

Neuropeptide Y antagonizes development of pulmonary fibrosis through IL-1 β inhibition

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AT A GLANCE COMMENTARY

1

2 **Scientific Knowledge on the Subject:** NPY has previously been linked to airway
3 inflammation, but its role in pulmonary fibrosis is unclear.

4

5 **What This Study Adds to the Field:** NPY suppresses the development of pulmonary
6 fibrosis, and might be beneficial for treatment of pulmonary fibrosis.

7

8 This article has an online data supplement, which is accessible from this issue's table of
9 content online at www.atsjournals.org.

10

1 **Abbreviations**

2	α -SMA:	α -smooth muscle actin
3	AT2:	Alveolar type 2 epithelial cells
4	BAL:	Bronchoalveolar lavage
5	BLM:	Bleomycin sulfate
6	EMT:	Epithelial-mesenchymal transition
7	EpCAM:	Epithelial cell adhesion molecule
8	H&E:	Hematoxylin and eosin
9	IF:	Immunofluorescence
10	IL:	Interleukin
11	IPF:	Idiopathic pulmonary fibrosis
12	mAb:	monoclonal antibody
13	MCP:	Monocyte chemoattractant protein
14	NPY:	Neuropeptide Y
15	PBS:	Phosphate-buffered saline
16	pro-SPC:	pro-surfactant protein C
17	TGF:	Transforming growth factor
18	Th17:	T-helper type 17 cell

- | | | |
|---|------|----------------------------|
| 1 | TNF: | Tumor necrosis factor |
| 2 | WT: | Wild type |
| 3 | Y1R: | neuropeptide Y Y1 receptor |
| 4 | Y2R: | neuropeptide Y Y2 receptor |
| 5 | Y4R: | neuropeptide Y Y4 receptor |
| 6 | Y5R: | neuropeptide Y Y5 receptor |

1 **Abstract**

2 Neuropeptide Y, a 36-amino acid residue polypeptide, distributed throughout the nervous
3 system, acts on various immune cells in many organs, including the respiratory system.
4 However, little is known about its role in the pathogenesis of pulmonary fibrosis. This study
5 was performed to determine the effects of neuropeptide Y on pulmonary fibrosis. Neuropeptide
6 Y-deficient and wild-type mice were intratracheally administered bleomycin. Inflammatory
7 cells, cytokine levels, and morphological morphometry of the lungs were analyzed. Serum
8 neuropeptide Y levels were also measured in idiopathic pulmonary fibrosis patients and healthy
9 controls. Neuropeptide Y-deficient mice exhibited significantly enhanced pulmonary fibrosis
10 and higher IL-1 β levels in the lungs compared to wild-type mice. Exogenous neuropeptide Y
11 treatment suppressed the development of bleomycin-induced lung fibrosis and decreased IL-
12 1 β levels in the lungs. Moreover, IL-1 β neutralization in neuropeptide Y-deficient mice
13 attenuated the fibrotic changes. Neuropeptide Y decreased IL-1 β release, and Y1 receptor
14 antagonists inhibited IL-1 β release and induced epithelial mesenchymal transition in human
15 alveolar epithelial cells. Patients with idiopathic pulmonary fibrosis had lower neuropeptide Y
16 and greater IL-1 β levels in the serums compared to healthy controls. Neuropeptide Y
17 expression was mainly observed around bronchial epithelial cells in human idiopathic
18 pulmonary fibrosis lungs. These data suggest that neuropeptide Y plays a protective role against

1 pulmonary fibrosis by suppressing IL-1 β release and manipulating the neuropeptide Y-Y1
2 receptor axis could be a potential therapeutic strategy for delaying disease progression.

3

4 **Keywords:** idiopathic pulmonary fibrosis, neuropeptide Y, IL-1 β , bleomycin, bronchial
5 epithelial cells

6

7 **Abstract word count:** 228 (less than 250)

8

9

1 **Introduction**

2 Idiopathic pulmonary fibrosis (IPF) has a poor prognosis with a median survival time of
3 approximately 2–5 years after diagnosis (1–3). The development of fibrosis is characterized by
4 recurrent damage to pulmonary epithelium and ineffective repair following aberrant
5 fibroblastic response (4–6). Alveolar epithelial cells acquire a mesenchymal phenotype through
6 epithelial-mesenchymal transition (EMT), which causes fibroblast activation (7, 8). Several
7 chemokines, cytokines, and growth factors, such as transforming growth factor (TGF)- β 1,
8 interleukin (IL)-17A, and IL-1 β , have been reported to mediate EMT in pulmonary fibrosis (9–
9 16).

10 Neuropeptide Y (NPY) is a 36 amino-acid residue polypeptide widely distributed in
11 the central and peripheral nervous systems (17, 18). NPY also affects immune cells and
12 inflammation in several organs (19, 20). Recent studies have reported the role of NPY in airway
13 inflammation, such as asthma (21–23). However, the role of NPY in pulmonary fibrosis is
14 poorly understood.

15 Evidence has shown that NPY could inhibit nitric oxide production and IL-1 β release
16 in lipopolysaccharide-stimulated microglial cells (24). NPY also exerts anticonvulsant effects
17 by reducing serum levels of pro-inflammatory cytokines, such as IL-1 β , tumor necrosis factor
18 (TNF)- α , and IL-6 (25). These studies suggest the potential involvement of NPY in the

1 pathogenesis of pulmonary fibrosis by regulating the production or release of pro-inflammatory
2 cytokines.

3 The present study aimed to investigate the effects of NPY on bleomycin (BLM)-
4 induced pulmonary fibrosis in NPY-deficient (NPY^{-/-}) mice. We demonstrated that NPY exerts
5 a protective effect against BLM-induced pulmonary fibrosis by suppressing the release of IL-
6 1 β .

