WNT/β-Catenin Signaling Induces IL-1β Expression by Alveolar Epithelial Cells in Pulmonary Fibrosis

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Idiopathic pulmonary fibrosis (IPF) is a lethal lung disease of unknown etiology. It is characterized by alterations of the alveolar epithelium, myofibroblast activation, and increased extracellular matrix deposition. Recently, reactivation of the developmental WNT/β-catenin pathway has been linked with pulmonary fibrosis. The cell-specific mechanisms and mediators of WNT/β-catenin signaling in the lung, however, remain elusive. Here, we applied an unbiased gene expression screen to identify epithelial cell–specific mediators of WNT/β-catenin signaling. We found the proinflammatory cytokine IL-1β to be one of the most up-regulated genes in primary murine alveolar epithelial Type II (ATII) cells after WNT3a treatment. Increased transcript and protein expression of IL-1β upon WNT3a treatment was further detected in primary ATII cells by quantitative RT-PCR (log fold change, 2.0 ± 0.5) and ELISA (1.8-fold increase). We observed significant up-regulation of IL-1β and IL-6 in bronchoalveolar lavage fluid (BALF) in bleomycin-induced lung fibrosis *in vivo*. Importantly, primary fibrotic ATII cells isolated from lungs subjected to bleomycin secreted enhanced IL-1β and IL-6 *in vitro*. Furthermore, the orotracheal application of recombinant WNT protein in the Tcf optimal promoter (TOP)-β-galactosidase reporter animals led to WNT/β-catenin activation in epithelial cells, along with significant increases in IL-1β and IL-6 *in vivo* (2.7-fold and 6.0-fold increases, respectively). Finally, we found increased WNT3a protein in fibrotic alveolar epithelia, accompanied by enhanced IL-1β and IL-6 concentrations in BALF from patients with IPF. Taken together, our findings reveal that the alveolar epithelium is a relevant source of proinflammatory cytokines induced by active WNT/β-catenin in pulmonary fibrosis. Thus, WNT/interleukin signaling represents a novel link between developmental pathway reactivation and inflammation in the development of pulmonary fibrosis.

Keywords: WNT/β-catenin signaling; pulmonary fibrosis; proinflammatory cytokines; inflammation

CLINICAL RELEVANCE

Idiopathic pulmonary fibrosis (IPF) represents a progressive and lethal lung disease with limited responsiveness to current therapies. The aberrant activation of the developmental WNT/β-catenin pathway has been linked to the pathogenesis of IPF. Our study demonstrates that increased WNT/β-catenin signaling leads to the expression of the proinflammatory cytokines IL-1β and IL-6 in alveolar epithelial type cells *in vitro* and *in vivo*. Our findings reveal a novel link between the reactivation of developmental pathways and inflammation in IPF, leading to the perturbation of profibrotic effects. Thus, the WNT/interleukin axis may represent a suitable therapeutic target for future treatment options for patients with IPF.

Idiopathic pulmonary fibrosis (IPF) is a progressive chronic lung disease of unknown etiology. IPF exhibits a poor prognosis and is subject to only limited treatment options, because the causative mechanisms underlying its development remain unknown (1–3). It is well described that repetitive alveolar epithelial cell injury and impaired repair, along with the activation of mesenchymal cells, lead to excessive extracellular matrix (ECM) deposition. Finally, these fibrotic processes result in lung parenchymal destruction and respiratory failure. The role of inflammation within the pathogenesis of IPF remains open to debate, and although inflammation is not assumed to be a driving force in the development of IPF, its relevance in impaired cellular crosstalk remains a topic of research (4, 5).

Recently, the reactivation of developmental pathways, such as WNT/β-catenin, has been demonstrated in experimental and human idiopathic pulmonary fibrosis (6–8). The WNT/β-catenin pathway constitutes a large family of secreted glycoproteins that signals via membrane-bound receptors and its main cellular mediator β-catenin. Activation of the pathway leads to cytoplasmic accumulation and the nuclear translocation of β-catenin, subsequently regulating target gene expression via interactions with members of the T cell–specific transcription factor/lymphoid enhancer–binding factor (TCF/LEF) family. Nuclear β-catenin has been reported in epithelial and mesenchymal cells in pulmonary fibrosis. Thus, WNT/β-catenin may present a major molecular hallmark in impaired epithelial–mesenchymal crosstalk during the pathogenesis of IPF (9). Several cellular functions, such as epithelial cell proliferation and differentiation, the epithelial-to-mesenchymal transition, and fibroblast migration and myofibroblast differentiation, have been linked to WNT/β-catenin signaling (10, 11). In lung epithelial cells, WNT/β-catenin activation has been reported to lead to a proliferative response along with increased WNT target gene expression, indicating the involvement of WNT/β-catenin signaling in epithelial cell repair mechanisms *in vitro* and *in vivo* (12, 13). Importantly, it was recently shown that interfering with WNT/β-catenin signaling may serve as a suitable approach to novel treatment options for IPF (12, 14, 15). The cell-specific mechanism by which WNT/β-catenin signaling exerts its effects on cellular function and potentially drives fibrosis,

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however, remains elusive. Therefore, the aim of our study involved characterizing cell-specific mediators of WNT/β-catenin signaling in alveolar epithelial Type II (ATII) cells. To that end, we performed whole-genome arrays of WNT3a-stimulated primary mouse ATII cells and identified the proinflammatory cytokines IL-1β and IL-6 as WNT/β-catenin downstream targets, thereby establishing a novel link between developmental pathway reactivation and inflammation in IPF.

MATERIALS AND METHODS

Animals

Eight-week-old, pathogen-free female C57BL/6N mice (Charles River Laboratories, Sulzfeld, Germany) were used for the experiments, which were performed in accordance with the guidelines of the Ethics Committee at the School of Medicine of the University of Giessen, and which were approved by the Regierungspräsidium Giessen (Giessen, Hessen, Germany). Mice had free access to water and rodent laboratory chow. The Tcf optimal promoter (TOP)-β-galactosidase (TOPGAL) (Fos-LacZ)34Efu/J WNT reporter mice (16) were purchased from Jackson Laboratories. Recombinant mouse WNT3a or PBS was administered orotracheally (500 ng in 80 μl total volume). Bronchoalveolar lavage (BAL) was performed after 24 hours with 1 ml of PBS, and mouse lungs were excised after 24 hours for the detection of β-galactosidase in the challenged reporter animals. Bleomycin sulfate (Almirall, Barcelona, Spain) was dissolved in sterile saline and applied using the MicroSprayer Aerosolizer, Model IA-1C (Penn-Century, Wyndmoor, PA), as a single dose of 0.08 mg in 200 μl solution per animal (5 U/kg body weight). Control mice received 200 μl PBS. Lung tissues were excised and inflated with 4% (mass/volume) paraformaldehyde (PAA Laboratories, Cölbe, Germany) at 21 cm H_2O pressure for histological analyses.

