Online Supplement

Neuropeptide Y Antagonizes Development of Pulmonary Fibrosis Through IL-1β Inhibition

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Materials and Methods

Animal procedures

All experimental procedures were approved by the Animal Care and Use Committee of Okayama University. All C57BL/6J mice were maintained under a 12-h light/dark cycles with free access to murine chow and water.

Bleomycin administration

Female C57BL/6J wild-type (WT) mice were obtained from Charles River Laboratories (Yokohama, Japan). NPY-deficient ($NPY^{-/-}$) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and backcrossed with C57BL6/J mice (1). Several studies used female mice for bleomycin-induced lung fibrosis models (2–5). In addition, female mice are easy to handle; therefore, we used female mice in the present study.

Female C57BL/6J WT and $NPY^{-/-}$ mice (8–11 weeks old; body weight: 18–21 g) received 1.5 mg/kg of bleomycin (BLM; Nippon Kayaku, Tokyo, Japan) in 30 μL of normal saline, or phosphate-buffered saline (PBS) via intratracheal (i.t.) instillation on day 0 (6). Mice were anesthetized with 4% isoflurane, then administered BLM or PBS via the intratracheal route using MμltiFlex Round Tips (Sorenson BioScience, Salt Lake City, UT, USA). We carefully observed the breathing patterns of the mice under anesthesia; no special physiological

monitoring was performed, but the procedure was safely performed under anesthesia.

To evaluate lung fibrosis, all mice were sacrificed under anesthesia using ketamine (150 mg/kg) (Kyoritsu Seiyaku Corp., Tokyo, Japan) and xylazine (10 mg/kg) (Fujita Pharmaceutical Co. Ltd., Tokyo, Japan) injected intraperitoneally (i.p.). The lungs were harvested on days 0, 2, 7, 14, and 21 following i.t. BLM or PBS treatment. Lung fibrosis was assessed on day 21 because extensive fibrotic changes in mouse lungs reportedly develop by this stage (2–5). Mice without cardiac blood sampling were euthanized by incision of the inferior vena cava under ketamine and xylazine anesthesia.

NPY administration

NPY (4012616; Bachem AG, Bubendorf, Switzerland) was stored at −30°C before the experiment, then reconstituted with 1 mg NPY per 12.5 mL of PBS. WT mice received i.p. 50 μg/kg or 200 μg/kg of NPY daily between days −1 and 20, whereas control mice received PBS. The mice received i.t. 1.5 mg/kg of BLM or PBS on day 0, and the fibrotic changes were assessed on day 21 (Figure E4A). Based on lung-histology assessment, fibrotic changes appeared 7 days after BLM instillation (Figures E5A and E5B). The same NPY-treatment experiment was performed in $NPY^{-/-}$ mice (Figures E6A–E6I). The protective effect of NPY is reportedly dose-dependent; we selected NPY dosages based on previous findings (7, 8).

Anti-mouse IL-1β monoclonal antibody (mAb) administration in NPY$^{-/-}$ mice

NPY$^{-/-}$ mice received either 20 μg of anti-mouse IL-1β mAb (clone B122; BioLegend, San Diego, CA, USA) or control IgG (purified Armenian Hamster IgG Isotype, HTK888; BioLegend) on days −1, 1, 3, and 7, and 1.5 mg/kg of BLM or PBS on day 0, via i.t. instillation (Figure 4A). We determined the dose and timing of anti-mouse IL-1β mAb administration based on the effects on acute inflammation described in previous reports (9, 10). Previous studies showed that the levels of inflammatory cytokine production in PBS-instilled mice did not significantly differ between treatments (control IgG or anti-IL-1β antibody) (11, 12). In the present study, PBS-instilled mice showed no differences in fibrotic changes between treatment with PBS and treatment with anti-IL-1β mAb (data not shown).

