The loss of IL-31 signaling attenuates bleomycin-induced pulmonary fibrosis

Dan JK Yombo^{1,2}, Varshini Odayar^{1,2}, Nishant Gupta³, Anil G. Jegga^{2,4}, and Satish K

Madala^{1,2}

¹Division of Pulmonary Medicine, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio USA.

²Department of Pediatrics, University of Cincinnati, College of Medicine, Cincinnati, Ohio USA.

³Division of Pulmonary, Critical Care and Sleep Medicine, University of Cincinnati, Cincinnati, Ohio USA.

⁴Division of Biomedical Informatics, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio USA

*** Correspondence:** Dr. Satish K. Madala, Cincinnati Children's Hospital Medical Center, Division of Pulmonary Medicine, 3333 Burnet Avenue, MLC 2021, Cincinnati, OH 45229. E-mail: satish.madala@cchmc.org

Keywords: Interleukin 31, Interleukin 31 receptor alpha, lung, idiopathic pulmonary fibrosis, bleomycin

IL-31 regulation of pulmonary fibrosis

Abstract

Idiopathic Pulmonary Fibrosis (IPF) is a severe fibrotic lung disease characterized by excessive collagen deposition and progressive decline in lung function. Th2 T cell-derived cytokines including IL-4 and IL-13 have been shown to contribute to inflammation and fibrotic remodeling in multiple tissues. Interleukin-31 (IL-31) is a newly identified cytokine that is predominantly produced by CD4 Th2 T cells, but its signaling receptor IL-31RA is primarily expressed by non-hematopoietic cells. However, the potential role of the IL-31-IL31RA axis in pulmonary inflammation and fibrosis has remained largely unknown. To determine the role of IL-31 signaling in pulmonary fibrosis, wildtype, and IL-31RA knockout mice were treated with bleomycin and measured changes in collagen deposition and lung function. Notably, the loss of IL-31 signaling attenuated collagen deposition and lung function decline during bleomycin-induced pulmonary fibrosis. However, the loss of IL-31RA signaling did not affect inflammation in the lungs. The total lung transcriptome analysis showed a significant reduction in fibrosis-associated gene transcripts including extracellular matrix and epithelial cell-associated gene networks. Furthermore, the lungs of human IPF showed an elevated expression of IL-31 when compared to healthy subjects. In support, the percentage of IL-31 producing CD4⁺ T cells was greater in the lungs and PBMCs from IPF patients compared to healthy controls. Our findings suggest a pathogenic role for IL-31/IL-31RA signaling during bleomycin-induced pulmonary fibrosis. Thus, therapeutic targeting the IL-31-IL-31RA axis may prevent collagen deposition, improve lung function, and have therapeutic potential in pulmonary fibrosis.

Introduction

Pulmonary fibrosis is a chronic heterogeneous lung disease characterized by an uncontrolled injury and repair process in the lung parenchyma. This process includes excessive deposition of collagen and extracellular matrix (ECM) protein components in the distal areas of the lung [1; 2]. Patients with severe fibrotic lung diseases including idiopathic pulmonary fibrosis (IPF) develop irreversible decline of lung function characterized by a decrease in forced expiratory volume in one second (FEV1) and forced vital capacity (FVC) in part due to the progressive accumulation of ECM producing myofibroblasts. Currently, the United States Food and Drug Administration (FDA) approved therapies to treat IPF, Nintedanib or Pirfenidone, slow the rate of FVC decline but do not reverse ongoing fibrosis [3; 4]. While the etiology of IPF is not well established, multiple pathways underlying the pathogenesis of pulmonary fibrosis have been identified [2; 5]. The development of fibrotic lesions, specifically, is regulated by signaling pathways that are driven by multiple pro-fibrotic growth factors, including TGF- β , TGF- α , CTGF, and cytokines such as IL-1 β , IL-17, IL-4, and IL-13 [6; 7; 8]. Th2 cytokines IL-4 and IL-13 have been found to play a major role in the pathophysiology of fibrotic diseases and are considered potential therapeutic targets [8]. IL-13 signaling can induce activation of fibroblasts and macrophages to produce ECM and constitutive factors such as proline, which are required for the biogenesis of collagen. IL-4 and IL-13 signal through a type II IL-4 receptor alpha (IL-4R α); this receptor is expressed in multiple cell types, including macrophages, epithelial cells, and fibroblasts [9].

Interleukin-31 (IL-31) is a recently described cytokine in the gp130/IL-6 cytokine family and is mainly produced by type 2 helper T cells. It plays a major role in the pathogenesis of chronic inflammatory diseases including atopic dermatitis (AD) or eczema [10; 11; 12]. IL-31 signaling is mediated through the heterodimeric receptor composed of the IL-31 receptor A

IL-31 regulation of pulmonary fibrosis

(IL-31RA) and the Oncostatin M receptor β (OSMR β). IL-31RA is expressed by various cells including immune cells, epithelial cells, and fibroblasts; these cells can secrete pro-inflammatory cytokines and chemokines following stimulation by IL-31[11; 13; 14; 15; 16]. The role of IL-31 in the development of inflammatory diseases including AD has been described and potential therapeutic agents have been generated to target IL-31/IL-31RA signaling [10; 11; 17; 18]. Recent studies have shown elevated levels of IL-31 in patients with AD or asthma and these levels have been correlated with disease severity [19; 20]. In fibrotic diseases, several IL-6 family of cytokines including IL6 and OSM have been shown to induce pulmonary fibrosis, but the contribution of IL-31/IL-31RA signaling in lung fibrosis has remained unexplored [20; 21]. Our previous work has depicted the effector mechanism of IL-4/IL-13 signaling in the pathogenesis of pulmonary fibrosis and the upregulation of IL-31RA via the IL-4R α /STAT6 signaling axis [8; 22]. However, the role of IL-31/IL-31RA signaling in the pathogenesis of pulmonary fibrosis has not been definitively determined.

The current study aimed to determine the role of IL-31/IL-31RA signaling in the pathogenesis of pulmonary fibrosis using a bleomycin-induced lung fibrosis model and IL-31RA deficient mice. We observed a significant decrease in collagen deposition and the expression of profibrotic genes in the lungs of IL-31RA deficient mice compared to wildtype mice treated with bleomycin. Notably, the loss of IL-31RA signaling was sufficient to attenuate worsening lung function. Further, we observed increased expression of IL-31 in the lung tissue and CD4-positive T cells of IPF patients compared to healthy subjects.