7

1 **Materials and Methods**

2 Additional details of the materials and methods can be found in the online supplement.

3 **Animal procedures**

4 All experimental procedures were approved by the Animal Care and Use Committee of
5 Okayama University.

6

7 **BLM administration**

8 Female C57BL/6J wild-type (WT) and NPY-deficient (NPY^{-/-}) mice (23) were administered
9 either BLM or phosphate-buffered saline (PBS) by intratracheal instillation on day 0. To assess
10 the fibrotic changes, lungs were harvested on days 0, 2, 7, 14, and 21.

11

12 **NPY administration**

13 WT and NPY^{-/-} mice received daily intraperitoneal NPY injections from day -1 to 20, or day
14 7 to 20, while control mice received PBS injections. Airway inflammation and lung fibrotic
15 changes were assessed 21 days after BLM instillation.

16

17 **Anti-mouse IL-1 β monoclonal antibody (mAb) administration**

18 NPY^{-/-} mice were treated using anti-mouse IL-1 β mAb or control IgG, along with BLM or

1 PBS. Lung inflammation and fibrosis were evaluated 21 days after BLM instillation.

2

3 **Bronchoalveolar lavage (BAL) fluid analysis**

4 The lungs were lavaged and the number of cells counted on days 0, 2, 7, 14, and 21 (26).

5

6 **Blood collection**

7 Murine blood samples were collected for serum analysis on days 2 and 21.

8

9 **Collagen assay**

10 Collagen concentration in the BAL fluid was measured using a collagen assay kit.

11

12 **Lung histology**

13 Right-lung tissue sections were stained with hematoxylin and eosin (H&E), and Masson's

14 trichrome stains to analyze the fibrosis (27, 28).

15

16 **Lung homogenates**

17 Left-lung tissues were used to prepare lung homogenates (26).

18

1 **Cytokine and chemokine measurement**

2 Cytokine and chemokine levels in the BAL fluid and lung homogenates were measured using
3 enzyme-linked immunosorbent assay (ELISA) (23).

4

5 **Flow cytometry**

6 Lung cells isolated by collagenase digestion were stained as previously described (29).

7

8 **Cell viability assays**

9 A549 cells were seeded with the medium or various BLM concentrations for 24 h. Cell viability
10 was assessed using Cell Counting Kit-8.

11

12 **NPY or Y1 receptor (Y1R) antagonist treatment**

13 After seeding, A549 cells were treated concurrently with the medium, BLM, NPY, and Y1R
14 antagonist BIBP3226. The culture supernatant was collected after another 24 h, and stored for
15 further assessment.

16

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

1 RNAs were extracted from A549 cells using TRIzol reagent. Reverse transcription was
2 performed using Super Script™ II Reverse Transcriptase (Thermo Fisher Scientific, Waltham,
3 MA, USA) according to the manufacturer's instructions. qPCR was performed using TaqMan
4 and SYBR Green methods. Primer sequences and probes are shown in Table E1.

6 **Human samples**

7 Serum samples were collected from IPF patients and healthy controls, and plasma samples
8 were obtained as previously described (30). Human lung tissues were obtained from transplant
9 patients. The study was approved by the Okayama Medical School and Hospital's Research
10 Ethics Committee.

12 **Immunofluorescence staining of murine and human lungs**

13 Mouse- and human-lung tissues were stained using antibodies (Table E3). Images were
14 captured using the BZ-X700 fluorescence microscope (Keyence, Osaka, Japan).

16 **Statistical analyses**

17 The results were expressed as means $\pm$ standard errors of the mean (SEMs). Comparisons
18 among multiple groups were performed by one-way or two-way analysis of variance (ANOVA)

- 1 with Tukey's multiple comparison test. Two-group comparisons were performed using two-
- 2 tailed Student's t-test for equal variance and the Mann–Whitney U test for unequal variance. P
- 3 values < 0.05 were considered significant.

4

Results

Development of pulmonary fibrosis is enhanced in NPY^{-/-} mice

We assessed airway inflammation and fibrosis after BLM instillation in NPY^{-/-} mice to determine the role of NPY in the development of pulmonary fibrosis. Although no pathological differences were observed between WT and NPY^{-/-} mice following PBS instillation, BLM-instilled NPY^{-/-} mice exhibited significantly greater fibrotic areas compared to BLM-instilled WT mice on day 21 (Figures 1A and 1B). BLM-instilled NPY^{-/-} mice also had higher collagen levels in the BAL fluid and higher hydroxyproline levels in the lungs compared to BLM-instilled WT mice on day 21 (Figures 1C and 1D). Cellular differentiation in BAL fluid was analyzed to determine the influence of inflammatory-cell recruitment in NPY^{-/-} mice. BLM-instilled NPY^{-/-} mice had significantly greater numbers of total cells, neutrophils, and lymphocytes in the BAL fluid compared to BLM-instilled WT mice on day 21 (Figure 1E). The levels of IL-1 β in lung homogenate and TGF- β 1 in BAL fluid were also significantly higher in BLM-instilled NPY^{-/-} mice compared to BLM-instilled WT mice on day 21 (Figures 1E and 1F). These data suggest that NPY is protective against BLM-induced pulmonary inflammation and fibrosis.

Pro-inflammatory cytokine levels in BLM-instilled WT and NPY^{-/-} mice

WT mice were administered either PBS or BLM, and assessed on days 0, 2, 7, 14, and 21, to examine the influence of NPY on airway inflammation and pulmonary fibrosis. NPY levels in BAL fluids of BLM-instilled WT mice peaked on day 2 and then gradually decreased, and were completely suppressed by day 21 (Figure 2A). Consistent with these results, serum NPY levels in BLM-instilled WT mice were significantly higher on day 2 (Figure 2B) and were significantly lower on day 21, compared to those of PBS-instilled WT mice (Figure 2C).