Bronchoalveolar lavage (BAL) fluid analysis

On days 0, 2, 7, 14, and 21, the lungs were lavaged with Hanks' balanced salt solution (1 mL, 37 °C) using a tracheal tube, and the BAL fluid was collected. The samples were then centrifuged at 1500 rpm for 15 min at 4 °C, and stored at −30 °C. The samples for analysis of NPY were stored at −80 °C. BAL-fluid cells were counted and differentiated using blinded May-Giemsa staining by counting at least 200 cells under a light microscope (13).

Blood collection

On days 2 and 21, murine blood samples were collected by cardiac puncture antemortem, immediately after the induction of ketamine and xylazine anesthesia. We inserted a needle 5 mm from the center of the thorax using a 1-mL syringe and a 26-gauge needle, then obtained the blood samples in non-anticoagulant tubes. The samples were centrifuged at 3000 rpm for 20 min at 4 °C, and stored at −30 °C. The samples for analysis of NPY were stored at −80 °C.

Collagen assay

The collagen concentrations in BAL fluids were measured by colorimetric assay performed using a collagen assay kit (Sircol™ soluble collagen assay; Biocolor Life Science Assays, Carrickfergus, UK) according to the manufacturer's protocol. The absorbance of each well was measured on a multimode reader at 570 nm. The sensitivity limit was 1.0 μg of collagen in a volume of 1 mL. All samples were analyzed in duplicate, and the results were within ± 5% of their mean values.

Hydroxyproline assay

Hydroxyproline concentrations were measured using a QuickZyme Hydroxyproline Assay kit

(QuickZyme BioSciences, Leiden, The Netherlands) in accordance with the manufacturer's protocol. The left lung tissues were incubated in 6 M HCl at 95°C for 20 h. After the samples had been cooled to room temperature and centrifuged at 13,000 × *g* for 10 min, the supernatants were collected and diluted with deionized water to 4 M HCl. The absorbance of each well was measured on a multimode reader at 570 nm. Data were pooled from three independent experiments. The sensitivity limit was 2.4 μM (0.3 μg/mL).

Lung histology

The right lung was inflated, fixed by i.t. instillation of 10% buffered formalin (14), and embedded in paraffin. Sections measuring 3.5 μm were stained with hematoxylin-eosin and Masson's trichrome, and observed under a microscope. Five sections from each mouse were selected at random by ≥ 2 researchers to assess fibrotic changes with the same magnification and brightness, then analyzed using Adobe Photoshop (Adobe Inc., San Jose, CA, USA) (15). The blue pixel area was measured as the percentage of fibrotic area using Adobe Photoshop CC 2018 (32-bit). The Color Range tools allowed all pixels in the image to be selected, and the fibrotic area was shown as blue pixels relative to all pixels using the Histogram tool. The fibrotic area was defined as the mean percentage of blue pixels captured in the five sections.

Lung homogenates

Left lung tissue was immediately frozen at −80 °C, and lung homogenates were prepared using 300 μL of PBS-containing 0.1% Triton-X100 solution and 0.2% proteinase inhibitors (P8340; Sigma-Aldrich Merck KGaA, Darmstadt, Germany). The specimens were then homogenized and centrifuged at 14,000 rpm for 30 min (16). The resulting supernatant was collected and frozen at −30°C until analysis using enzyme-linked immunosorbent assay (ELISA).

Cytokine and chemokine levels

Cytokine and chemokine levels in BAL fluids and homogenized lung tissues were measured using ELISA according to the manufacturers' instructions. The detection limits were 0.68, 15.4, 4.8, 5.64, and 5 pg/mL for monocyte chemoattractant protein-1 (MCP-1), transforming growth factor (TGF)-β1, mice IL-1β, human IL-1β, and IL-17A, respectively. Except for human IL-1β kit (ab214025; Abcam, Cambridge, UK), all other kits were purchased from R&D Systems (Minneapolis, MN, USA). All cytokine and chemokine levels were measured in duplicate by ELISA.