Material and methods

IL-31 regulation of pulmonary fibrosis

Mice. IL31RA knockout (IL-31RA^{-/-}) mice and their littermates wildtype (WT) mice of C57BL/6 background were used in this study to investigate the role of IL-31RA signaling in the development of pulmonary fibrosis [11]. Both male and female mice at 10–18 weeks of age were used for all of the experiments and were housed in the Cincinnati Children's Hospital Medical Center animal facility under conditions approved by the American Association for the Accreditation of Laboratory Animal Care. All mice were maintained under aseptic conditions and received sterile food and water. The experiments were approved by the Institutional Animal Care and Use Committee.

Bleomycin-induced pulmonary fibrosis model. IL-31RA^{-/-} and littermate wildtype mice were intradermally injected with bleomycin to induce lung fibrosis as previously described [8; 23]. Briefly, mice were injected with saline or bleomycin (6 U/kg of body weight) in 50 μ l saline, once a day and during 5 consecutive days per week, for a total of 4 weeks, to induce pulmonary fibrosis. Mice were euthanized on day 28 and lung samples were collected to assess fibrosis using biochemical and molecular methods.

Histology and lung hydroxyproline. The lungs were fixed with 10% neutral formalin and paraffin embedded. The five-micron thick lung sections were prepared and stained with Masson's Trichrome to evaluate the deposition of collagen in lung tissues, a key feature of the fibrotic process. The deposition of collagen was assessed by measuring hydroxyproline levels in lung lysates using a colorimetric detection assay as previously described [24]. In brief, the right lung lobes were collected, weighed, and hydrolyzed in 4 ml of 6 N HCl overnight at 110°C. Hydrolyzed samples were neutralized with 1 N NaOH. For colorization, chloramine-T (0.05 M) and the aldehyde-perchloric acid reagent were added and samples were placed in a hot water bath at 60°C for 25 min. The concentration of hydroxyproline in the samples was determined using the standard curve. The hydroxyproline level was normalized to the lung

IL-31 regulation of pulmonary fibrosis

weight and expressed in comparison to the level measured in the saline-treated wildtype control group [8].

Lung function measurements. Murine lung function was measured using a computerized FlexiVent system (Flexiware version 7.5, SCIREQ, Montreal Canada) as previously described [25; 26; 27]. Briefly, mice were anesthetized with Ketamine/Xylazine and an incision was performed on the anterior area of the neck to expose the trachea. The trachea was then cannulated with a 20G cannula and the mouse was connected to the FlexiVent system. Following deep inflation, three consecutive measures were collected for each mouse and the average value was used to measure resistance, elastance, and compliance.

Immunohistochemistry. Formalin-fixed and paraffin-embedded human lung tissue sections of IPF (n=8) and healthy (n=7) controls were immunostained with antibodies against IL-31 as previously described [28; 29]. Briefly, rabbit anti-human IL-31 monoclonal antibody (R&D Systems) was used as the primary antibody (1:25 dilution). Goat anti-rabbit Ig was conjugated with a peroxidase enzyme to form a brown precipitate in the presence of hydrogen peroxide and DAB. Nuclei were stained in blue using Hematoxylin counterstain as described in previous reports [23; 30]. The staining of the lung section using control IgG showed no detectable immunostaining (data not shown). All images were obtained using a Leica DM2700 M bright-field microscope. High-magnification images (X40) were captured with a 3CCD color video camera. The number of IL-31-positive cells and total cells was counted using MetaMorph imaging software and expressed as the percentage of IL-31-positive cells in total cells in the field.

RNA preparation and real-time PCR. Lungs were homogenized using Qiagen tissue homogenizer and beads (Qiagen), and total RNA was extracted from lung tissues using

IL-31 regulation of pulmonary fibrosis

RNeasy mini kit (Qiagen Science, Valencia, CA) as previously described [10; 22]. Lung RNA samples were reverse transcribed into cDNA using Superscript III (Invitrogen), and the relative transcript expression of select genes was measured using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and the CFX384 Touch Real-Time PCR detection system (Bio-Rad, Hercules, CA) as previously described [31]. Gene transcripts were normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) or β actin and were expressed as the relative fold-induction change compared to the gene expression level of the control wildtype mice treated with saline solution. Data was analyzed with Bio-Rad CFX maestro software version 4.2 (Bio-Rad Laboratories, Hercules, CA). The list of primers used for real-time PCR to analyze the relative expression of the genes is included in Supplementary Table 1.

Whole lung transcriptome shotgun sequencing (RNAseq) Total lung RNA samples were obtained from IL-31RA^{-/-} and wildtype mice treated with bleomycin as described above. Four samples were prepared from each experimental group and subjected to RNA sequencing using an Illumina HiSeq-1000 Sequencer (Illumina, San Diego, CA), as described previously [32]. A comparative analysis between groups was realized to identify genes with differential changes in expression following treatment with bleomycin or saline. Genes with statistically significant changes were selected based on a p-value cut-off of 0.05 and differential expression (defined as a 1.5-fold increase or decrease in expression). Heatmaps of genes with differential expression change were generated to highlight clusters of genes up-or downregulated in IL-31RA^{-/-} and wildtype mice treated with bleomycin. Functional enrichment analyses were performed using the ToppFun application of the ToppGene Suite [33]. The IL-31RA-dependent genes were also compared with differentially expressed genes (DEGs) from

IL-31 regulation of pulmonary fibrosis

158 IPF patients (GSE53845) to identify common genes between IL-31RA regulated genes and
159 IPF DEGs as described previously [34; 35].

160 **In vitro epithelial cell treatments.** Human bronchial epithelial BEAS-2B cells were obtained
161 from the American type culture collection (Manassas, VA) and cultured in DMEM medium
162 supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. To assess the effects
163 of IL-31, airway epithelial cells were seeded in 12 or 24 well plates in low serum media
164 (1%FBS) for 16 hrs. Under low serum conditions, cells were stimulated with media
165 supplemented with increasing doses (0, 100, 250, or 500 ng/ml) of recombinant IL-31(R&D
166 Systems) for 18 hours. Total RNA was extracted from the cultured cells using the Qiagen
167 RNA shredder and the RNeasy mini kit (Qiagen). cDNA was prepared and applied for real-
168 time RT-PCR to measure gene transcript levels as described above. Gene expression was
169 normalized to the housekeeping gene human β -actin.