Since we observed increased NPY levels in serums and BAL fluids of BLM-instilled WT mice on day 2 (Figures 2A and 2B), the acute inflammation caused by BLM instillation may have been induced systemically and locally in the lungs, resulting in increased NPY in serum and BAL fluid. Next, we analyzed NPY expression in the lungs on day 2 following BLM or PBS instillation using immunofluorescent (IF) staining. NPY-positive cells were mainly the bronchial epithelial cells in both PBS-instilled mice and BLM-instilled mice; they overlapped with epithelial cell adhesion molecule (EpCAM)-positive cells around bronchial epithelial cells, and some inflammatory cells and alveolar cells in BLM-instilled WT mice on day 2 (Figures 2D and E2). Absence of NPY expression was confirmed in NPY^{-/-} mice (Figures 2D; a and d).

To assess the contribution of inflammatory cytokines toward lung fibrosis in NPY^{-/-} mice, we measured IL-1 β and IL-17A levels in lungs, and monocyte chemoattractant protein-1

(MCP-1) levels in BAL fluids. BLM-instilled NPY^{-/-} mice had significantly higher lung IL-1 β levels compared to BLM-instilled WT mice on day 2 (Figure 2E). BLM-instilled NPY^{-/-} mice also had higher MCP-1 levels in BAL fluids, and IL-17A levels in the lungs, compared to BLM-instilled WT mice on day 2, but the differences were not statistically significant (Figures 2F and 2G).

IL-1 β has been reported to induce T helper 17 (Th17) cells (31, 32). BLM-instilled NPY^{-/-} mice had higher airway IL-1 β levels compared to BLM-instilled WT mice. We used flow cytometry to analyze the numbers of Th17 cells in the lungs (Figure E3A); BLM-instilled NPY^{-/-} mice had significantly higher numbers on day 2 compared to BLM-instilled WT mice (Figure 2H). To investigate the recruitment and proliferation of Th17 cells, we used flow cytometry to analyze the numbers of Th17 cells in blood and the numbers of Ki67-positive Th17 cells in the lungs using flow cytometry (Figure E3A). BLM-instilled NPY^{-/-} mice had significantly higher numbers of Ki67-positive Th17 cells in the lungs on day 2 compared to BLM-instilled WT mice (Figures 2H and E3B). There were no significant differences among groups in the numbers of Th17 cells in blood (Figure E3C). These findings imply that increased IL-1 β secretion in BLM-instilled NPY^{-/-} mice did not affect recruitment of Th17 cells into the lungs but enhanced Th17 cell proliferation in the lungs.

NPY treatment ameliorates pulmonary fibrosis in BLM-instilled WT and NPY^{-/-} mice

WT mice with BLM-induced pulmonary fibrosis were treated with NPY to determine if it prevented pulmonary fibrosis. Prophylactic NPY administration was started 24 h prior to BLM instillation (Figure E4A). Prophylactic NPY significantly reduced the fibrotic changes in mice in a dose-dependent manner, compared to PBS (Figures E4B and E4C). Fibrotic changes in PBS-instilled mice did not significantly differ between treatment with PBS or NPY. Next, we examined whether NPY supplementation could improve established fibrosis in the BLM-induced mice model (Figure 3A). NPY therapy was initiated 7 days after BLM instillation, which was the time taken for fibrotic changes to develop in this model (Figures 3A, E5A, and E5B). NPY-treated mice showed markedly reduced lung fibrosis compared with PBS-treated mice (Figures 3B and 3C). Collagen levels in BAL fluids of NPY-treated mice were significantly lower than PBS-treated mice (Figure 3D). NPY-treated mice had significantly lower white blood cell numbers in BAL fluids, including lymphocytes, neutrophils, and eosinophils, compared to PBS-treated mice on day 21 (Figure 3E).

Furthermore, NPY-treated mice had significantly lower IL-1 β levels in the lungs and TGF- β 1 levels in BAL fluids compared to PBS-treated mice on day 21 (Figures 3F and 3G). NPY-treated mice showed higher NPY levels in BAL fluids and serums compared to PBS-treated mice (Figures 3H and 3I). These data suggest that exogenous NPY treatment prevents

pulmonary fibrosis. The effects of exogenous NPY supplementation were also investigated in BLM-instilled NPY^{-/-} mice. Consistent with the findings in WT mice, NPY supplementation suppressed the development of pulmonary fibrosis in BLM-instilled NPY^{-/-} mice (Figures E6A–E6I).

Neutralization efficacy of anti-IL-1 β mAb

We demonstrated that greater pulmonary fibrosis and inflammation in NPY^{-/-} mice were associated with higher IL-1 β levels in the lungs, and NPY treatment suppressed pulmonary fibrosis and decreased IL-1 β levels. We sought to determine whether IL-1 β neutralization suppressed fibrotic changes and airway inflammation in NPY^{-/-} mice (Figure 4A). NPY^{-/-} mice treated with anti-IL-1 β mAbs had significantly milder fibrotic changes compared to those treated with control IgG, following BLM instillation (Figures 4B and 4C). Collagen levels in BAL fluids were also significantly lower in anti-IL-1 β mAb-treated mice compared to IgG-treated mice on day 21 (Figure 4D). TGF- β 1 and IL-1 β are profibrotic mediators that lead to the development of fibrosis (33); the inhibition of IL-1 β reduces TGF- β 1 expression and fibrotic changes (34). Therefore, we evaluated the levels of TGF- β 1 in our mouse model. Anti-IL-1 β mAb-treated mice showed significantly lower white cell numbers in BAL fluids, including lymphocytes and neutrophils (Figure 4E), as well as considerably lower IL-1 β levels

in lungs and TGF- β 1 levels in BAL fluids, compared to IgG-treated mice on day 21 (Figures 4F and 4G). These data suggested that the inhibition of IL-1 β reduces the development of pulmonary fibrosis in BLM-instilled NPY^{-/-} mice.