Human Tissue

IPF tissue specimens were obtained as described previously (12, 17). The study protocol was approved by the Ethics Committee at the Justus-Liebig-University School of Medicine. Informed consent was obtained in written form from each subject for the study protocol.

ATII Cell Isolation

ATII cells were isolated as previously described (12, 18). For the collection of supernatants and for gene expression analyses, attached cells were washed with PBS, and starved with medium containing 0.1% FBS for 12 hours for synchronization. Cells were treated with recombinant WNT3a (R&D Systems) or control for the indicated time periods (8 h or 24 h). Supernatants were collected and centrifuged for 5 minutes and 4°C at 1,500 × *g*, and stored for analysis.

Microarray Experiments

Primary ATII cells were pooled from six mice and treated with either control or recombinant WNT3a (100 ng/ml; R&D Systems). Three groups of control and WNT3a-treated samples were used for RNA extraction. Total RNA was extracted with RNeasy columns (Qiagen), according to the manufacturer's instructions. RNA quality was assessed by capillary electrophoresis, using a Bioanalyzer 2100 (Agilent Technologies). Samples contained 0.3–1.0 μg RNA, and were preamplified and labeled using a Low Input RNA T7 kit (Agilent Technologies). Three samples each (of control-treated and WNT3a-treated cells) were labeled with cyanine (Cy)3 and Cy5. The labeled RNA was hybridized overnight to 44K 60 mer oligonucleotide-spotted microarray slides (Mouse Whole Genome 44K; Agilent Technologies). Slides were washed at different stringencies, dried by gentle centrifugation, and scanned using a GenePix 4100A scanner (Axon Instruments, Union City, CA). Data analysis was performed with GenePix Pro 5.0 software (Axon Instruments). Data were evaluated using the limma package from the R/Bioconductor framework (http://www.bioconductor.org).

ELISA

Experiments were performed according to the manufacturer's protocols, using the DuoSet ELISA Development System for IL-1β (DY401), IL-6 (DY406), and WNT3a (DY1324) from R&D Systems.

Statistical Analysis

Results are presented as means ± SEMs or SDs, and were considered statistically significant when $P < 0.05$. Means of indicated groups were compared using a two-tailed Student *t* test or one-way ANOVA, followed by the Dunnet *post hoc* test.

RESULTS

Initially, we determined the localization of the canonical WNT ligand WNT3a in a murine model of bleomycin-induced pulmonary fibrosis. Immunohistochemical staining for WNT3a and the ATII cell marker surfactant protein–C (SP-C) on serial lung-tissue sections revealed that WNT3a was localized to bronchial and alveolar epithelial cells in PBS-treated control animals (Figure 1A, *left*, *arrows*). Upon the development of lung fibrosis, WNT3a was particularly increased in fibrotic regions in the parenchyma, in areas of bronchiolization, and in hypertrophic alveolar epithelial

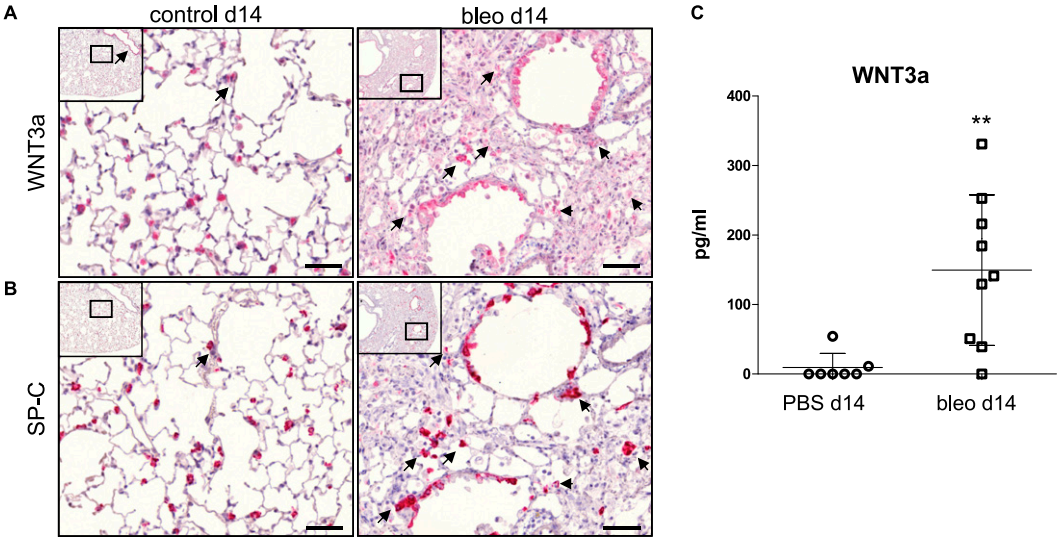

Figure 1. Expression of WNT3a in bleomycin-induced pulmonary fibrosis. Immunohistochemical staining was performed on serial lung tissue sections of PBS-treated (*left*) and bleomycin-treated (bleo) mice (*right*) on Day 14 (d14) after instillation. (*A*) Staining for WNT3a. (*B*) Staining for surfactant protein–C (SP-C) (magnifications, ×20; *insets*, ×5). *Arrows* indicate WNT3a-positive alveolar epithelial Type II (ATII) cells. (*C*) WNT3a protein concentrations were quantified by ELISA in bronchoalveolar lavage fluid (BALF) from control or bleomycin-treated mouse lungs, 14 days after injury. Individual values are depicted as picograms/milliliters in a scatterplot, with *horizontal lines* representing means. Significance, $^{**}P < 0.01$ (Student *t* test; PBS, $n = 7$; bleomycin, $n = 9$).

cells, indicating an enhanced secretion of WNT3a (Figure 1B, *right*, *arrows*). These data are in accordance with previously published findings that canonical WNT ligands are expressed in epithelial cells in experimental lung fibrosis as well as in human IPF tissue specimens (7, 8, 19). Furthermore, we determined WNT3a protein concentrations in bronchoalveolar lavage fluid (BALF) from mouse lungs subjected to experimental fibrosis by ELISA. Importantly, we found significantly increased WNT3a protein in BALF upon the development of lung fibrosis (control versus bleomycin; mean values, 13 pg/ml versus 110 pg/ml, respectively; Figure 1C), whereas transcript levels were found to be decreased, as reported previously (7) (Figures E1 and E2 in the online supplement). We further analyzed the transcript level of several WNT ligands, and found increased WNT1 and WNT7b mRNA concentrations upon the development of fibrosis in whole-lung homogenates, but not in primary ATII cells (Figures E1 and E2).