170 **Flow Cytometry.** Peripheral blood mononuclear cells (PBMC) were isolated from peripheral
171 venous blood of IPF patients (n=11) and healthy control donors (n=10) using Ficoll-paque
172 density gradient-based separation with centrifugation (GE Healthcare, Uppsala, Sweden).
173 Red blood cells were lysed using an ACK lysis buffer and cell viability was confirmed with
174 trypan blue. Isolated PBMCs were frozen with DMSO and stored in liquid nitrogen until used.
175 To measure the production of IL-31 and IL-4, PBMCs were thawed from liquid nitrogen and
176 plated in a 96-well round-bottom plate (1×10^6 cells/well) in RPMI 1640 medium
177 supplemented with L-glutamine, antibiotics (penicillin/streptomycin), and 10% heat-
178 inactivated fetal bovine serum (FBS). Cells then were allowed to rest overnight. PBMCs were
179 stimulated with phorbol 12-myristate 13-acetate (PMA) 10 ng/ml with Ionomycin (1ug/ml), and
180 Golgi stop (1:1500) for 5 hours to induce cytokine production. Control samples were cultured
181 with PMA and ionomycin for the same period. Cells were then stained with Zombie

IL-31 regulation of pulmonary fibrosis

182 UV(BioLegend) for 30 min at room temperature in the dark to allow for discrimination between
183 living and dead cells. Non-specific binding of fluorochromes was prevented by incubating cells
184 with Human BD Fc Block™ (BD Biosciences), followed by surface marker staining with anti-
185 CD3-V500 (UCHT1) and anti-CD4-APC(RPA-T4) (Bio Legend). Cells were then fixed and
186 permeabilized using Cytofix/Cytoperm (BD Biosciences) and stained with antibodies against
187 IL-31- PE (U26-947) (BD Biosciences) and IL-4 -PE-Cy7 (MP4-25D2) (BioLegend). Data was
188 acquired using LSR Fortessa flow cytometer and analyzed using FlowJo 10 (FlowJo, Tree
189 Star).

190 **Statistical analysis.** All data were analyzed using GraphPad PRISM 8. One-way ANOVA
191 with Turkey's multiple comparison test was used to compare different experimental groups.
192 Student's *t* test was used to compare between two experimental groups. Quantitative data
193 were presented as mean $\pm$ SEM, and $p < 0.05$ was considered statistically significant.

194

195 Results

196 The loss of IL-31RA signaling attenuates collagen deposition and lung function decline

197 To determine whether the loss of IL-31RA has any effect on pulmonary fibrosis, IL-
198 31RA knockout mice and their littermate wildtype mice were treated intradermally with
199 bleomycin (to induce pulmonary fibrosis) or treated with saline as a non-fibrosis control. To
200 assess collagen deposition, the lung sections were stained with Masson Trichrome. There
201 were no significant differences in collagen staining between wildtype and IL-31RA knockout
202 mice treated with saline. A significant increase in collagen deposition was observed in the
203 lungs of both wildtype mice and IL-31RA knockout mice treated with bleomycin (Figure 1A).

IL-31 regulation of pulmonary fibrosis

However, there was a modest decrease in collagen staining in mice deficient for IL-31RA compared to wildtype mice treated with bleomycin (Figure 1A). To further evaluate collagen deposition, the level of hydroxyproline was quantified in the lungs of IL-31RA knockout mice and wildtype mice treated with bleomycin or saline. Both wildtype and IL-31RA knockout mice treated with bleomycin had increased expression of lung hydroxyproline compared with saline-treated mice. However, there was a modest but significant reduction in lung hydroxyproline levels in IL-31RA knockout mice compared to wildtype mice treated with bleomycin (Figure 1B). Our published studies have demonstrated that repetitive bleomycin-induced fibrotic remodeling decreases lung function [36; 37]. Therefore, we assessed whether the loss of IL-31RA signaling has any effect on the decline in lung function following repetitive bleomycin treatment. Consistent with previous findings, there was an increase in resistance, and elastance, and a significant decrease in compliance in wildtype mice treated with bleomycin compared to saline-treated wildtype or IL-31RA knockout mice (Figure 1C-1E). However, the decline in lung function was attenuated with the loss of IL-31RA signaling compared to wildtype mice treated with bleomycin (Figure 1C-1E). These findings suggest that the loss of IL-31RA has a partial protective effect against collagen deposition, and the subsequent decline in lung function, during bleomycin-induced pulmonary fibrosis.

The role of IL-31RA signaling in fibrosis-associated gene expression

To identify IL-31RA dependent gene expression and associated signaling pathways involved in bleomycin-induced pulmonary fibrosis, whole lung transcriptome analysis was performed using next-generation RNA-seq analysis in wildtype and IL-31RA knockout mice treated with bleomycin. The comparison of differentially expressed genes, with 1.5-fold up-or down-regulation in wildtype and IL31RA knockout lungs, highlighted two clusters of genes as illustrated in the heatmap (Figure 2). A total of 458 genes were differentially expressed in the

IL-31 regulation of pulmonary fibrosis

lungs of IL-31RA knockout mice compared to wildtype mice treated with bleomycin. About 228 genes were up-regulated in IL-31RA knockout mice, and 230 genes were down-regulated, with the loss of IL-31RA signaling (Supplementary Table 2). Gene ontology enrichment analysis of down-regulated genes suggested IL-31RA is involved in the expression of several ECM-associated genes (*Col1a1*, *Col3a*, *Fn1*, *Arg1*, *Timp1*, *Mmp13*, *Ereg*, and *IL-6*), Protein G coupled receptor Gα(i) signaling (*Ccl28*, *Rgs5*, *Rgs4*, and *Rgs16*), Epithelium-associated genes (*Krt4*, *Krt5*, *Krt14*, *Krt20*), Adenylate cyclase activity (*Calcb*, *Calcr*) and chemotaxis (*Ccl28*, *Mcp1*, *IL6*). Enrichment analysis of genes that were upregulated with the loss of IL-31RA suggested an increase in the expression of genes associated with the G protein-coupled receptor, and synaptic and calcium signaling.

Next, to identify the IL-31RA-dependent genes that are also dysregulated and relevant to IPF pathogenesis, differentially expressed genes in IPF lungs were obtained and compared to IL-31RA-dependent genes (Figure 2B). In total, 181 IPF differentially expressed genes were identified that were also dysregulated with the loss of IL-31RA (Figure 2B). This IL-31RA dependent gene list includes 31 genes that were up-regulated, and 46 down-regulated genes, in IPF lungs (Figure 2B and Supplementary Figure 1). We performed an enrichment analysis of these negatively correlated genes using the ToppFun application and visualized the gene networks using Cytoscape. As shown in Figure 2C, our gene functional enrichment analysis further supports alterations in fibroblast- and epithelial cell-associated genes and also a decrease in multiple collagen gene transcripts.