NPY-Y1 receptor axis inhibits IL-1 β secretion in alveolar type-2 epithelial cells

To explore further the mechanisms underlying IL-1 β suppression with NPY therapy, we investigated the effects of NPY in alveolar type-2 epithelial cells (AT2) using the A549 cell line. First, we determined the median lethal dose of BLM in A549 cells using the CCK-8 assay (Figure E7A), and calculated the appropriate BLM concentration as 80 μ M. This concentration led to a significant increase in α -smooth muscle actin (α -SMA) expression and a decrease in E-cadherin expression compared to controls (Figures E7B and E7C). The cell viabilities were unaffected by diluent (data not shown). There were no significant differences in NPY values based on BLM concentrations (Figure E7D).

NPY treatment significantly inhibited IL-1 β secretion in the culture supernatant of A549 cells exposed to BLM (Figure 5A), suggesting that exogenous NPY treatment might inhibit IL-1 β secretion in AT2. Next, we investigated the expression of NPY receptors, Y1R, Y2R, Y4R, and Y5R, in A549 cells using qPCR. Y1R had the highest expression (Figure 5B), so we examined the effects of the Y1 receptor antagonist, BIBP3226. BIBP3226 antagonized

the effects of NPY on IL-1 β levels in the supernatant (Figure 5C). BIBP3226 also nullified the inhibitory effects of NPY on α -SMA and E-cadherin gene expression in A549 cells exposed to BLM (Figures 5D and 5E). These data suggest that NPY inhibits IL-1 β through the NPY–Y1R axis to exert protective effects against pulmonary fibrosis.

Serum and lung NPY levels in IPF patients

As stated previously, serum NPY levels were decreased in mice with established fibrosis. This prompted us to investigate NPY levels in the serums of IPF patients. Table 1 summarizes the characteristics of the enrolled patients. Compared to healthy controls, IPF patients had significantly lower serum NPY levels (Figure 6A) and higher serum IL-1 β levels (Figure 6B). We further analyzed the plasma NPY levels in IPF patients prior to lung transplantation. The characteristics of enrolled patients are summarized in Table E2. IPF patients had significantly lower plasma NPY levels compared to healthy controls (Figure E8).

Next, we analyzed NPY expression in human lungs using IF staining. The characteristics of IPF patients and a donor are shown in Table 2. NPY expression was detected mainly around bronchial epithelial cells in the donor lung, whereas few NPY-positive cells were detected in IPF lungs (Figures 6C and 6D). To detail NPY distribution further, we investigated the relationship between NPY, and EpCAM-positive epithelial cells and pro-

1 surfactant protein C (pro-SPC)-positive AT2 cells (35) and CD68-positive alveolar
2 macrophages. In the donor lung, NPY-positive cells were mainly detected around EpCAM-
3 positive epithelial cells (Figure 6E). NPY was also found in pro-SPC-positive cells and CD68-
4 positive cells in the donor lung (Figures 6F and E9). On the other hand, few NPY-positive cells
5 were detected in IPF lungs although some EpCAM-positive epithelial cells, pro-SPC-positive
6 cells, and CD68-positive cells expressed NPY (Figures 6 and E9). These data imply that NPY
7 is expressed mainly in the bronchial epithelial cells in the normal lungs, and may influence
8 AT2. We next assessed IL-1 β -positive cells in human lungs. IL-1 β -positive cells were detected
9 around the alveolar epithelial cells and CD68-positive macrophages in the alveolar space of the
10 donor lung. IL-1 β -positive cells were also seen around the alveolar epithelial metaplasia and
11 CD68-positive macrophages in the alveolar space of the IPF lung sections (Figures E10A and
12 E10B). These histological data, together with in the vivo and in vitro findings, imply that NPY
13 is expressed mainly in bronchial epithelial cells, and affects IL-1 β secretion from alveolar
14 epithelial cells, at least in part, and influences the development of pulmonary fibrosis.

1 **Discussion**

2 NPY is a neurotransmitter involved in the regulation of immune response (36). It has been
3 reported that NPY suppresses the secretion of pro-inflammatory cytokines, such as IL-1 β , TGF-
4 β , and IL-6 (24, 25), which are thought to be involved in the pathogenesis of pulmonary fibrosis
5 (37). These studies have suggested an association between NPY and pulmonary fibrosis, but
6 the role of NPY remains unclear.

7 In the present study, we demonstrated that NPY is protective against pulmonary
8 fibrosis. Using the BLM-induced pulmonary fibrosis model, NPY^{-/-} mice exhibited
9 significantly enhanced pulmonary fibrosis and higher IL-1 β levels in the lungs, compared to
10 WT mice. Mice with established fibrosis had lower NPY levels in BAL fluids and serums
11 compared to healthy mice. In vitro studies have suggested that NPY reduces fibrosis through
12 the suppression of IL-1 β . We demonstrated the efficacy of NPY therapy as a novel therapeutic
13 option for pulmonary fibrosis. Serum NPY levels in IPF patients were significantly lower
14 compared to healthy controls. These data confirmed our hypothesis in a murine pulmonary
15 fibrosis model and in vitro.