Given that mouse lung epithelial cells express WNT3a *in vivo* and that WNT3a protein was increased in BALF upon the development of lung fibrosis, we aimed to identify further WNT3a-induced epithelial cell–specific mediators in ATII cells. Therefore, we used primary murine ATII cells from C57BL/6N mice, as previously described (purity, 95% ± 3%; positive for prosurfactant protein–C, pan-cytokeratin, and tight-junction protein–1; Figure 2A; negative for α–smooth muscle actin; Figure E3A) (12, 20). ATII cells exhibited a serum-dependent capacity to proliferate after isolation, as assessed by [^{3}H]-thymidine incorporation on Day 1 after isolation (Figure E3B). Primary ATII cells were stimulated with WNT3a for 24 hours and subjected to whole-genome expression profiling by microarray analysis. Differential expression analysis revealed several genes to be significantly regulated (induced or repressed) by WNT3a after 24 hours, including the WNT target genes Axin2 and Dickkopf (Dkk) 2, thereby confirming WNT/β-catenin activation in primary ATII cells (*volcano plot*, Figure 2B; target genes are highlighted in *green*). The heat map in Figure 2C depicts the changes in gene expression level of the 50 most regulated genes after WNT3a stimulation (*red*, decreased expression; *green*, increased expression). Interestingly, we found the proinflammatory cytokine IL-1β to be one of the most upregulated genes (Figure 2C; 10 probe sets are highlighted by *brackets*; 0.64-fold on the log scale), whereas the IL-1 family