The loss of IL-31RA signaling attenuates the expression of pro-fibrotic genes

To validate the findings using RNA-seq, fibrosis-associated gene transcripts were quantified in the total lung RNA of IL-31RA knockout and wildtype mice treated with bleomycin

IL-31 regulation of pulmonary fibrosis

or saline. The major ECM gene transcripts including *Col1 α* , *Col3 α* , and *Fn1* were upregulated in lungs of wildtype mice treated with bleomycin compared to wildtype mice treated with saline. This increase in ECM gene expression was significantly attenuated in IL-31RA knockout mice compared to wildtype mice treated with bleomycin (Figure 3A). Similarly, genes associated with ECM production and remodeling were significantly decreased with the loss of IL-31RA; these genes included MMP13 and TIMP1, as well as IL-6 which increased in wildtype mice during bleomycin-induced pulmonary fibrosis (Figure 3B). The total lung RNA-seq analysis also suggests altered expression of several epithelial cell-associated genes with the loss of IL-31RA during bleomycin-induced pulmonary fibrosis (Figure 2). To validate the differential expression of genes associated with epithelial cells, we measured transcript levels of several epithelium-associated genes including *Krt5*, and *Krt14*. As shown in Figure 4A, we observed a significant increase in the transcripts of *Krt5*, and *Krt14* in wildtype mice treated with bleomycin compared to saline-treated wildtype mice. This increase in epithelial gene transcripts was attenuated with the loss of IL-31RA compared to wildtype mice treated with bleomycin (Figure 4A). These findings suggest a critical role for airway epithelium in the pathophysiology of IL-31RA-driven pulmonary fibrosis and decline in lung function. Therefore, we studied the effect of IL-31 on airway epithelial cells in the production of pro-fibrotic cytokines associated with pulmonary fibrosis. Bronchial epithelial cell line BEAS-2B, or primary bronchial epithelial cells, were stimulated with IL-31 cytokine and the transcript levels of *Mcp1* and *IL-6* were determined using RTPCR. The production of pro-fibrotic cytokines, including *Mcp1* and *IL-6*, increased with IL_31 stimulation in both BEAS-EB and HBEC cells (Fig. 4B & 4C). These findings suggest that IL-31-driven signaling increases the production of pro-fibrotic cytokines by epithelial cells in the pathogenesis of pulmonary fibrosis.

Increased frequency of IL-31 producing cells in IPF patients

To determine whether IL-31 is involved in human IPF, IL-31 staining was performed in the lung sections obtained from healthy controls and IPF patients. In contrast to normal lungs, we observed prominent immunostaining for IL-31 in cells populated in the thickened parenchymal areas of IPF lungs (Figure 5A). We quantified the number of IL-31-positive cells and total cells in lung images to assess the accumulation of IL-31-positive cells in IPF lungs compared to healthy lungs. There was a significant increase in the percent of IL-31-positive cells in the lungs from IPF patients compared with healthy controls (Figure 5B). Previous studies have described type 2 CD4 T cells as the main source of IL-31 production in chronic allergic diseases such as AD [11; 38]. To determine the frequency of IL-31 producing CD4 T cells in IPF, PBMCs were stimulated with PMA-ionomycin or medium for 6 h and fixed. Cells were fluorescently stained with IL-31 and IL-4 and analyzed by flow cytometry with a gating strategy as illustrated in Supplementary Figure 2. Notably, the majority of IL-31 producing cells in PBMCs were T cells. CD4 T cells producing IL-31, or IL-4 and IL-31, were significantly increased in PBMCs of IPF patients compared to healthy controls (Figure 5D & 5E).

Discussion

In this study, we have demonstrated that bleomycin-induced pulmonary fibrosis is dependent on IL-31RA-driven signaling. The loss of IL-31RA signaling was associated with a decrease in hydroxyproline and downregulation of several pro-fibrotic gene transcripts. In addition, our findings demonstrate that the loss of IL-31RA attenuates lung function decline as observed during bleomycin induced-pulmonary fibrosis. We also showed that IL-31-positive cells are increased in fibrotic lung lesions of IPF patients and IL-31 is predominantly

IL-31 regulation of pulmonary fibrosis

298 produced by CD4 T cells. These findings illustrate the potential utility of targeting the IL-31/IL-
299 31RA axis to treat IPF.

300 Previous studies have identified important roles for Th2 T cell-derived IL-4 and/or IL-
301 13 in the development of pulmonary fibrosis and other fibrotic diseases. However, the role of
302 IL-31/IL-31RA signaling in pulmonary fibrosis has remained unexplored. Our previous study
303 along with other research studies have shown that the administration of exogenous IL-31, or
304 overexpression of IL-31, in transgenic mice induces skin thickening and alters the skin barrier.
305 These findings suggest that IL-31/IL-31RA signaling plays an integral role in inflammation and
306 fibrosis [10; 11]. Previous studies have suggested a potential role for IL-31-driven signaling
307 in the development of fibrosis in various organs including the liver [39] and intestine [40].
308 Shish and colleagues reported increased expression of IL-31RA on intestinal myofibroblasts
309 following treatment with T11a, a TNF superfamily 15 protein, and established an association
310 between IL-31RA signaling and colonic fibrosis [40]. Here, we dissected the pro-fibrotic role
311 of the IL-31/IL-31RA axis using a mouse model of bleomycin-induced pulmonary fibrosis. Our
312 studies which were conducted using IL-31RA knockout mice revealed a significant decrease
313 in hydroxyproline and subsequent improvement in lung function. In addition, whole lung
314 transcriptome analysis revealed a decrease in the expression of ECM-related genes, which
315 thus supports the role of IL-31/IL-31RA in lung fibrosis. Our findings align with a recent
316 publication demonstrating that in scleroderma IL-31 contributes to skin fibrosis; in this study,
317 a sub-cutaneous mini-pump was implanted in the skin to deliver IL-31 [41]. Together, these
318 studies indicate that IL-31-driven signaling is a key factor driving pulmonary fibrosis. However,
319 additional studies are needed to identify specific mechanisms by which IL-31 contributes to
320 ECM production and decline in lung function during bleomycin-induced pulmonary fibrosis.

IL-31 regulation of pulmonary fibrosis

Previous studies have identified CD4 helper T cells as a source of IL-31 [11; 12] which is consistent with our findings. We demonstrated a significant increase in IL-31 and IL-4/IL-31-producing CD4⁺ T cells in PBMCs of IPF patients compared with control healthy subjects. The expression of IL-31 was significantly increased in lung tissues of IPF patients compared to control subjects. These findings may indicate that during pulmonary fibrosis development, the TH2 CD4⁺ T cell population contributes to the increased expression of IL-31 in fibrotic lung lesions; within these lung lesions, IL-31 interacts with the unique receptor IL-31RA that is expressed in stromal cells. Collectively, these observations make a compelling case that hematopoietic cell-derived IL-31 might play a role in the pathogenesis of pulmonary fibrosis by activating non-hematopoietic cells such as epithelial cells and mesenchymal cells. Indeed, stimulation of airway epithelial cells with IL-31 induced an increased production of MCP1 and IL-6 cytokines known to play a role in the development of pulmonary fibrosis [42].