16 IL-1 β , a pro-inflammatory cytokine of the IL-1 family, is involved in the pathogenesis
17 of acute and chronic inflammatory diseases (38), with several reports suggesting that IL-1 β
18 induces lung fibrosis (6, 39, 40). In this study, NPY^{-/-} mice exhibited significantly higher IL-

1 1β levels in the lungs compared to WT mice following BLM instillation. NPY supplementation
2 suppressed the development of pulmonary fibrosis and decreased IL- 1β levels in the lungs.
3 Moreover, IL- 1β neutralization in NPY^{-/-} mice attenuated fibrotic changes. NPY also
4 suppressed BLM-induced IL- 1β secretion from alveolar epithelial cells, while Y1 receptor
5 antagonists antagonized these effects. This in vitro study confirms IL- 1β inhibition as the
6 mechanism underlying the protective effects of NPY against pulmonary fibrosis.

7 The primary sources of IL- 1β in IPF lungs have not yet been identified. Macrophages
8 (41, 42) and epithelial cells (16, 43) are reported to release IL- 1β . In the present study,
9 numerous IL- 1β -positive cells were detected around airway epithelium and CD68-positive
10 macrophages in the alveolar space in IPF lung sections. These findings imply that the
11 expression of NPY in bronchial epithelial cells and IL- 1β secretion from alveolar epithelium
12 and alveolar macrophages were related to the development of fibrosis in IPF. Our study also
13 demonstrated that alveolar-epithelium-derived IL- 1β exacerbates EMT, which causes fibrotic
14 changes, and that NPY suppresses IL- 1β secretion through Y1 receptors.

15 NPY is secreted by several cells, including nerve, immune, and structural cells (36,
16 44). Previous studies have shown that NPY is produced by nerves in the airway epithelium (45)
17 and has ectopic expression (46). We detected NPY-positive cells mainly in the bronchial
18 epithelial cells, confirmed by IF staining in mouse and human lung tissue. Therefore, NPY may

1 be secreted by both airway epithelium cells and nerves in the airway epithelium. NPY is
2 reported to regulate immune-cell function in a paracrine or autocrine manner (47). NPY
3 secreted by bronchial epithelial cells may suppress IL-1 β release from these AT2 cells (Figure
4 7).

5 In the present study, experiments using A549 cells, which share several characteristics
6 with AT2 cells, revealed that NPY inhibited IL-1 β production and release. The Y1 receptor
7 expression was higher compared to other receptors in a normal state, indicating that Y1
8 receptors are related to IPF pathogenesis. NPY is also reported to act on Y1 receptors to inhibit
9 IL-6 release in splenic tissues (48). This suggests that Y1 receptor controls the secretion of
10 NPY from AT2 and pro-inflammatory cytokines, including IL-1 β , to suppress pulmonary
11 fibrosis. We also detected Y4 receptor expression in A549 in a normal state. However, little is
12 known about the role of Y4 receptors in lung diseases, such as pulmonary fibrosis.

13 We also measured serum and plasma NPY levels in IPF patients. Serum NPY levels
14 of IPF patients were significantly lower, while serum IL-1 β levels were higher, compared to
15 healthy controls. These findings were similar to the murine IPF model. Serum and plasma
16 NPY levels were found to be reliable biomarkers for IPF. However, further investigation is
17 required to corroborate these findings.

18 In agreement with previous reports, we demonstrated that the NPY-Y1 axis contributes

to the development of type-2 immune responses, allergen-induced airway hyperresponsiveness, and inflammation (23, 47, 49). NPY may enhance eosinophilic inflammation by inducing type-2 cells. The results of the present study demonstrated that inhibition of IL-1 β signaling reduced fibrosis in BLM-instilled NPY^{-/-} mice. IL-1 β is essential for differentiation and proliferation of Th17, which is required for IL-17A production (31, 50). IL-17A reportedly also promotes lung fibrosis by stimulating fibroblast proliferation and activation, and extracellular matrix production (4). We found higher Th17-cell numbers in NPY^{-/-} mice compared to WT mice following BLM instillation. NPY-induced IL-1 β inhibition may suppress Th17 proliferation, resulting in lower Th17-cell numbers in NPY^{-/-} mice compared to WT mice. The effects of NPY on Th-cell differentiation may vary with the disease phenotype. NPY supplementation in asthmatic IPF patients may exacerbate the allergic asthma. Further assessment of the role of NPY, including in asthma-associated IPF, is required.

Our study had some limitations. First, serum and plasma NPY levels were assessed retrospectively in a small number of IPF patients and healthy volunteers without matching. Second, the relationship between serum and plasma NPY levels and fibrotic changes in IPF patients was not assessed. However, IPF patients did exhibit significantly lower serum and plasma NPY levels compared to healthy controls, suggesting the potential of serum and plasma NPY levels as a novel biomarker for IPF. Third, only female mice were used in all experiments,

1 which may represent a minor limitation of the study.

2 In conclusion, the current study demonstrated that NPY plays a critical role in BLM-
3 induced pulmonary fibrosis by suppressing IL-1 β release. This suggests that NPY could be a
4 novel therapeutic agent for pulmonary fibrosis.

5

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1 **Figure Legends**

2 **Figure 1. Worsening fibrosis in NPY^{-/-} mice following intratracheal bleomycin (BLM)** 3 **instillation.**