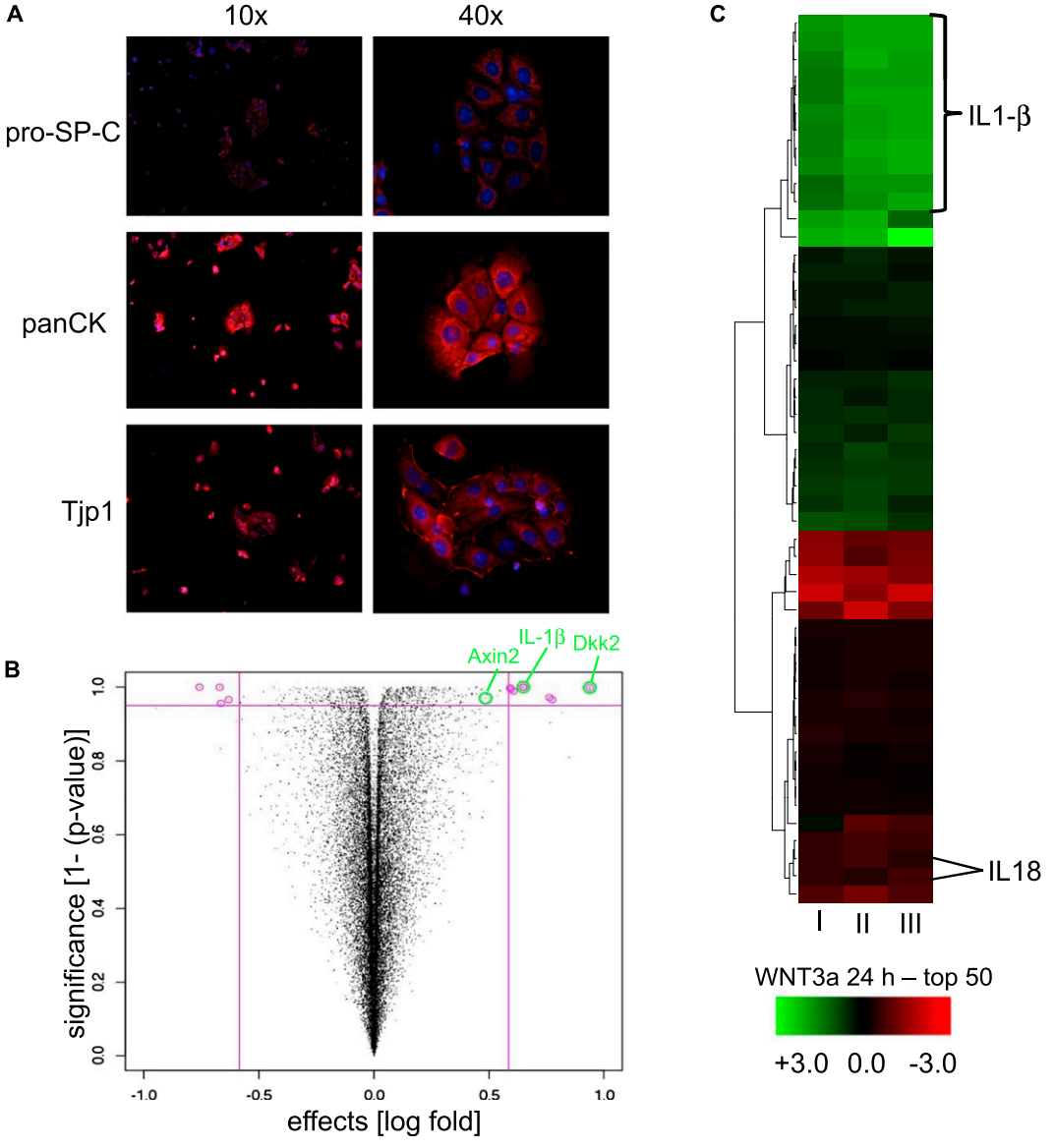

Figure 2. Gene expression profiling of WNT3a-treated ATII cells by microarrays. (*A*) Freshly isolated primary mouse ATII cells were fixed after 24 hours of attachment. The expression and localization of prosurfactant protein C (pro–SP-C), pan-cytokeratin (panCK), and tight-junction protein–1 (Tjp1) were determined by immunofluorescence. Nuclei were visualized by 4′6-diamidino-2-phenylindole staining. Stainings are representative of five independent experiments (magnifications, ×10, *left*; ×40, *right*). (*B*) Gene expression profiles were analyzed by microarrays, using RNA from primary ATII cells after 24 hours of WNT3a (100 ng/ml) or control stimulation. The *x* axis of the volcano plot depicts the estimated effects (log folds) in the differential expression analysis (WNT3a versus control treatment). The *y* axis displays the significance (1 − (*P* values)). The *horizontal line* marks the significance threshold of $P < 0.05$. The two *vertical lines* indicate induction/repression at 1.5-fold. Genes that are significantly induced or repressed are depicted in *magenta*. For IL-1β, the WNT targets Axin2 and Dickkopf 2 (Dkk2) are highlighted in *green*. (*C*) The heat map shows the top 50 increased and decreased genes, respectively, in WNT3a-stimulated versus unstimulated ATII cells. *Green* and *red* indicate increased or decreased gene expression levels, respectively. Different columns in the heat map represent individual samples. The *brackets* highlight different IL-1β probe sets.

member IL-18 was down-regulated. In addition, the proinflammatory cytokine IL-6 was up-regulated (0.53-fold on the log scale). WNT3a treatment also increased the proliferative capacity of primary ATII cells in a dose-dependent manner (by 1.8-fold and 2.2-fold, compared with control values; Figure E4). To validate the microarray results, we confirmed the gene expression changes by quantitative RT-PCR (Figures 3A and 3B). Here, six independent isolations of primary ATII cells were analyzed upon WNT3a stimulation. As shown in Figure 3A, the significantly increased expression of IL-1β (1.96 ± 0.51-fold on the log scale), IL-6 (0.92 ± 0.21-fold on the log scale), Axin2 (0.77 ± 0.46-fold on the log scale), and Dkk2 (1.74 ± 0.32-fold on the log scale) was observed after 24 hours of WNT3a stimulation. Interestingly, IL-1β was already highly induced by WNT3a after 8 hours, whereas IL-6 expression was only significantly elevated at the later time point (Figure 3B). This indicated that IL-1β may contribute to IL-6 expression. In addition, we confirmed the gene expression changes of Naked1, Lipocalin2, Fibronectin 1, and T-cell factor 7 by single quantitative RT-PCR (Figure E5).

Interleukins are secreted signaling molecules that mediate immune responses and promote the proliferation and differentiation of many cell types. The IL-1 cytokine family is mainly associated with the innate immune system and has been well-studied in autoinflammatory diseases, as reviewed elsewhere (21). In the context of fibrotic diseases, IL-1β has been shown to act as a profibrotic cytokine in the lung, and the adenoviral overexpression of IL-1β led to persistent fibrosis (22). In addition, the IL-1 receptor has been shown to be essential for the development of fibrosis, suggesting a major role of IL-1 signaling in lung fibrosis (23).

To elucidate whether ATII cells secrete IL-1β and IL-6 upon WNT3a stimulation, we determined IL-1β and IL-6 concentrations after WNT3a stimulation in supernatants of cultured primary ATII cells by ELISA. As depicted in Figure 3C, we observed a significant increase of IL-1β (control versus WNT3a-treated, mean values, 47 pg/ml versus 83 pg/ml, respectively), as well as IL-6 (control versus WNT3a-treated, mean values, 312 pg/ml versus 699 pg/ml, respectively) upon 24 hours of WNT3a stimulation of ATII cells *in vitro*. To analyze the combined effects of active WNT/β-catenin and IL-1β signaling, we stimulated mouse lung epithelial cell line-12 (MLE-12) cells with WNT3a, IL-1β, or a combination thereof. The mRNA concentrations of IL-6 and Axin2 were determined by quantitative RT-PCR (Figures 3D and E6C). Both WNT3a and IL-1β significantly induced IL-6 expression, and we observed an additive effect on IL-6 expression upon combined treatment with WNT3a and IL-1β (log fold

