Phospho-Y654-β-catenin (green)

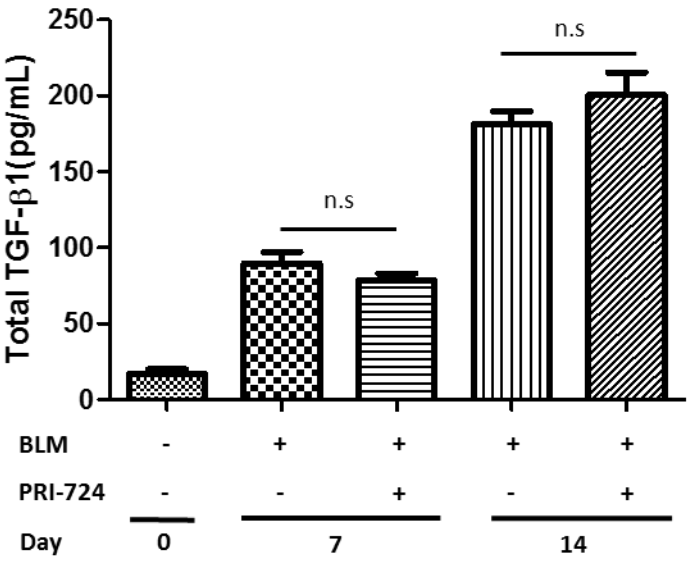
**B**




CD68 (red)
Nonphospho-β-catenin (green)

**C**




Pro-SPC (red)
Nonphospho-β-catenin (green)

Supplementary Figure 6.