4 (A) Lung photomicrographs of wild type (WT) and NPY^{-/-} mice on day 21. Lung tissues were
5 stained with hematoxylin and eosin (left) and Masson's trichrome (right) stains. Scale bars: 200
6 μm. (B) Quantitative image analysis of Masson's-trichrome-stained fibrotic areas. WT PBS;
7 WT mice instilled with PBS (n = 4), NPY^{-/-} PBS; NPY^{-/-} mice instilled with PBS (n = 4), WT
8 BLM; WT mice instilled with BLM (n = 10), and NPY^{-/-} BLM; NPY^{-/-} mice instilled with
9 BLM (n = 10). (C) Collagen levels in BAL fluids of BLM- and PBS-instilled mice on day 21.
10 WT PBS (n = 4), NPY^{-/-} PBS (n = 4), WT BLM (n = 6), NPY^{-/-} BLM (n = 6). (D) Cellular
11 composition of BAL fluids on day 21. WT PBS (n = 4), NPY^{-/-} PBS (n = 4), WT BLM (n = 7),
12 NPY^{-/-} BLM (n = 7). (E, F) IL-1β levels in the lungs, WT PBS (n = 6), NPY^{-/-} PBS (n = 6),
13 WT BLM (n = 6), NPY^{-/-} BLM (n = 6) and TGF-β1 levels in BAL fluids, WT PBS (n = 6),
14 NPY^{-/-} PBS (n = 6), WT BLM (n = 8), NPY^{-/-} BLM (n = 8), on day 21 following intratracheal
15 BLM or PBS instillation, measured using enzyme-linked immunosorbent assay (ELISA). Data
16 are represented as mean ± SEM. Eos, eosinophils; Lym, lymphocytes; Mac, macrophages; Neut,
17 neutrophils; TGF, transforming growth factor. *p < 0.05, **p < 0.01, ***p < 0.001, ****p <
18 0.0001 (two-way ANOVA with Tukey's multiple comparisons).

Figure 2. NPY and pro-inflammatory-cytokine levels in BLM-instilled WT and NPY^{-/-} mice.

(A) NPY levels in BAL fluids of WT mice on days 0, 2, 7, 14, and 21 following BLM or PBS instillation, WT PBS (n = 4), WT BLM (n = 6). (B, C) Serum NPY levels in WT mice on day 2 (B) and day 21 (C) after BLM or PBS instillation, WT PBS (n = 8), WT BLM (n = 8). (D) The immunofluorescence staining of NPY in mouse lung tissues (a–c: 10×; d–f: 40×). Magnified view (bottom) of the area enclosed by the square (top). NPY-positive cells (green) in PBS- (b, e) and BLM- (c, f) instilled WT mice. NPY-positive cells were not detected in NPY^{-/-} mice (a, d). Representative images from three mice. Scale bars: 200 μm (a–c); 50 μm (d–f). Thin and bold arrows indicate alveolar epithelium and infiltrated inflammatory cells, respectively. Arrowheads depict bronchial endothelial cells. (E–G) Pro-inflammatory cytokine and chemokine levels in the BAL fluids or lung homogenates of WT and NPY^{-/-} mice on day 2. (E) IL-1β levels in lungs, WT PBS (n = 8), NPY^{-/-} PBS (n = 8), WT BLM (n = 8), NPY^{-/-} BLM (n = 9), (F) Monocyte chemoattractant protein (MCP)-1 levels in the BAL fluids, WT PBS (n = 4), NPY^{-/-} PBS (n = 4), WT BLM (n = 11), NPY^{-/-} BLM (n = 11), and (G) IL-17A levels in the lungs, WT PBS (n = 6), NPY^{-/-} PBS (n = 6), WT BLM (n = 16), NPY^{-/-} BLM (n = 16). (H) The number of T helper (Th) 17 cells in the lungs of WT and NPY^{-/-} mice on day

after PBS or BLM instillation, assessed using flow cytometry, WT PBS (n = 6), NPY^{-/-} PBS (n = 3), WT BLM (n = 6), NPY^{-/-} BLM (n = 5). Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (two-way ANOVA with Tukey's multiple comparisons).

Figure 3. NPY treatment suppresses pulmonary fibrosis in BLM-instilled WT mice.

(A) Experimental NPY administration in BLM-instilled WT mice. (B) Lung photomicrographs of BLM- and PBS-instilled WT mice treated with PBS or NPY on day 21. Lung tissues were stained with hematoxylin and eosin (left), and Masson's trichrome (right) stains. Representative images from three mice. Scale bars: 200 µm. (C) Quantitative image analysis of Masson's-trichrome-stained fibrotic areas, PBS/PBS; PBS-instilled WT mice treated with PBS (n = 4), BLM/PBS; BLM -instilled WT mice treated with PBS (n = 6), WT mice treated with NPY (n = 6). (D) Collagen levels in the BAL fluids of BLM- and PBS-instilled WT mice treated with PBS or NPY on day 21, PBS/PBS (n = 4), BLM/PBS (n = 6), BLM/NPY (n = 6). (E) Cellular composition of BAL fluids of BLM- and PBS-instilled WT mice treated with PBS or NPY on day 21, PBS/PBS (n = 4), BLM/PBS (n = 6), BLM/NPY (n = 6). (F, G) IL-1β levels in the lungs (F) and TGF-β1 levels in BAL fluids (G) in BLM- and PBS-instilled WT mice treated with PBS or NPY on day 21, PBS/PBS (n = 4), BLM/PBS (n = 6), BLM/NPY (n = 6).

(H) NPY levels in the BAL fluids of BLM- and PBS-instilled WT mice treated with PBS or NPY on day 21, PBS/PBS (n = 4), BLM/PBS (n = 6), BLM/NPY (n = 6). (I) Serum NPY levels in BLM- and PBS-instilled WT mice treated with PBS or NPY on day 21, PBS/PBS (n = 4), BLM/PBS (n = 6), BLM/NPY (n = 6). Data are represented as mean $\pm$ SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Figure 4. Neutralization efficacy of anti-IL-1 β monoclonal antibody (mAb) in BLM-instilled NPY^{-/-} mice.