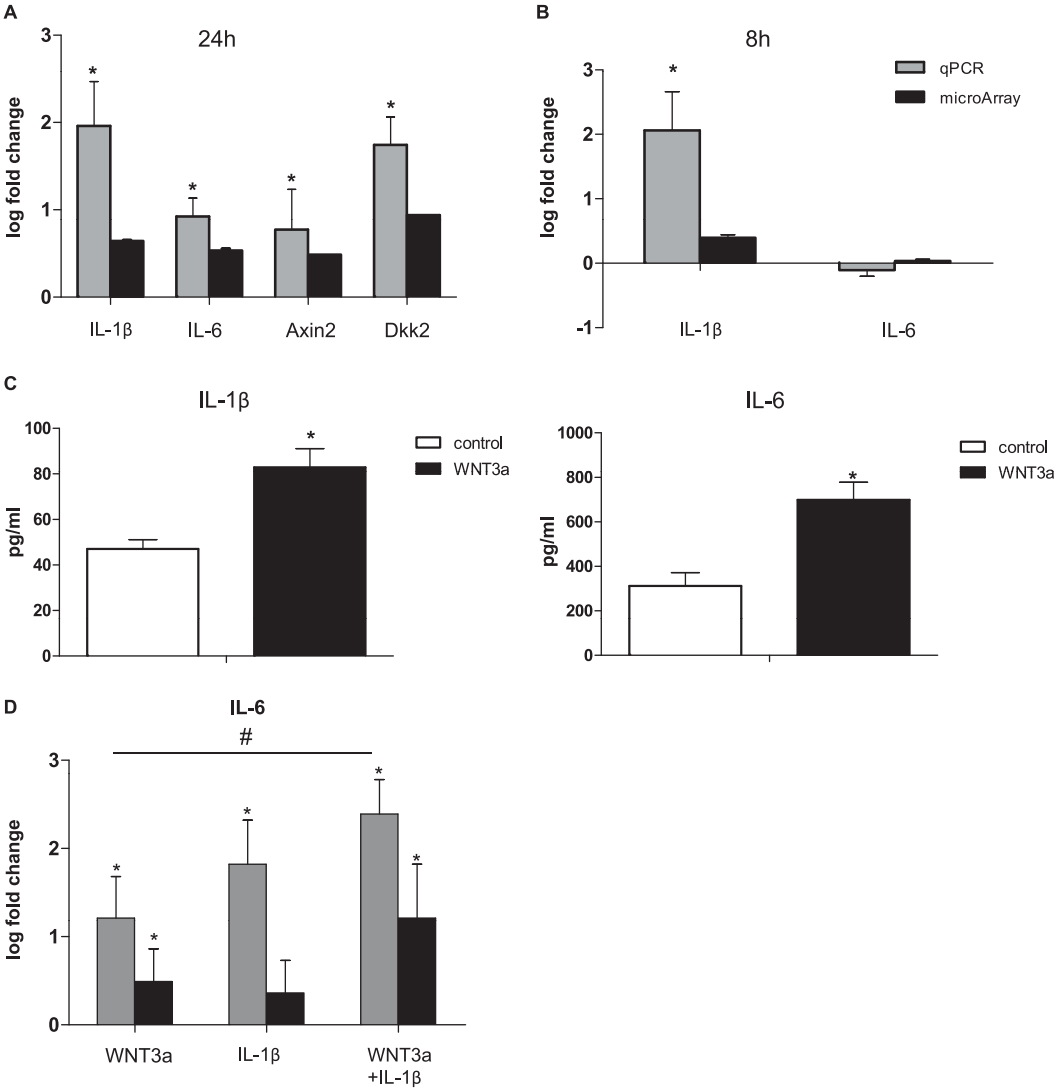

Figure 3. Validation of IL-1β and IL-6 gene and protein expression in WNT3a-treated ATII cells. (*A*) Confirmation of microarray results was performed for selected genes in isolated primary ATII cells after 24 hours of WNT3a stimulation by quantitative RT-PCR (qRT-PCR). The mRNA concentrations of IL-1β and IL-6 and of the WNT targets Axin2 and Dickkopf 2 (Dkk2) were measured and plotted as log-fold changes of mRNA concentrations, comparing qRT-PCR results (*gray bars*) with microarray results (*black bars*). Depicted data represent means ± SEMs. *$P < 0.05$ (Student *t* test, $n = 6$). (*B*) IL-1β and IL-6 mRNA concentrations were measured by quantitative RT-PCR after 8 hours of WNT3a stimulation, comparing quantitative RT-PCR results (*gray bars*) with microarray results (*black bars*). Depicted data represent means ± SEMs. *$P <$ 0.05 (Student *t* test, $n = 6$). (*C*) Cytokine concentrations of IL-1β and IL-6 in supernatants of WNT3a-stimulated (*solid bars*) and control stimulated primary ATII cells (*open bars*) were determined by ELISA. Data are shown as picograms/milliliters, and represent the mean ± SEM. *$P < 0.05$ ($n =$ 6). (*D*) IL-6 mRNA concentrations were measured after 6 and 24 hours of WNT3a (100 ng/ml), IL-1β (1 ng/ml), or WNT3a + IL-1β stimulation in mouse epithelial cell line-12 cells by qRT-PCR. Depicted data represent the means ± SDs. *$P < 0.05$ (one-way ANOVA, followed by the Dunnett *post hoc* test; $n = 5$–6). $^{\#}P < 0.05$ (single WNT3a treatment versus WNT3a + IL-1β treatment).

changes for WNT3a, 1.2 ± 0.47; for WNT3a + IL-1β, 2.4 ± 0.39). WNT3a stimulation did not induce IL-18 or IL-1α, which is in accordance with the microarray results (Figures E6A and E6B, respectively). Taken together, these results indicate that WNT/β-catenin and IL-1β signaling may potentiate the profibrotic effects mediated by IL-6 in pulmonary fibrosis.

We next asked whether WNT3a-induced IL-1β and IL-6 secretion may be involved in the development of lung fibrosis *in vivo*. To this end, we monitored cytokine release *in vivo*, and analyzed BALF obtained from bleomycin-treated and PBS-treated mouse lungs (Figures 4A and 4B). Both IL-1β and IL-6 concentrations were elevated on Day 14 and Day 21 after bleomycin instillation (Figures 4A and 4B). These data validate previous reports (23–25). However, whether ATII cells present a significant source of IL-1β and IL-6 in this context has not been investigated thus far. To address this question, we isolated primary ATII cells from fibrotic and control mouse lungs (14 d after bleomycin or PBS treatment, respectively). ATII cells were cultured for 24 hours, and the release of IL-1β and IL-6 was assessed in cell supernatants. Notably, IL-1β and IL-6 were significantly increased in conditioned media of fibrotic ATII cells (control versus bleomycin, mean values, 79 pg/ml versus 195 pg/ml, respectively, for IL-1β; 838 pg/ml versus 1,224 pg/ml, respectively, for IL-6; Figure 4C). To strengthen the point further that the induction of interleukins by WNT3a is specific for the epithelium, we treated primary human fibroblasts and the murine alveolar macrophage cell line (MH-S) with WNT3a, and we did not observe increased expression of either IL-1β or IL-6 (Figure E7, and data not shown).

Next, we aimed to corroborate that WNT/β-catenin signal activation leads to the release of IL-1β or IL-6 *in vivo*, and we mimicked WNT/β-catenin signal activation *in vivo* using TOPGAL reporter animals, that is, (Fos-LacZ)34Efu/J (16). Mice were treated orotracheally with recombinant WNT3a, and 24 hours after the treatment, BALF and lung tissue were analyzed. Previously, we showed that mainly epithelial cells stained positive for β-galactosidase in response to WNT3a, and a similar staining pattern has been reported in lungs upon bleomycin challenge, corroborating that epithelial cells are sites of active WNT/β-catenin signaling during the development of lung fibrosis (12, 19). Remarkably, IL-1β and IL-6 concentrations were significantly increased in BALF obtained from WNT3a-treated TOPGAL mice *in vivo*, as analyzed by ELISA (Figure 5A, IL-1β, mean values of control versus WNT3a, 1,130 pg/ml versus 3,070 pg/ml, respectively; Figure 5B, IL-6, 11 pg/ml versus 64 pg/ml, respectively).

Finally, we sought to determine WNT3a and interleukin expression in tissue and BALF samples of patients with IPF, compared with transplant donors. WNT3a expression was localized mainly to hyperplastic alveolar epithelial cells in IPF tissue specimens, as analyzed by immunohistochemistry (Figure 6A, *black arrows*). Moreover, we found increased WNT3a protein concentrations in whole-lung homogenates of patients with IPF compared with transplant donors, as analyzed by Western blotting (Figure 6B). Further, we detected significantly increased IL-1β and elevated IL-6 protein concentrations in the BALF of patients with IPF (Figure 6C, mean values, donor versus IPF, 11 pg/mg versus 17 pg/mg, respectively, for IL-1β; 60 pg/mg versus 340 pg/mg, respectively, for IL-6), thereby further indicating that an aberrant WNT/interleukin axis is involved during disease progression in IPF.