We applied the whole lung transcriptome through RNA seq analysis to identify IL-31/IL-31RA -dependent genes and pathways that might participate in IL-31-driven pulmonary fibrosis. Expression of genes associated with the ECM formation was significantly downregulated in IL-31RA^{-/-} mice treated with bleomycin when compared to wildtype mice. Notably, genes associated with epithelial tissue integrity (*Krt5* and *Krt14*), protein Gα signaling, as well as previously reported genes associated with lung function and remodeling (*Arg1*, *Ccl28* and *Rgs4*) were down regulated in IL-31RA^{-/-} fibrotic mice [43; 44; 45]. Weathington and colleagues have recently reported an association between increased expression of the genes *KCNJ2* and *KRT18* in the setting of asthma, suggesting a strong relationship between lung function and tissue remodeling [46]. In support, genes related to membrane potential regulation, including genes for potassium channels *Kcna2*, *Kcnh4*, and *Kcnu1*, were upregulated in IL-31RA^{-/-} mice compared to wildtype mice. These findings

IL-31 regulation of pulmonary fibrosis

support a mechanistic pathway that mediates improved lung function in the absence of IL-31RA signaling. Additional investigation is needed to explore whether IL-31/IL-31RA signaling contributes to the regulation of potassium channel activity and whether altered keratin gene expression in epithelial cells could mediate collagen deposition and lung function.

In conclusion, our data indicates that genetic deletion of IL-31RA significantly attenuates collagen deposition and lung function decline induced during repetitive bleomycin-induced injury and pulmonary fibrosis. In recent years, several cytokines have been implicated in the pathogenesis of pulmonary fibrosis. While IL-31 has been identified in several inflammatory and remodeling diseases, mechanistic studies evaluating IL-31 producing cells in IPF, and the role of IL-31 in pulmonary fibrosis have been missing. Our results provide new evidence that supports the pathogenic role of IL-31RA-driven signaling in bleomycin-induced pulmonary fibrosis. The IL-31-IL31RA axis may serve as a novel therapeutic target for the treatment of pulmonary fibrosis.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

SKM conceived and designed the research. DJY, and SKM performed the experiments and wrote the manuscript. AGJ performed bioinformatic analysis and edited the manuscript. NG

366 provided human IPF samples and edited the manuscript. VO analyzed data and edited the
367 manuscript.

368

369 **Funding**

370 This work was supported by NIH [1R01 HL134801, 1R21 AI137309, and W81XWH-17-1-
371 0666) (SKM)].

372

373 **Acknowledgments**

374 The authors thank Dr. Brijendra Singh, Dr. Edukulla Ramakrishna, and the veterinary
375 services and pathology research core at Cincinnati Children's Hospital Medical Center for
376 help in this study.

377

378 **Data Availability Statement**

379 The datasets analyzed in this study can be found here:

380 <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53845>.

381 <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE163740>.

382

383 Supplementary Table 1. List of primers used for RT-PCR

384 Supplementary Table 2: List of IL-31RA-dependent genes

385

386 **Figures legend**

387 **Figure 1. The loss of IL-31RA signaling attenuates collagen deposition and lung**
 388 **function decline during bleomycin-induced pulmonary fibrosis.** wildtype (IL-31RA^{+/+})
 389 and IL-31RA knockout (IL31RA^{-/-}) mice were treated intradermally with bleomycin or saline
 390 for four weeks to induce pulmonary fibrosis. (A) Lung sections of saline or bleomycin-treated
 391 mice were stained with Masson Trichrome. Scale bar 300 μ m. (B) Total lung hydroxyproline
 392 was compared between IL-31RA^{-/-} and IL31RA^{+/+} mice treated with bleomycin or saline. (D-
 393 F) Lung function measurements including resistance, elastance and compliance were
 394 measured in all four groups using FlexiVent. Data are presented as means $\pm$ SEM. The above
 395 data is cumulative of two independent experiments (n=12-19/group). Statistical analysis was
 396 performed using one-way ANOVA with Tukey's multiple comparisons test. **p < 0.01;
 397 ****p<0.001.

398 **Figure 2. The IL-31-IL-31RA axis regulated gene networks during bleomycin-induced**
 399 **pulmonary fibrosis and IPF.** Total lung RNA was isolated from bleomycin or saline-treated
 400 mice and performed RNA-seq analysis. (A) The heat map shows two clusters of differentially
 401 expressed genes that were up-or down-regulated by at least 1.5-fold upon bleomycin
 402 treatment of genetic knockdown of IL-31RA compared to wildtype mice. Gene Ontology
 403 Enrichment analysis for IL-31RA-dependent gene transcripts was performed using ToppFun.
 404 (B) Venn diagram of IL31RA-regulated genes compared with IPF-differentially expressed
 405 genes in the lungs of IPF patients. (C) IL-31RA-driven gene networks that are activated in
 406 IPF were analyzed using ToppFun and visualized using Cytoscape. Red- and violet-colored
 407 oval shapes represent genes that are IL31RA-regulated and up-or down-regulated

IL-31 regulation of pulmonary fibrosis

respectively in IPF lungs. Both square and rectangle shapes represent different enriched biological processes and cell types for the inversely correlated genes between the IL-31-IL-31RA axis and IPF.

Figure 3. The loss of IL-31RA signaling attenuates the expression of fibrosis-associated genes during bleomycin-induced pulmonary fibrosis. Total lung RNA was isolated from wildtype and IL-31RA^{-/-} mice treated with saline and bleomycin and fibrosis-associated genes quantified using real-time RTPCR. (A) Quantification of ECM gene transcripts including Col1 α , Col3 α 1, and Fn1. (B) Quantification of gene transcripts involved in ECM remodeling and production including Mmp13, Timp1, and IL-6. Data presented as Mean $\pm$ SEM (n=4/group). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test * p<0.05; ** p<0.01; *** p< 0.005; **** p<0.001.

Figure 4: The loss of IL-31RA signaling alters the expression of epithelial cell-specific gene expression during bleomycin-induced pulmonary fibrosis. (A) Reduced expression of epithelial cell-specific genes including KRT5 and KRT14 in the lungs during bleomycin-induced pulmonary fibrosis. (B) Bronchial epithelial cell line BEAS-2B was stimulated with recombinant IL-31 for 24 hours and the transcripts of MCP1 and IL-6 measured using RT-PCR. (C) primary human bronchial epithelial (NHBE) cells were stimulated with recombinant IL-31 for 24 hours and the transcripts of MCP1 and IL-6 measured using RT-PCR. Data presented as Mean $\pm$ SEM (n=4 mice/group). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test * p<0.05; ** p<0.01; *** p< 0.005; **** p<0.001.