(A) Experimental anti-IL-1 β mAb administration in BLM-instilled NPY^{-/-} mice. (B) Lung photomicrographs of BLM- and PBS-instilled NPY^{-/-} mice treated with anti-IL-1 β mAb or control IgG. Lung tissues were stained with hematoxylin and eosin (left) and Masson's trichrome (right) stains. Representative images from three mice. Scale bars: 200 μ m. (C) Quantitative image analysis of Masson's-trichrome-stained fibrotic areas, PBS/IgG; PBS-instilled NPY^{-/-} mice treated with control IgG (n = 5), BLM/IgG; BLM-instilled NPY^{-/-} mice treated with control IgG (n = 9), BLM/anti-IL-1 β mAb; BLM-instilled NPY^{-/-} mice treated with anti-IL-1 β mAb (n = 9). (D) Collagen levels in BAL fluids of BLM- and PBS-instilled NPY^{-/-} mice treated with anti-IL-1 β mAb or control IgG, PBS/IgG (n = 5), BLM/IgG (n = 9), BLM/anti-IL-1 β mAb (n = 9). (E) Cellular composition of BAL fluids of BLM- and PBS-

instilled NPY^{-/-} mice treated with anti-IL-1 β mAb or control IgG, PBS/IgG (n = 5), BLM/IgG (n = 9), BLM/anti-IL-1 β mAb (n = 9). (F, G) IL-1 β levels in the lungs (F) and TGF- β 1 levels in the BAL fluids (G) in BLM- and PBS-instilled WT mice treated with anti-IL-1 β mAb or control IgG, PBS/IgG (n = 5), BLM/IgG (n = 9), BLM/anti-IL-1 β mAb (n = 9). Data are represented as mean $\pm$ SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Figure 5. NPY-Y1 receptor axis inhibits IL-1 β release and production in alveolar type-2 epithelial cells.

(A) IL-1 β levels in BLM-exposed A549 cells treated with various doses of NPY (0.01, 0.1, and 1 μ M). (B) qPCR analysis of A549 cells in steady state (n = 3 in each group). Data were normalized to GAPDH expression. Gene Expression Array Cards are shown in Table E1. (C) IL-1 β levels in the culture supernatant of BLM-exposed A549 cells with concurrent NPY (0.1 μ M) and Y1R antagonist (1 μ M) treatment, determined using ELISA (n = 3 in each group). (D and E) qPCR analysis of BLM-exposed A549 cells with concurrent NPY (0.1 μ M) and Y1R antagonist (1 μ M) treatment (n = 3 in each group). Data from three independent experiments were normalized to GAPDH expression. The primer sequences are shown in Table E1. Data are represented as mean $\pm$ SEM. α -SMA, α -smooth muscle actin; Y1R, neuropeptide Y Y1 receptor; Y2R, neuropeptide Y Y2 receptor; Y4R, neuropeptide Y Y4 receptor; Y5R,

neuropeptide Y Y5 receptor. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 6. Serum NPY levels and NPY expression in the lungs of IPF patients.

(A) Serum NPY levels in healthy controls and IPF patients, assessed using ELISA (healthy controls: $n = 8$, and IPF patients: $n = 9$). (B) Serum IL-1 β levels in healthy controls and IPF patients, assessed using ELISA (healthy controls: $n = 8$, and IPF patients: $n = 9$). Data are represented as mean $\pm$ SEM. *** $p < 0.001$, **** $p < 0.0001$.

(C, D) Lung tissue sections from a lung-transplant donor and IPF patients were stained with anti-NPY antibody and DAPI. Clinical characteristics of the donor and IPF patients are presented in Table 2. (C, a–d; D, a–d: $\times 10$; C, e–h; D, e–h: $\times 40$). Magnified view (bottom) of the area enclosed by the square (top) is shown. As a negative control, IF images without primary antibodies for NPY are shown (C: d, h; D: d, h). Representative images from donors ($n = 1$), IPF patients ($n = 3$), and negative controls ($n = 1$) are shown. Scale bars: 200 μm (C and D, a–d), 50 μm (C and D, e–h). Arrowheads indicate bronchial endothelial cells. (E) Donor lung sections co-immunostained with NPY and epithelial cell adhesion molecule (EpCAM) antibodies ($40\times$). Scale bars: 50 μm . Arrowheads indicate NPY-positive cells around bronchial endothelial cells. (F) Donor lung sections co-immunostained with NPY and pro-surfactant protein C (pro-SPC) ($40\times$). NPY-positive cells overlap with pro-SPC-positive cells around

alveolar walls (arrowheads). Representative IF staining images of donors and IPF patients (n = 3) are shown. Scale bars: 50 μ m. EpCAM, Epithelial cell adhesion molecule; IPF, Idiopathic pulmonary fibrosis; pro-SPC, pro-surfactant protein C.

Figure 7. Graphical representation of the protective effects of NPY against pulmonary fibrosis.

NPY exerts a protective role by inhibiting IL-1 β , which induces epithelial–mesenchymal transition (EMT) and pulmonary fibrosis via the NPY-Y1 receptor (Y1R) axis. Irritants, such as bleomycin, infiltrate the airway structural cells, including bronchial epithelial cells, followed by IL-1 β production and release from type-2 alveolar epithelial cells (AT2), which induces EMT and lung fibrosis. Our findings suggest that NPY is secreted from bronchial epithelial cells in response to BLM stimulation, and acts on AT2 via Y1R. NPY-Y1R axis suppresses IL-1 β secretion from AT2, leading to the amelioration of pulmonary fibrosis. AT2, alveolar type 2 epithelial cells, Y1R, neuropeptide Y Y1 receptor.