DISCUSSION

IPF is a fatal lung disease characterized by the progressive and irreversible destruction of lung architecture. It is well-accepted that repetitive injury of the alveolar epithelium, in the presence

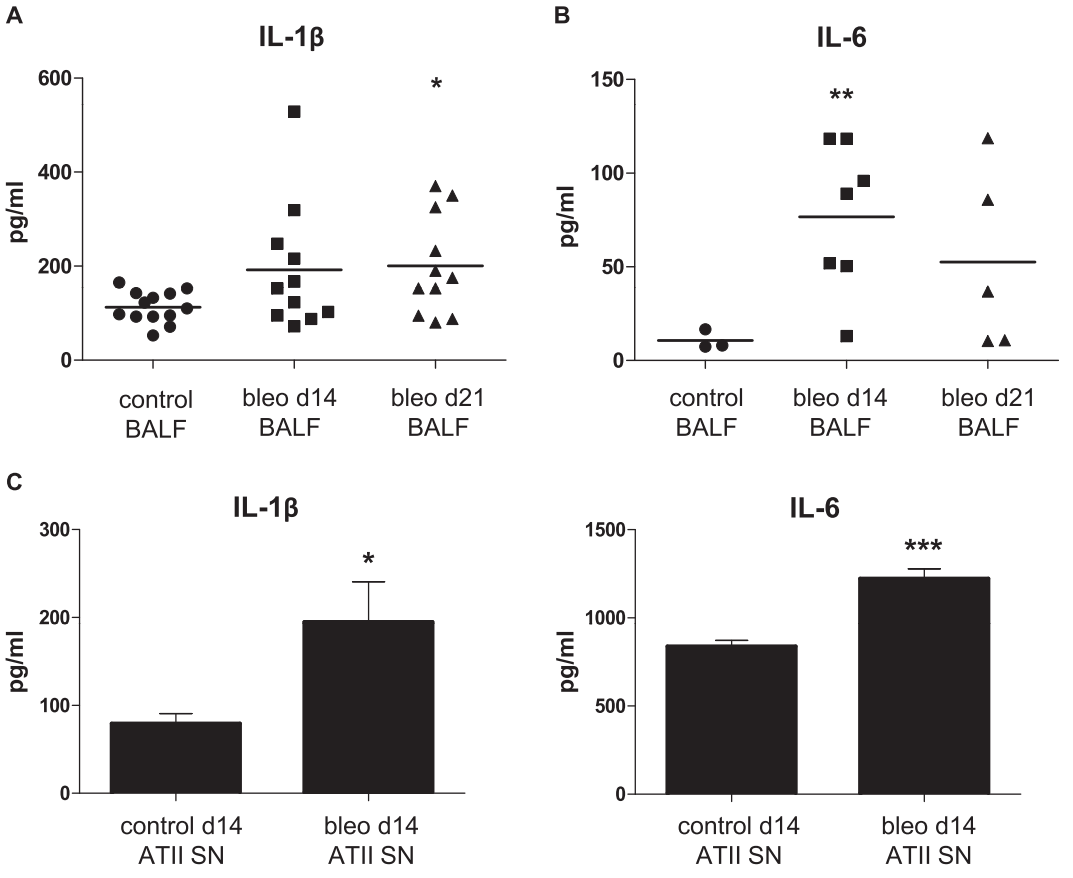

Figure 4. IL-1β and IL-6 secretion by ATII cells in bleomycin-induced pulmonary fibrosis. The IL-1β (*A*) and IL-6 (*B*) protein concentrations were quantified in BALF from control or bleomycin-treated mouse lungs after 14 and 21 days by ELISA. Individual values are depicted as a scatterplot, with *horizontal lines* representing means. Significance, $*P < 0.05$ and $**P < 0.01$. (*C*) Cytokine concentrations released by primary ATII cells to the supernatants (ATII SN), isolated from control or fibrotic lungs (Day 14 after bleomycin), were measured by ELISA. Data are presented as picograms/milliliters, and represent means ± SEMs. $*P < 0.05$ and $***P < 0.001$ ($n = 6$).

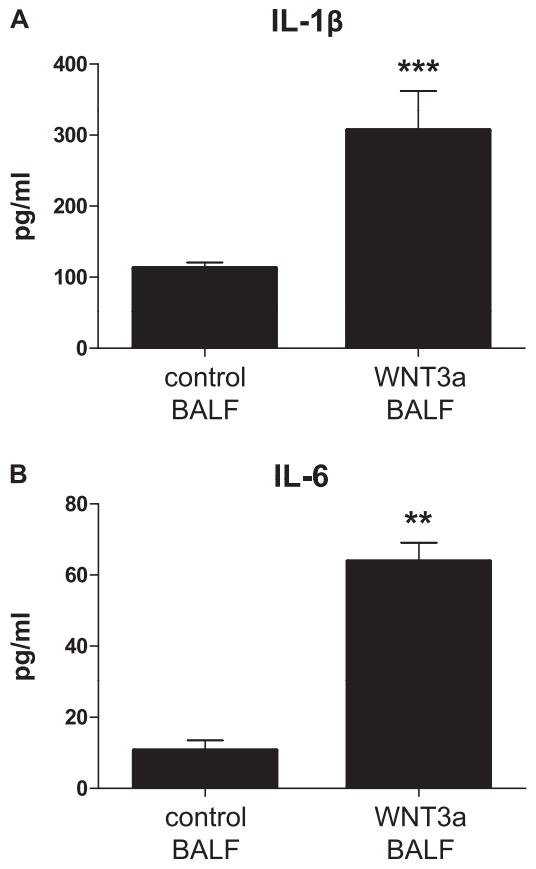

Figure 5. IL-1β and IL-6 expression upon WNT/β-catenin signal activation *in vivo*. The Tcf optimal promoter-β-galactosidase (Fos-LacZ)34Efu/J WNT reporter mice were treated orotracheally with recombinant WNT3a, as described in detail in MATERIALS AND METHODS. The IL-1β (*A*) and IL-6 (*B*) protein concentrations in mouse BALFs from control or WNT3a-treated reporter mice were quantified by ELISA. Data are presented as picograms/milliliters, and represent means ± SEMs. $^{**}P < 0.05$ and $^{***}P < 0.001$ ($n = 3$).

or absence of local inflammation, leads to deregulated wound-repair processes and an accumulation of extracellular matrix. One of the mediators linked to the impaired wound-healing response in IPF is the WNT/β-catenin signaling pathway (6, 10). Here, we report that active WNT/β-catenin signaling leads to the production of the proinflammatory cytokines IL-1β and IL-6 in alveolar epithelial cells. Using *in vitro* and *in vivo* experiments, we provide evidence that these interleukins are induced on the mRNA as well as protein level. To the best of our knowledge, our data link for the first time the reactivation of developmental pathways to an inflammatory response in pulmonary fibrosis. Thus, this WNT/interleukin axis may present a novel path contributing to the persisting phenotype of lung fibrosis.