Mouse and human NPY levels

Mouse NPY levels in mice were measured using the EZRMNPY-27K ELISA kit (Merck & Co., Inc., Kenilworth, NJ, USA), whereas human NPY levels were measured using the EZHNPY-25K ELISA kit (Merck & Co., Inc.), according to the manufacturer's instructions. The detection limits were 0.002 ng/mL and 3 pg/mL for mice and humans, respectively. Mouse NPY levels were measured in duplicate, while human NPY levels in serum were measured in triplicate. Human NPY levels in plasma were measured only once because of the limited sample volume.

Flow cytometry

Peripheral blood was collected; red blood cells were lysed with Red Blood Cell Lysis Solution (Miltenyi Biotec, Bergisch Gladbach, Germany), then stained with antibodies. Lung cells were isolated following collagenase digestion as previously described (6). Whole lungs were collected and placed in a petri dish, and the tissue was minced with scissors into pieces ≤ 3 mm. PBS in a volume of 5 mL containing 0.025 mg of collagenase (type I; Gibco, Paisley, Scotland, UK), 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), and 1% antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin) was added and mixed well. Then, 0.01% DNase (D4263; Sigma-Aldrich, St. Louis, MO, USA) was added and incubated at 37°C with rotation at a speed of 225 rpm for 40 min. After incubation, the remaining tissue

and all fluid in the dishes were drawn into 5-mL syringes through a 20-gauge needle, then discharged into the digestion dishes to facilitate tissue disruption. The cell suspensions were carefully placed onto Histopaque-1077 (Sigma-Aldrich) and centrifuged. After centrifugation, the upper layer was aspirated and suspended cells were collected in RPMI-1640 medium (Sigma-Aldrich). Intranuclear staining with Foxp3/Transcription Factor Staining Buffer (eBioscience Inc., San Diego, CA, USA) was then performed. The cells were stained with Pacific-blue-conjugated anti-CD3ε (145-2C11, BioLegend), fluorescein-isothiocyanate-conjugated anti-CD4 (GK1.5, BD bioscience), and phycoerythrin-conjugated anti-RORγt antibody (Q31-378; BD Biosciences), and APC-conjugated anti-Ki67 antibody (16A8; BioLegend). The gating strategy for T helper 17 (Th17) cells and Ki67-positive Th17 cells is shown in Figure E3A. Cells were then analyzed on a MACS Quant flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) using the FlowJo software (FlowJo, LLC, Ashland, OR, USA).

Cell line and reagents

The RIKEN BioResource Research Center provided A549 cells through the National Bio-Resource Project of MEXT, Japan. The cells were grown in the DMEM D-6429 medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (Thermo Fisher Scientific,

Waltham, MA, USA) and 1% antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin). All cells were cultured at 37 °C in a humidified incubator with 5% carbon dioxide. In all experiments, cells were passaged weekly and used at 4–10 passages. At each passage, cells seeded onto plastic plates (Falcon culture dishes; Becton Dickinson and Company, Franklin Lakes, NJ, USA) reached 80%–100% confluence.

Bleomycin (Selleck Chemicals, Houston, TX, USA), NPY (4012616; Bachem AG), and Y1R antagonist BIBP3226 (Bachem AG) were dissolved in distilled water, then adjusted to the required final concentration in serum-free DMEM for in vitro studies.

Cell viability assays

A549 cells (1.0×10^4/well) were seeded into 96-well plates. After 24 h, the medium was replaced with a serum-free medium or various concentrations of BLM (Selleck Chemicals, Houston, TX, USA) for another 24 h. Then, 0, 10, 20, 40, 80, 160, and 320 μM of BLM were added to the A549 cells. Cell viability was assessed using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD, USA). The same volume of CCK-8 solution was added to each well for another 2 h, after which the absorbance of each well was measured on a multimode reader at 450 nm (Figure E7A). Data were pooled from three independent experiments.

NPY or Y1R antagonist treatment in BLM-exposed A549 cells

After seeding the A549 cells (1.0×10^4/well), they were concurrently treated with serum-free medium, BLM (80 μM), NPY (0.01, 0.1, and 1 μM), and Y1receptor antagonist BIBP3226 (1 μM). The dose of NPY and Y1 receptor antagonist BIBP3226 were determined based on the previous report (17), and all the reagents were dissolved by distilled water. Distilled water did not affect cell viability (Data not shown). The A549-culture supernatant was collected 24 h later. The samples were then centrifuged at 1500 rpm for 5 min at room temperature, and stored at −80 °C before they were assayed. Data were pooled from three independent experiments.