Figure 5: Increased staining and frequency of IL-31-positive CD4 T cells in IPF. Lung sections from IPF and non-IPF control subjects were probed to detect the expression of IL-

IL-31 regulation of pulmonary fibrosis

31 by immunohistochemistry, (A) Representative images of IL-31 expression in non-IPF (top) and IPF (bottom) lung sections; (B) Percent of IL-31⁺ cells quantified in IHC stained lung sections. Data are shown as mean $\pm$ SEM., ** $p < 0.01$. Peripheral blood mononuclear cells (PBMC) of IPF patients (n=7) and healthy control subjects (n=8) were isolated and stimulated with PMA and ionomycin, intracellular cytokine expression of IL-31, and IL-4 were assessed by flow cytometry. (C) Representative images of CD3 CD4 cells expressing IL-31 and IL-4, unstimulated (top) or stimulated cells with PMA(bottom) from Non-IPF and IPF patients are shown ; (D) Percent of IL-31 and (E) IL4/IL-31- producing CD4 T cells are shown. Data presented as mean $\pm$ SEM with n=6-9, with * $p < 0.05$, ** $p < 0.01$. Student *t*-test was used to compare the mean between groups.

References

- [1] G. Sgalla, B. Iovene, M. Calvello, M. Ori, F. Varone, and L. Richeldi, Idiopathic pulmonary fibrosis: pathogenesis and management. *Respir Res* 19 (2018) 32.
- [2] P.J. Wolters, H.R. Collard, and K.D. Jones, Pathogenesis of idiopathic pulmonary fibrosis. *Annu Rev Pathol* 9 (2014) 157-79.
- [3] L. Richeldi, R.M. du Bois, G. Raghu, A. Azuma, K.K. Brown, U. Costabel, V. Cottin, K.R. Flaherty, D.M. Hansell, Y. Inoue, D.S. Kim, M. Kolb, A.G. Nicholson, P.W. Noble, M. Selman, H. Taniguchi, M. Brun, F. Le Maulf, M. Girard, S. Stowasser, R. Schlenker-Herceg, B. Disse, H.R. Collard, and I.T. Investigators, Efficacy and safety of nintedanib in idiopathic pulmonary fibrosis. *The New England journal of medicine* 370 (2014) 2071-82.

IL-31 regulation of pulmonary fibrosis

- 453 [4] T.E. King, Jr., W.Z. Bradford, S. Castro-Bernardini, E.A. Fagan, I. Glaspole, M.K.
454 Glassberg, E. Gorina, P.M. Hopkins, D. Kardatzke, L. Lancaster, D.J. Lederer, S.D.
455 Nathan, C.A. Pereira, S.A. Sahn, R. Sussman, J.J. Swigris, P.W. Noble, and A.S.
456 Group, A phase 3 trial of pirfenidone in patients with idiopathic pulmonary fibrosis. *N*
457 *Engl J Med* 370 (2014) 2083-92.
- 458 [5] V. Sontake, P.R. Gajjala, R.K. Kasam, and S.K. Madala, New therapeutics based on
459 emerging concepts in pulmonary fibrosis. *Expert Opin Ther Targets* 23 (2019) 69-81.
- 460 [6] M.S. Wilson, S.K. Madala, T.R. Ramalingam, B.R. Gochuico, I.O. Rosas, A.W. Cheever,
461 and T.A. Wynn, Bleomycin and IL-1beta-mediated pulmonary fibrosis is IL-17A
462 dependent. *J Exp Med* 207 (2010) 535-52.
- 463 [7] K.K. Kim, D. Sheppard, and H.A. Chapman, TGF-beta1 Signaling and Tissue Fibrosis.
464 *Cold Spring Harb Perspect Biol* 10 (2018).
- 465 [8] B. Singh, R.K. Kasam, V. Sontake, T.A. Wynn, and S.K. Madala, Repetitive intradermal
466 bleomycin injections evoke T-helper cell 2 cytokine-driven pulmonary fibrosis. *Am J*
467 *Physiol Lung Cell Mol Physiol* 313 (2017) L796-L806.
- 468 [9] R.L. Gieseck, 3rd, M.S. Wilson, and T.A. Wynn, Type 2 immunity in tissue repair and
469 fibrosis. *Nat Rev Immunol* 18 (2018) 62-76.
- 470 [10] B. Singh, A.G. Jegga, K.S. Shanmukhappa, R. Edukulla, G.H. Khurana Hershey, M.
471 Medvedovic, S.R. Dillon, and S.K. Madala, IL-31-Driven Skin Remodeling Involves
472 Epidermal Cell Proliferation and Thickening That Lead to Impaired Skin-Barrier
473 Function. *PLoS One* 11 (2016) e0161877.
- 474 [11] S.R. Dillon, C. Sprecher, A. Hammond, J. Bilsborough, M. Rosenfeld-Franklin, S.R.
475 Presnell, H.S. Haugen, M. Maurer, B. Harder, J. Johnston, S. Bort, S. Mudri, J.L.

IL-31 regulation of pulmonary fibrosis

- 476 Kuijper, T. Bukowski, P. Shea, D.L. Dong, M. Dasovich, F.J. Grant, L. Lockwood,
477 S.D. Levin, C. LeCiel, K. Waggle, H. Day, S. Topouzis, J. Kramer, R. Kuestner, Z.
478 Chen, D. Foster, J. Parrish-Novak, and J.A. Gross, Interleukin 31, a cytokine
479 produced by activated T cells, induces dermatitis in mice. *Nature immunology* 5
480 (2004) 752-60.
- 481 [12] E. Maier, D. Werner, A. Duschl, B. Bohle, and J. Horejs-Hoeck, Human Th2 but not Th9
482 cells release IL-31 in a STAT6/NF-kappaB-dependent way. *J Immunol* 193 (2014)
483 645-54.
- 484 [13] U. Raap, M. Gehring, S. Kleiner, U. Rudrich, B. Eiz-Vesper, H. Haas, A. Kapp, and B.F.
485 Gibbs, Human basophils are a source of - and are differentially activated by - IL-31.
486 *Clin Exp Allergy* 47 (2017) 499-508.
- 487 [14] N. Kunsleben, U. Rudrich, M. Gehring, N. Novak, A. Kapp, and U. Raap, IL-31 Induces
488 Chemotaxis, Calcium Mobilization, Release of Reactive Oxygen Species, and CCL26
489 in Eosinophils, Which Are Capable to Release IL-31. *J Invest Dermatol* 135 (2015)
490 1908-1911.
- 491 [15] Y. Yagi, A. Andoh, A. Nishida, M. Shioya, T. Nishimura, T. Hashimoto, T. Tsujikawa, Y.
492 Saito, and Y. Fujiyama, Interleukin-31 stimulates production of inflammatory
493 mediators from human colonic subepithelial myofibroblasts. *Int J Mol Med* 19 (2007)
494 941-6.
- 495 [16] W.K. Ip, C.K. Wong, M.L. Li, P.W. Li, P.F. Cheung, and C.W. Lam, Interleukin-31
496 induces cytokine and chemokine production from human bronchial epithelial cells
497 through activation of mitogen-activated protein kinase signalling pathways:
498 implications for the allergic response. *Immunology* 122 (2007) 532-41.