The WNT/β-catenin signaling pathway has been implicated in the development of experimental and human pulmonary fibrosis (7, 8, 12). The cell-specific mechanism by which WNT/β-catenin signaling exerts its effect on cellular function, however, remains elusive. In epithelial cells, WNT/β-catenin reactivation was reported to be a prosurvival factor in ATII cells after bleomycin injury, and to drive ATII-to-ATI cell differentiation *in vitro* (19, 26). Similarly, active WNT/β-catenin signaling was recently demonstrated to attenuate experimental emphysema, indicating that this pathway drives alveolar epithelial cell repair (27). Moreover, Zemans and colleagues observed that neutrophil transmigration led to activated β-catenin signaling, thereby regulating lung epithelial-cell repair (13). Interestingly, IL-1β was shown to induce alveolar epithelial-cell repair mechanisms in injured epithelia (28, 29). These findings indicate that WNT and IL-1β may function as important mediators for the autocrine induction of alveolar epithelial-cell repair mechanisms. In pulmonary fibrosis, however, alveolar epithelial cell repair and/or differentiation are impaired because alveolar epithelial cells appear hyperplastic and hypertrophic, indicating that the WNT/β-catenin–driven attempt to repair and regenerate is insufficient (10). This raises the question of whether other mediators and mechanisms are involved either in the reduced capacity of fibrotic alveolar epithelial cells to undergo regular wound healing in general, or WNT/β-catenin signaling in particular.

Although the role of WNT/β-catenin signaling in general has been investigated in several recent studies, the specific role of distinct WNT ligands remains elusive. Here, we report that the canonical WNT ligand WNT3a is expressed by ATII cells, and increased WNT3a concentrations were detected in BALF from fibrotic lungs. Our comprehensive analysis of the WNT ligand transcript level in lung homogenates, as well as in primary ATII cell isolates, suggests that other canonical (or noncanonical) WNT ligands might be further differentially regulated in ATII or other cell types, and might thereby be active during lung fibrosis. This will be further investigated in future studies, depending on the availability of recombinant WNT isoforms.

Furthermore, WNT/β-catenin signaling may drive fibrogenesis via paracrine actions on nonepithelial cells, and in particular on fibroblasts. Active WNT/β-catenin reportedly led to increased fibroblast migration and proliferation, leading in turn to aggravated systemic sclerosis pulmonary fibrosis (30). Similarly, active β-catenin signaling has been demonstrated to exert proliferative effects on airway smooth muscle cells, which is crucial within the development of asthma and chronic obstructive pulmonary disease (31). Notably, the contradictory effects of WNT3a on ECM production have been reported for adult lung fibroblast lines and primary mesenchymal cells, thereby highlighting the heterogeneity of fibroblasts based on their origin (11, 30, 32). Our finding that interleukin signaling is connected to WNT/β-catenin activation in alveolar epithelial cells provides further explanation for the disturbed autocrine and paracrine signaling during pulmonary fibrosis.

The relevance of inflammation in the development of IPF remains controversial (4, 5). Although inflammatory events were initially assumed to lead to a fibrotic response in IPF, this hypothesis has been challenged by the observation that signs of inflammation have been detected only rarely in IPF tissue specimens, and further challenged by the fact that patients with IPF do not respond to immunosuppressive therapy (33). Nevertheless, proinflammatory cytokines and chemokines such as IL-1, TNF-α, chemokine (C-C motif) ligand 2 (CCL-2), and CCL-3 have been described as profibrotic stimuli during the development of IPF, as reviewed elsewhere (34, 35). In our transcriptome array, we found that IL-1β was one of the most strongly induced genes in ATII cells after WNT3a stimulation. Furthermore, we identified IL-6 as a novel target of the WNT/β-catenin pathway. IL-1β is well known as a strong inducer of IL-6 in alveolar epithelial cells *in vitro* (36, 37), and thus both WNT3a and IL-1β can induce IL-6 in primary ATII cells. Interestingly, our data regarding lung epithelial cells (MLE-12) favor a direct mechanism of IL-6 induction by WNT3a, because MLE-12 cells did not express IL-1β transcripts, whereas WNT3a induced IL-6 expression in this cell line. Furthermore, IL-6 expression was significantly increased upon IL-1β and WNT3a cotreatment.

To the best of our knowledge, this is the first study reporting IL-1β and IL-6 as novel downstream targets of the WNT/β-catenin