RNA extraction, reverse transcription, and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from A549 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific); in all experiments, 1000 ng of RNA were used as the template for reverse transcription with Super Script™ II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. qPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific) and TaqMan Gene Expression Master Mix (Applied Biosystems). Primer sequences and probes are shown in Table E1. Analyses were

performed on the LightCycler 96 using the comparative delta cycle threshold method. Data, pooled from three independent experiments, were normalized to GAPDH expression.

Human samples

All blood samples were collected from patients and healthy controls who had provided informed consent to participate in the study. Blood samples for plasma NPY analysis were collected from IPF patients immediately before lung transplantation; blood samples for serum NPY and IL-1β analysis were collected from IPF patients in an outpatient setting. Serum samples from IPF patients and healthy controls were collected into non-anticoagulant tubes, centrifuged at 3000 rpm for 5 min at 4 °C, and stored at −80 °C before performing assays. Plasma samples from IPF patients and healthy controls were collected using a previously described method (18). Human lung tissue samples were obtained from transplant patients. The characteristics of serum, plasma, and lung tissue samples are shown in Tables 1, 2, and E2. Lung tissue samples were obtained from IPF patients who had already undergone lung transplantation. Donor lung tissues were obtained following brain death because the lung sections had slight congestion. The remaining normal sections of the donor lung were used. The characteristics of the lung tissues are shown in Table 2. IPF was diagnosed by at least three respiratory physicians independently, based on the 2018 ATS/ERS/JRS/ALAT criteria (19).

Okayama University Graduate School approved all human-sample experiments in the present study (Okayama University Hospital Ethics Committee #1908-045).

Immunohistochemistry

Human lung tissues were fixed in 10% buffered formalin, embedded in paraffin, and cut into 3-μm-thick sections for immunohistochemical analysis. Heat-mediated antigen retrieval was performed by incubating the sections in 1 mM EDTA buffer in a Pascal pressure chamber (DakoCytomation, Glostrup, Denmark). The sections were blocked, then incubated overnight with primary antibodies: mouse anti-IL-1β antibody (1:300; ab156791; Abcam), rabbit anti-EpCAM antibody (1:200; ab223582; Abcam), and rabbit anti-CD68 antibody (1:800; #76437; Cell Signaling Technologies). Secondary antibodies were from the ImmPRESS Duet Double Staining Polymer Kit (MP-7714; Vector Laboratories, Burlingame, CA, USA), which was used according to the manufacturer's instructions. Anti-rabbit IgG antibody and anti-mouse IgG antibody were conjugated with horseradish peroxidase and alkaline phosphatase, respectively. 3-3'-diaminobenzidine and Vector Red (Vector Laboratories) were used as chromogens. Tissues were then counterstained with hematoxylin.

Immunofluorescence staining of murine and human lungs

Murine and human lung tissues were fixed in 10% buffered formalin and embedded in paraffin, and 3-μm thick sections were used for immunofluorescence (IF) staining. Bond Max stainer (Leica Biosystems, Washington, D.C., USA) was used for antigen retrieval. The primary and secondary antibodies are shown in Table E3. The sections were followed by blocking and primary antibodies were incubated overnight. Primary antibodies (Table E3) for mouse lungs included anti-rabbit-NPY antibody (1:200; NBP2-33423; Novus Biologicals, Littleton, CO, USA) and anti-mouse EpCAM antibody (1:10; 323/A3; Invitrogen). Primary antibodies used for human lungs included anti-sheep-NPY antibody (1:200; ab6173; Abcam), anti-rabbit-epithelial cell adhesion molecule (EpCAM) antibody (1:200; ab223582; Abcam), and anti-rabbit-pro-surfactant protein C (pro-SPC) antibody (1:2000; ab90716; Abcam), and anti-mouse-CD68 antibody (1:200; ab955; Abcam). Secondary antibodies included anti-rabbit-IgG antibodies conjugated with Alexa Fluor 488 (1:400; ab150077; Abcam), anti-sheep-IgG antibodies conjugated with Alexa Fluor 488 (1:400; A11015; Invitrogen), and anti-rabbit-IgG antibodies conjugated with Alexa Fluor 555 (1:400; ab150078; Abcam), and anti-mouse-IgG antibodies conjugated with Alexa Fluor 555 (1:400; ab150114; Abcam). Sections were mounted using the ImmunoSelect Antifading Mounting Medium DAPI (SCR-038448; Dianova, Hamburg, Germany), viewed using fluorescence microscope (BZ-X700; Keyence, Osaka, Japan), and analyzed using the BZ-X Analyzer software program (Keyence).