1 **Tables**2 **Table 1. Clinical characteristics of IPF patients and controls.**

		Healthy controls	IPF	p-Value
Serum				
<i>n</i>		8	9	
Sex	Male/female	6/2	9/0	0.206
Age, years	Median, (range)	48, (29–62)	71, (47–84)	0.224

3 IPF: Idiopathic pulmonary fibrosis.

4

1 **Table 2. Clinical characteristics of lung transplant patients and donor.**

Lung tissue		IPF	Donor
<i>n</i>		3	1
Sex	Male/female	3/0	1/0
Age, years	Median, (range)	60, (55–61)	60

2

1 **Online Supplement**

2 **Neuropeptide Y antagonizes development of pulmonary fibrosis through**
3 **IL-1 β inhibition**

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5
6 Junko Itano, Akihiko Taniguchi, Satoru Senoo, Noboru Asada, Yuka Gion, Yuria Egusa,

7 Lili Guo, Naohiro Oda, Kota Araki, Yasuharu Sato, Shinichi Toyooka,

8 Katsuyuki Kiura, Yoshinobu Maeda, Nobuaki Miyahara
9

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 MultiFlex 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 $\mu\text{g/kg}$ or 200 $\mu\text{g/kg}$ 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 $\text{NPY}^{-/-}$ 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-1 β monoclonal antibody (mAb) administration in NPY^{-/-} mice

NPY^{-/-} mice received either 20 μ g of anti-mouse IL-1 β 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-1 β 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-1 β 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-1 β 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).

1

2 **Blood collection**

3 On days 2 and 21, murine blood samples were collected by cardiac puncture antemortem,
4 immediately after the induction of ketamine and xylazine anesthesia. We inserted a needle 5
5 mm from the center of the thorax using a 1-mL syringe and a 26-gauge needle, then obtained
6 the blood samples in non-anticoagulant tubes. The samples were centrifuged at 3000 rpm for
7 20 min at 4 °C, and stored at –30 °C. The samples for analysis of NPY were stored at –80 °C.

8

9 **Collagen assay**

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

16

17 **Hydroxyproline assay**

18 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 $13,000 \times g$ 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 μ M (0.3 μ g/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 μ m 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)- β 1, mice IL-1 β , human IL-1 β , and IL-17A, respectively. Except for human IL-1 β 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

1 and all fluid in the dishes were drawn into 5-mL syringes through a 20-gauge needle, then
2 discharged into the digestion dishes to facilitate tissue disruption. The cell suspensions were
3 carefully placed onto Histopaque-1077 (Sigma-Aldrich) and centrifuged. After centrifugation,
4 the upper layer was aspirated and suspended cells were collected in RPMI-1640 medium
5 (Sigma-Aldrich). Intranuclear staining with Foxp3/Transcription Factor Staining Buffer
6 (eBioscience Inc., San Diego, CA, USA) was then performed. The cells were stained with
7 Pacific-blue-conjugated anti-CD3 ϵ (145-2C11, BioLegend), fluorescein-isothiocyanate-
8 conjugated anti-CD4 (GK1.5, BD bioscience), and phycoerythrin-conjugated anti-ROR γ t
9 antibody (Q31-378; BD Biosciences), and APC-conjugated anti-Ki67 antibody (16A8;
10 BioLegend). The gating strategy for T helper 17 (Th17) cells and Ki67-positive Th17 cells is
11 shown in Figure E3A. Cells were then analyzed on a MACS Quant flow cytometer (Miltenyi
12 Biotec, Bergisch Gladbach, Germany) using the FlowJo software (FlowJo, LLC, Ashland, OR,
13 USA).

14 15 **Cell line and reagents**

16 The RIKEN BioResource Research Center provided A549 cells through the National Bio-
17 Resource Project of MEXT, Japan. The cells were grown in the DMEM D-6429 medium
18 (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 μ M 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 μ M), NPY (0.01, 0.1, and 1 μ M), and Y1receptor antagonist BIBP3226 (1 μ M). 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-1 β 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- μ m-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-1 β 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

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

1

2 **Statistical analyses**

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

9

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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 μ m. 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⁺, ROR γ t⁺, 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 $\pm$ 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 μ m. (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 $\pm$ 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 μ m. (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-1 β in the lungs and TGF- β 1 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 $\pm$ 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 $\pm$ 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 ($\times 40$). Scale bars: 50 μ m. (B) IPF lung sections co-immunostained with NPY and pro-surfactant protein C (pro-SPC) ($\times 40$). Scale bars: 50 μ m. NPY-positive cells overlap with pro-SPC-positive cells around

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

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

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

9 Lung tissue sections from the donor and IPF patients were subjected to immunohistochemical
10 staining with anti-IL-1 β antibody. Clinical characteristics of the donor and IPF patients are
11 presented in Table 2. (A) Donor and IPF lung tissue sections co-immunostained with anti-IL-
12 1 β and EpCAM antibodies. Higher magnification (bottom) of the area enclosed by the square
13 (top). IL-1 β -positive cells (magenta) overlapped with EpCAM (brown)-positive cells around
14 the alveolar epithelium and airway epithelial metaplasia (arrowheads). Scale bars: 200 μm
15 (top); 50 μm (bottom). (B) Donor and IPF lung tissue sections co-immunostained with anti-IL-
16 1 β and anti-CD68 antibodies. Scale bars: 200 μm (top); 50 μm (bottom). Higher magnification
17 (bottom) of the area enclosed by the square (top). CD68-positive macrophages (brown) were
18 observed in the alveolar space in donor and IPF patients. IL-1 β -positive cells (magenta)

- 1 overlapped with CD68-positive macrophages (brown) in the alveolar space (arrowheads).
- 2 Representative images from the donor (n = 1) and IPF patients (n = 3). Scale bars: 200 μ m
- 3 (top); 50 μ m (bottom).
- 4 EpCAM, epithelial cell adhesion molecule.
- 5

1 Supplemental Tables

2 Supplemental Table E1: Primer sequences and gene-expression array cards for real-time 3 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

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

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

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

2 IPF: Idiopathic pulmonary fibrosis.

3

1 **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.