IL-31 regulation of pulmonary fibrosis

- 499 [17] F. Cevikbas, X.D. Wang, T. Akiyama, C. Kempkes, T. Savinko, A. Antal, G. Kukova, T.
500 Buhl, A. Ikoma, J. Buddenkotte, V. Soumelis, M. Feld, H. Alenius, S.R. Dillon, E.
501 Carstens, B. Homey, A. Basbaum, and M. Steinhoff, A sensory neuron-expressed IL-
502 31 receptor mediates T helper cell-dependent itch: Involvement of TRPV1 and
503 TRPA1. *J Allergy Clin Immun* 133 (2014) 448-+.
- 504 [18] S. Oyama, H. Kitamura, T. Kuramochi, Y. Higuchi, H. Matsushita, T. Suzuki, M. Goto,
505 H. Adachi, K. Kasutani, A. Sakamoto, Y. Iwayanagi, A. Kaneko, M. Nanami, E. Fujii,
506 K. Esaki, Y. Takashima, S. Shimaoka, K. Hattori, and Y. Kawabe, Cynomolgus
507 monkey model of interleukin-31-induced scratching depicts blockade of human
508 interleukin-31 receptor A by a humanized monoclonal antibody. *Exp Dermatol* 27
509 (2018) 14-21.
- 510 [19] K. Psachoulia, K.A. Chamberlain, D. Heo, S.E. Davis, J.D. Paskus, S.E. Nanesco, J.L.
511 Dupree, T.A. Wynn, and J.K. Huang, IL4I1 augments CNS remyelination and axonal
512 protection by modulating T cell driven inflammation. *Brain* 139 (2016) 3121-3136.
- 513 [20] W. Lin, Q. Zhou, C. Liu, M. Ying, and S. Xu, Increased plasma IL-17, IL-31, and IL-33
514 levels in chronic spontaneous urticaria. *Sci Rep* 7 (2017) 17797.
- 515 [21] L. Stawski, and M. Trojanowska, Oncostatin M and its role in fibrosis. *Connect Tissue*
516 *Res* 60 (2019) 40-49.
- 517 [22] R. Edukulla, B. Singh, A.G. Jegga, V. Sontake, S.R. Dillon, and S.K. Madala, Th2
518 Cytokines Augment IL-31/IL-31RA Interactions via STAT6-dependent IL-31RA
519 Expression. *J Biol Chem* 290 (2015) 13510-20.

IL-31 regulation of pulmonary fibrosis

- 520 [23] V. Sontake, R.K. Kasam, D. Sinner, T.R. Korfhagen, G.B. Reddy, E.S. White, A.G.
521 Jegga, and S.K. Madala, Wilms' tumor 1 drives fibroproliferation and myofibroblast
522 transformation in severe fibrotic lung disease. *JCI Insight* 3 (2018).
- 523 [24] S.K. Madala, R. Edukulla, K.R. Davis, S. Schmidt, C. Davidson, J.A. Kitzmiller, W.D.
524 Hardie, and T.R. Korfhagen, Resistin-like molecule alpha1 (Fizz1) recruits lung
525 dendritic cells without causing pulmonary fibrosis. *Respiratory research* 13 (2012) 51.
- 526 [25] S.K. Madala, S. Schmidt, C. Davidson, M. Ikegami, S. Wert, and W.D. Hardie, MEK-
527 ERK pathway modulation ameliorates pulmonary fibrosis associated with epidermal
528 growth factor receptor activation. *Am J Respir Cell Mol Biol* 46 (2012) 380-8.
- 529 [26] S.K. Madala, R. Edukulla, S. Schmidt, C. Davidson, M. Ikegami, and W.D. Hardie,
530 Bone marrow-derived stromal cells are invasive and hyperproliferative and alter
531 transforming growth factor-alpha-induced pulmonary fibrosis. *Am J Respir Cell Mol*
532 *Biol* 50 (2014) 777-86.
- 533 [27] R.K. Kasam, S. Ghandikota, D. Soundararajan, G.B. Reddy, S.K. Huang, A.G. Jegga,
534 and S.K. Madala, Inhibition of Aurora Kinase B attenuates fibroblast activation and
535 pulmonary fibrosis. *EMBO Mol Med* 12 (2020) e12131.
- 536 [28] S. Nobbe, P. Dziunycz, B. Muhleisen, J. Bilsborough, S.R. Dillon, L.E. French, and G.F.
537 Hofbauer, IL-31 expression by inflammatory cells is preferentially elevated in atopic
538 dermatitis. *Acta Derm Venereol* 92 (2012) 24-8.
- 539 [29] R.K. Kasam, P.R. Gajjala, A.G. Jegga, J.A. Courtney, S.H. Randell, E.L. Kramer, J.P.
540 Clancy, and S.K. Madala, Fibrocyte accumulation in the lungs of cystic fibrosis
541 patients. *J Cyst Fibros* 19 (2020) 815-822.

IL-31 regulation of pulmonary fibrosis

- 542 [30] V. Sontake, S.K. Shanmukhappa, B.A. DiPasquale, G.B. Reddy, M. Medvedovic, W.D.
543 Hardie, E.S. White, and S.K. Madala, Fibrocytes Regulate Wilms Tumor 1-Positive
544 Cell Accumulation in Severe Fibrotic Lung Disease. *J Immunol* 195 (2015) 3978-91.
- 545 [31] D.J.K. Yombo, M.M. Mentink-Kane, M.S. Wilson, T.A. Wynn, and S.K. Madala, Heat
546 shock protein 70 is a positive regulator of airway inflammation and goblet cell
547 hyperplasia in a mouse model of allergic airway inflammation. *J Biol Chem* 294
548 (2019) 15082-15094.
- 549 [32] S.K. Madala, R. Edukulla, M. Phatak, S. Schmidt, C. Davidson, T.H. Acciani, T.R.
550 Korfhagen, M. Medvedovic, T.D. Lecras, K. Wagner, and W.D. Hardie, Dual targeting
551 of MEK and PI3K pathways attenuates established and progressive pulmonary
552 fibrosis. *PloS one* 9 (2014) e86536.
- 553 [33] J. Chen, E.E. Bardes, B.J. Aronow, and A.G. Jegga, ToppGene Suite for gene list
554 enrichment analysis and candidate gene prioritization. *Nucleic Acids Res* 37 (2009)
555 W305-11.
- 556 [34] V. Sontake, Y. Wang, R.K. Kasam, D. Sinner, G.B. Reddy, A.P. Naren, F.X.
557 McCormack, E.S. White, A.G. Jegga, and S.K. Madala, Hsp90 regulation of fibroblast
558 activation in pulmonary fibrosis. *JCI Insight* 2 (2017) e91454.
- 559 [35] D.J. DePianto, S. Chandriani, A.R. Abbas, G. Jia, E.N. N'Diaye, P. Caplazi, S.E.
560 Kauder, S. Biswas, S.K. Karnik, C. Ha, Z. Modrusan, M.A. Matthay, J. Kukreja, H.R.
561 Collard, J.G. Egen, P.J. Wolters, and J.R. Arron, Heterogeneous gene expression
562 signatures correspond to distinct lung pathologies and biomarkers of disease severity
563 in idiopathic pulmonary fibrosis. *Thorax* 70 (2015) 48-56.