Statistical analyses

The results were expressed as means ± standard errors of the mean (SEMs). Statistical analysis of data was done using Prism 9 (GraphPad Software, Inc., San Diego, CA, USA). Comparisons among multiple groups were performed by one-way or two-way analysis of variance (ANOVA) with Tukey's multiple comparison test. Comparisons between two groups were analyzed for equal variance using two-tailed Student's t-test, and for unequal variance using the Mann–Whitney U test.

Supplemental references

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Supplemental Figure Legends

Supplemental Figure E1. BLM-instilled NPY$^{-/-}$ mice showed significantly higher hydroxyproline levels compared to BLM-instilled WT mice on day 21.

Hydroxyproline levels in lung of BLM- and PBS-instilled mice on day 21. WT PBS; WT mice instilled with PBS (n = 5), NPY$^{-/-}$ PBS; NPY$^{-/-}$mice instilled with PBS (n = 3), WT BLM; WT mice instilled with BLM (n = 9), and NPY$^{-/-}$ BLM; NPY$^{-/-}$ mice instilled with BLM (n = 9). Data are represented as mean ± SEM.WT PBS, WT mice, instilled with PBS; NPY$^{-/-}$ mice PBS, NPY$^{-/-}$ mice instilled with PBS; WT BLM, WT mice instilled with BLM; and NPY$^{-/-}$ mice BLM, NPY$^{-/-}$ mice instilled with BLM. *p < 0.05, **p < 0.01, ****p < 0.0001 (Two-way ANOVA with Tukey's multiple comparisons was used for Figure E1).

Supplemental Figure E2. NPY expression in mouse lung tissues.

Immunofluorescence staining with anti-NPY and anti-epithelial cell adhesion molecule (EpCAM) antibodies (40×) in mouse lung tissues. NPY-positive cells in PBS(A)- and BLM-instilled WT mice (B) on day 2: WT PBS (n = 3) and WT BLM (n = 3). Thin and bold arrows indicate alveolar epithelial cells and inflammatory cells, respectively. Arrowheads indicate bronchial epithelial cells. NPY-positive cells overlapped with EpCAM-positive cells around bronchial epithelial cells (arrowheads). Representative images from three mice are shown.

Scale bars: 50 μm. WT PBS, WT mice, instilled with PBS; WT BLM, WT mice instilled with BLM (n = 3).

Supplemental Figure E3. Flowcytometry for T helper 17 (Th17) cells and Ki67-positive Th17 cells.

(A) The Gating strategy of T helper 17 (Th17) and Ki67-positive Th17 cells. The Th17 and Ki67-positive Th17 cells in the lungs were CD3+, CD4+, RORγt+, and Ki67+ cells on flow cytometry.

(B, C) Th17 cells and Ki67-positive Th17 cells in the lungs and Th17 cells in the blood.

(B) The percent of Ki67-positive Th17 cells in lungs BLM- and PBS-instilled mice on day 2. WT PBS; WT mice instilled with PBS (n = 4), $NPY^{-/-}$ PBS; $NPY^{-/-}$ mice instilled with PBS (n = 4), WT BLM; WT mice instilled with BLM (n = 4), and $NPY^{-/-}$ BLM; $NPY^{-/-}$ mice instilled with BLM (n = 4). (C) Th17 cells in the blood. WT PBS (n = 4), $NPY^{-/-}$ PBS (n = 4), WT BLM (n = 4), and $NPY^{-/-}$ BLM (n = 3). Data are represented as mean ± SEM.WT PBS, WT mice, instilled with PBS; $NPY^{-/-}$ mice PBS, $NPY^{-/-}$ mice instilled with PBS; WT BLM, WT mice instilled with BLM; and $NPY^{-/-}$ mice BLM, $NPY^{-/-}$ mice instilled with BLM. *$p < 0.05$, **$p < 0.01$ (Two-way ANOVA with Tukey's multiple comparisons was used for Figures E3B and E3C).

Supplemental Figure E4. NPY treatment suppressed pulmonary fibrosis in BLM-instilled WT mice.

(A) Timing of the experiments and NPY treatment in WT mice with BLM-induced pulmonary fibrosis. (B) Lung photomicrographs of PBS-instilled WT mice treated with PBS or 200 μg/kg of NPY, and BLM-instilled WT mice treated with PBS, 50 μg/kg of NPY, or 200 μg/kg of NPY on day 21, stained with hematoxylin and eosin (top) and Masson's trichrome stains (bottom). Representative images from three mice. Scale bars: 200 μm. (C) Quantitative evaluation of Masson's-trichrome-stained fibrotic areas in PBS-instilled WT mice treated with PBS (n = 4) or 200 μg/kg of NPY (n = 4) and BLM-instilled WT mice treated with PBS (n = 4), 50 μg/kg of NPY (n = 3), or 200 μg/kg of NPY (n = 5). Data are represented as mean ± SEM. PBS/PBS, PBS-instilled WT mice treated with PBS, PBS/NPY, PBS-instilled WT mice treated with NPY, BLM/PBS, BLM-instilled WT mice treated with PBS, and BLM/NPY, BLM-instilled WT mice treated with NPY. i.p.: intraperitoneal injections, i.t.: intratracheal instillation. ***$p < 0.001$ and ****$p < 0.0001$.

Supplemental Figure E5. Fibrotic changes in WT mice.

(A) Masson's trichrome staining in BLM- (bottom) and PBS- (top) instilled WT mice on days

2 and 7. Scale bars: 200 μm. (B) Fibrotic areas were measured by quantitative image analysis, WT PBS, WT mice instilled with PBS, and WT BLM, WT mice instilled with BLM (n = 3 in each group). Data are represented as mean ± SEM. WT PBS, WT mice, instilled with PBS and WT BLM, WT mice instilled with BLM. **$p < 0.01$.

Supplemental Figure E6. Effects of NPY in $NPY^{-/-}$ mice with established fibrosis.

(A) Timing of experimental NPY administration in $NPY^{-/-}$ mice with established fibrosis following BLM or PBS instillation on day 0. (B) Lung photomicrographs of PBS- and BLM-instilled $NPY^{-/-}$ mice treated with NPY or PBS on day 21 were stained with H&E (left) and Masson's trichrome stains (right). Scale bars: 200 μm. (C) Quantitative image analysis of Masson's-trichrome-stained fibrotic areas, PBS/PBS, PBS-instilled $NPY^{-/-}$ mice treated with PBS (n = 5), BLM/PBS, BLM-instilled $NPY^{-/-}$ mice treated with PBS (n = 7), and BLM/NPY, BLM-instilled $NPY^{-/-}$ mice treated with NPY (n = 7). (D) Collagen levels in the BAL fluids of BLM- and PBS-instilled $NPY^{-/-}$ mice treated with PBS or NPY on day 21, PBS/PBS (n = 5), BLM/PBS (n = 7), BLM/NPY (n = 7). (E) Cellular composition of BAL fluids of BLM- and PBS-instilled $NPY^{-/-}$ mice treated with PBS or NPY on day 21, PBS/PBS (n = 5), BLM/PBS (n = 7), BLM/NPY (n = 7). (F, G) IL-1β in the lungs and TGF-β1 in BAL fluids of BLM- and PBS-instilled $NPY^{-/-}$ mice treated with PBS or NPY on day 21, PBS/PBS (n = 5), BLM/PBS