IL-31 regulation of pulmonary fibrosis

- [36] J.H. Ryu, T. Moua, C.E. Daniels, T.E. Hartman, E.S. Yi, J.P. Utz, and A.H. Limper, Idiopathic pulmonary fibrosis: evolving concepts. *Mayo Clin Proc* 89 (2014) 1130-42.
- [37] T.A. Wynn, and T.R. Ramalingam, Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat Med* 18 (2012) 1028-40.
- [38] H.J. Kim, M. Zeidi, D. Bonciani, S.M. Pena, J. Tiao, S. Sahu, and V.P. Werth, Itch in dermatomyositis: the role of increased skin interleukin-31. *Br J Dermatol* 179 (2018) 669-678.
- [39] D. Ming, X. Yu, R. Guo, Y. Deng, J. Li, C. Lin, M. Su, Z. Lin, and Z. Su, Elevated TGF-beta1/IL-31 Pathway Is Associated with the Disease Severity of Hepatitis B Virus-Related Liver Cirrhosis. *Viral Immunol* 28 (2015) 209-16.
- [40] D.Q. Shih, L. Zheng, X. Zhang, H. Zhang, Y. Kanazawa, R. Ichikawa, K.L. Wallace, J. Chen, C. Pothoulakis, H.W. Koon, and S.R. Targan, Inhibition of a novel fibrogenic factor T11a reverses established colonic fibrosis. *Mucosal Immunol* 7 (2014) 1492-503.
- [41] B. Yaseen, H. Lopez, Z. Taki, S. Zafar, H. Rosario, B.A. Abdi, S. Vigneswaran, F. Xing, N. Arumalla, S. Black, S. Ahmad, K. Kumar, R. Gul, L. Scolamiero, S. Morris, A. Bowman, A. Stainer, A. Rice, C. Stock, E. Renzoni, C.P. Denton, C. Venturini, M. Brown, S. O'Reilly, and R. Stratton, Interleukin-31 promotes pathogenic mechanisms underlying skin and lung fibrosis in scleroderma. *Rheumatology (Oxford)* (2020).
- [42] E.A. Ayaub, A. Dubey, J. Imani, F. Botelho, M.R.J. Kolb, C.D. Richards, and K. Ask, Overexpression of OSM and IL-6 impacts the polarization of pro-fibrotic macrophages and the development of bleomycin-induced lung fibrosis. *Sci Rep* 7 (2017) 13281.

IL-31 regulation of pulmonary fibrosis

- 587 [43] G.S. Wong, J.L. Redes, N. Balenga, M. McCullough, N. Fuentes, A. Gokhale, C.
588 Koziol-White, J.A. Jude, L.A. Madigan, E.C. Chan, W.H. Jester, S. Biardel, N.
589 Flamand, R.A. Panettieri, Jr., and K.M. Druey, RGS4 promotes allergen- and aspirin-
590 associated airway hyperresponsiveness by inhibiting PGE2 biosynthesis. *J Allergy*
591 *Clin Immunol* (2020).
- 592 [44] J. Huang, H. Yue, T. Jiang, J. Gao, Y. Shi, B. Shi, X. Wu, and X. Gou, IL-31 plays dual
593 roles in lung inflammation in an OVA-induced murine asthma model. *Biol Open* 8
594 (2019).
- 595 [45] M.A. Thomas, B.J. Buelow, A.M. Nevins, S.E. Jones, F.C. Peterson, R.L. Gundry, M.H.
596 Grayson, and B.F. Volkman, Structure-function analysis of CCL28 in the
597 development of post-viral asthma. *J Biol Chem* 290 (2015) 4528-36.
- 598 [46] N. Weathington, M.E. O'Brien, J. Radder, T.C. Whisenant, E.R. Bleeker, W.W. Busse,
599 S.C. Erzurum, B. Gaston, A.T. Hastie, N.N. Jarjour, D.A. Meyers, J. Milosevic, W.C.
600 Moore, J.R. Tedrow, J.B. Trudeau, H.P. Wong, W. Wu, N. Kaminski, S.E. Wenzel,
601 and B.D. Modena, BAL Cell Gene Expression in Severe Asthma Reveals
602 Mechanisms of Severe Disease and Influences of Medications. *Am J Resp Crit Care*
603 200 (2019) 837-856.

604

605

Figure 1

IL-31RA^{+/+}

IL-31RA^{-/-}

A

Saline

Bleomycin

C

D

E

F

Figure 2

A

Down in IL31RA^{-/-} mice GO enrichment (example genes)

ECM - related genes

A2m, Arg1, Mmp13, Col1a1, Col3a1, Col5a2, Timp1, Eln, Nrg1, Ereg, Fn1, IL6

G $\alpha(i)$ signaling

Ccl28, Rgs5, Rgs4, Rgs16, Ptqdr2

Epithelium-associated genes

Nrg1, Krt20, Krt4, Krt5, Krt14, Sfrp1, Krt17,

Chemotaxis

Ccl28, Mcp1, Il6, Cxcl13

Adenylate cyclase activity

Calcb, Calcr, gper1, mtnr1a, Ptgdr2

Up in IL31RA^{-/-} mice GO enrichment (example genes)

regulation of membrane potential

Avpr1a, Adra1a, Kcna2, Kcnu1, Alox12, dpp6, gri1, Gri14, Rgs7, Kcnh4, Grip2

G protein-coupled receptor binding

Avpr1a, Adra2a, Agtr1, Gria1, Edn3, Tff2, Vipr2

Synaptic signaling

Avpr1a, Nrn1, Colq, Adra1a, Adra2a, Kcna2, Nr3c2, grla1, grikl4 ,slc1a2, edn3,

positive regulation of cytosolic calcium ion concentration

Avpr1a, Adra1a, Aqtr1, Ccr3, Ric3, Gpr18, Gpr33, Snca, Cxcr1

epithelial cell-associated genes

Chi3l1, clca1, Krt23, lamp3, Krt79

B

IL31RA-KO DN

IPF UP

C

Figure 3

Figure 4

Figure 5