(n = 7), BLM/NPY (n = 7). (H) NPY levels in the BAL fluids of BLM- and PBS-instilled $NPY^{-/-}$ mice treated with PBS or NPY on day 21, PBS/PBS (n = 5), BLM/PBS (n = 7), BLM/NPY (n = 7). (I) Serum NPY levels in BLM- and PBS-instilled $NPY^{-/-}$ mice treated with PBS or NPY on day 21, PBS/PBS (n = 5), BLM/PBS (n = 7), BLM/NPY (n = 7). The levels of NPY in BAL fluids and serum were zero in PBS/PBS and BLM/PBS in $NPY^{-/-}$ mice. Data are represented as mean ± SEM. Eos, eosinophils; Lym, lymphocytes; Mac, macrophages; Neut, neutrophils; TGF, transforming growth factor. WT PBS, WT mice, instilled with PBS and WT BLM, WT mice instilled with BLM. PBS/PBS, PBS-instilled $NPY^{-/-}$ mice treated with PBS; BLM/PBS: BLM-instilled $NPY^{-/-}$ mice treated with PBS; and BLM/NPY, BLM-instilled $NPY^{-/-}$ mice treated with NPY. **p< 0.01, ***p < 0.001, ****p < 0.0001.

Supplemental Figure E7. NPY-Y1 receptor axis inhibited IL-1β release and production in alveolar type-2 epithelial cells.

(A) Cell viability following BLM exposure. A549 cells were incubated with different concentrations of BLM for 24 h (n = 4 in each group) and analyzed from three independent experiments. (B and C) qRT-PCR analysis of A549 cells exposed to BLM (80 μM) (n = 3 in each group). Relative mRNA expression related with epithelial-mesenchymal transition (EMT) markers, α-smooth muscle actin (α-SMA) (B), and E-cadherin (C). Data from three

independent experiments were normalized to GAPDH expression. The primer sequences are shown in Table E1. (D) NPY levels in A549 culture supernatant following exposure to various BLM doses, measured using ELISA (n = 3 in each group). Data are represented as mean ± SEM. *p < 0.05, ***p < 0.001.

Supplemental Figure E8. Plasma NPY levels in IPF patients and healthy controls.

Plasma NPY levels of healthy controls and IPF patients were assessed using ELISA, healthy controls (n = 12), and IPF patients (n = 9). The characteristics of IPF patients and healthy controls are shown in Table E2. Data are represented as mean ± SEM. ****p < 0.0001.

IPF: Idiopathic pulmonary fibrosis.

Supplemental Figure E9. NPY expression in the lungs of Donor and IPF patients.

Immunofluorescence staining of NPY in lung tissue sections from the donor and IPF patients. Clinical characteristics of the donor and IPF patients are presented in Table 2. (A and B) The immunofluorescence staining of NPY and EpCAM (A) or pro-SPC (B) in IPF lung tissue sections. (A) IPF lung sections co-immunostained with NPY and EpCAM (×40). Scale bars: 50 μm. (B) IPF lung sections co-immunostained with NPY and pro-surfactant protein C (pro-SPC) (×40). Scale bars: 50 μm. NPY-positive cells overlap with pro-SPC-positive cells around

areas of thickening of the alveolar walls (arrowheads). (C and D) Immunofluorescence staining of NPY and CD68 (×40) in lung tissue sections from the donor (C) and IPF patients (D). Scale bars: 50 μm. NPY-positive cells overlapped with CD68-positive macrophages in the alveolar space (arrowheads). Representative images from the donor (n = 1) and IPF patients (n = 3) are shown. Scale bars: 50μm.

EpCAM: Epithelial cell adhesion molecule; pro-SPC: pro-surfactant protein C.

Supplemental Figure E10. IL-1β expression in the lungs of donor and IPF patients.

Lung tissue sections from the donor and IPF patients were subjected to immunohistochemical staining with anti-IL-1β antibody. Clinical characteristics of the donor and IPF patients are presented in Table 2. (A) Donor and IPF lung tissue sections co-immunostained with anti-IL-1β and EpCAM antibodies. Higher magnification (bottom) of the area enclosed by the square (top). IL-1β-positive cells (magenta) overlapped with EpCAM (brown)-positive cells around the alveolar epithelium and airway epithelial metaplasia(arrowheads). Scale bars: 200 μm (top); 50 μm (bottom). (B) Donor and IPF lung tissue sections co-immunostained with anti-IL-1β and anti-CD68 antibodies. Scale bars: 200 μm (top); 50 μm (bottom). Higher magnification (bottom) of the area enclosed by the square (top). CD68-positive macrophages (brown) were observed in the alveolar space in donor and IPF patients. IL-1β-positive cells (magenta)

overlapped with CD68-positive macrophages (brown) in the alveolar space (arrowheads). Representative images from the donor (n = 1) and IPF patients (n = 3). Scale bars: 200 μm (top); 50 μm (bottom).

EpCAM, epithelial cell adhesion molecule.

Supplemental Tables

Supplemental Table E1: **Primer sequences and gene-expression array cards for real-time quantitative polymerase chain reaction.**

Gene	Primer sequence (5′→3′; 3′→5′)
GAPDH	Fw: CTCCTCCACCTTTGACGCTG
	Rv: TCCTCTTGTGCTCTTGCTGG
E-cadherin	Fw: CGGGAATGCAGTTGAGGATC
	Rv: AGGATGGTGTAAGCGATGGC
α-SMA	Fw: CTATGAGGGCTATGCCTTGCC
	Rv: GCTCAGCAGTAGTAACGAAGGA
Gene	Gene Expression Array Cards
GAPDH	Hs02786624_g1
NPY1R	Hs00702150_s1
NPY2R	Hs01921296_s1
NPY4R	Hs00275980_s1
NPY5R	Hs01883189_s1

α-SMA: α-smooth muscle actin; NPY1R: neuropeptide Y Y1 receptor; NPY2R: neuropeptide Y Y2 receptor; NPY4R: neuropeptide Y Y4 receptor; NPY5R: neuropeptide Y Y5 receptor.

Supplemental Table E2: **Clinical characteristics of the IPF patients and controls.**

		Healthy controls	IPF	p-Value
Plasma				
n		12	**7**	
Sex	Male/female	9/3	7/0	0.263
Age, years	Median, (range)	35, (24–72)	58, (52–59)	0.256

IPF: Idiopathic pulmonary fibrosis.

Supplemental Table E3: **Antibodies used in the immunofluorescence analysis.**

A. Primary antibodies for mouse			
Antigen	**Code**	**Manufacture**	**Dilution**
NPY	NBP2-33423	Novus Biologicals, CO, USA	1:200
EpCAM	323/A3	Invitrogen, CA, USA	1:10
B. Primary antibodies for Human			
Antigen	**Code**	**Manufacture**	**Dilution**
NPY	ab6173	Abcam, Cambridge, UK	1:200
EpCAM	ab223582	Abcam, Cambridge, UK	1:200
pro-SPC	ab90716	Abcam, Cambridge, UK	1:2000
CD68	ab955	Abcam, Cambridge, UK	1:200
C. Secondary antibody			
Antibody	**Code**	**Manufacture**	**Dilution**
anti-sheep IgG Alexa Fluor 488	A11015	Invitrogen, CA, USA	1:400
anti-rabbit IgG Alexa Fluor 488	ab150077	Abcam, Cambridge, UK	1:400

anti-rabbit IgG Alexa Fluor 555	ab150078	Abcam, Cambridge, UK	1:400
anti-mouse IgG Alexa Fluor 555	ab150114	Abcam, Cambridge, UK	1:400

1 EpCAM: Epithelial cell adhesion molecule; pro-SPC: pro-surfactant protein C.