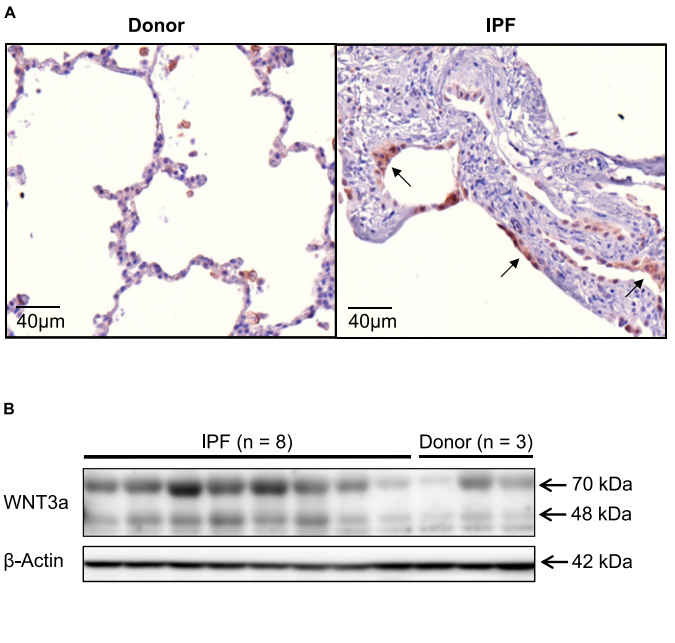

Figure 6. WNT3a and interleukin expression in idiopathic pulmonary fibrosis (IPF). (*A*) Immunohistochemistry of WNT3a in tissue specimens of patients with IPF and transplant donors. Positive cells are depicted in *red* and marked up with *black arrows* (magnification, ×20, *right*). (*B*) Western blot analysis of IPF (*lanes 1–8*) and transplant-donor (*lanes 9–11*) whole-lung homogenates for WNT3a (predicted molecular weight, 39 kD) (*above*) and β-actin (*below*). We observed bands with molecular weights of approximately 48 and 70 kD for WNT3a, indicating post-transcriptional modifications, such as glycosylation and palmitoylation. (*C*) BALFs obtained from patients with IPF and transplant donors were analyzed for IL-1β (*above*) and IL-6 (*below*) expression by ELISA. Data are presented as pg/mg, and represent the mean ± SEM. $*P < 0.05$ ($n = 5–8$).

pathway in alveolar epithelial cells. We first observed the induction of IL-1β and IL-6 gene expression and protein in isolated ATII cells upon WNT3a stimulation *in vitro*, and further by analyzing BALF from TOPGAL reporter animals after the orotracheal application of recombinant WNT protein *in vivo*. Future studies are needed to determine whether IL-1β and IL-6 represent direct targets of TCF/LEF-mediated gene regulation. Thus far, IL-1β is best known as an upstream mediator of WNT/β-catenin signaling in chondrocytes, because several studies showed that it was a potent inducer of the noncanonical WNT ligand WNT5a (38, 39) and the canonical ligand WNT7b (40). Similar to IL-1β, IL-6 has been reported to act upstream of noncanonical WNT signaling, as reviewed elsewhere (41).

The involvement of IL-6 and IL-1β in human fibrotic disease, and in the development of lung fibrosis in particular, has been investigated by several groups (24, 42–44). Kolb and colleagues demonstrated that the adenoviral-mediated overexpression of IL-1β led to acute inflammation and progressive lung fibrosis. These findings have mainly been linked with the long-term activation of transforming growth factor (TGF)-β signaling (22). The induction of TGF-β signaling by IL-1β was also demonstrated in kidney fibrosis. In that context, IL-1β was able to induce an epithelial-to-mesenchymal transition (EMT) in tubular epithelial cells *in vitro* (45). Furthermore, IL-1β was described to enhance the EMT induced by TGF-β in bronchial epithelial cells (46). Interestingly, in our study, we did not observe an EMT-like signature in the transcriptome array of WNT3a-stimulated ATII cells. Crosstalk between TGF-β and WNT/β-catenin signaling, however, has recently been demonstrated as crucial for the EMT and the development of lung fibrosis (47–49). These data indicate that whereas canonical WNT signaling alone may not drive EMT processes, other cytokines, such as TGF-β, exert their profibrotic effects via β-catenin activation.

In contrast to the downstream effects of IL-1β, namely, long-term TGF-β signaling induction, IL-6 is known to induce signal transducer and activator of transcription (STAT)–3 signaling. This led to worsened fibrosis in the bleomycin model, independent of Smad3-associated signaling (50). Recently, STAT3 was described as a putative central mediator in pulmonary fibrosis, as reviewed elsewhere (51). Therefore, we hypothesize that the WNT3a-dependent induction of IL-6 and subsequent STAT3 activation represent a possible mechanism whereby WNT/β-catenin signaling contributes to the persistent phenotype of IPF. Furthermore, in cancer, it has been shown that STAT3 signaling is able to induce tissue inhibitor of metalloproteinase–1 (TIMP-1) expression (52). TIMP proteins were reported to be up-regulated in fibroblastic foci during IPF (53). In addition, IPF fibroblasts are hyperresponsive for IL-6 (54). Therefore, IL-6 induction represents a possible positive feedback loop for excessive fibroblast activation, similar to IL-1β. Thus, our study provides evidence that reactivated WNT/β-catenin signaling in pulmonary fibrosis contributes to the pathology of the disease through both IL-1β and IL-6 secretion, which leads to TGF-β or STAT3 signaling, respectively, and finally to the paracrine activation of fibroblasts.

We report that IL-1β is not only regulated on the transcript level, but is also expressed in cell supernatants and BALF. Before secretion, IL-1β is further processed from pro–IL-1β to mature IL-1β by caspase-1. Caspase-1 itself can be activated by the NACHT, LRR, and PYD domains-containing protein 3 (NALP3) inflammasome. In systemic sclerosis, the caspase-1–dependent release of IL-1β and IL-18 was recently described to mediate fibroblast-to-myofibroblast differentiation (55). In experimental lung fibrosis, Gasse and colleagues showed that bleomycin administration leads to a release of uric acid in the lung, which activates the NALP3 inflammasome (25). To distinguish the general effects induced by bleomycin challenge from the specific gene regulatory mechanisms mediated by WNT ligands, we locally applied recombinant WNT3a protein *in vivo*. Although classic inflammasome proteins or markers, such as IL-18 or IL-33, were not up-regulated upon WNT/β-catenin signaling in our microarray analysis, it will be important to investigate the regulation of IL-1β processing in more detail in future studies.

Increased WNT3a expression in hyperplastic epithelial cells from IPF tissue specimens, as well as the increased protein concentrations of IL-1β and IL-6 in BALF from patients with IPF, indicates that our findings are relevant for future therapeutic treatment options. Therapeutic approaches in animal studies by interfering with IL-1β/MyD88 signaling, using IL-1 receptor–1 knockout animals or the application of the IL-1–receptor antagonist, demonstrated that bleomycin-induced lung fibrosis in mice was ameliorated or resolved (23, 56). In patients, IL-1β neutralization is achieved by the administration of anakinra, a specific drug already in clinical use for many years for autoinflammatory diseases such as arthritis (57), stroke (58), and Type 2 diabetes (59). In light of these findings, IL-1β neutralization using anakinra could be considered a therapeutic option for patients with IPF.

In conclusion, our study identified the proinflammatory cytokines IL-1β and IL-6 as novel WNT/β-catenin targets in alveolar epithelial cells *in vitro* and *in vivo*. Thus, we provide a novel link between WNT/β-catenin signaling and inflammation in pulmonary fibrosis, which may perturb profibrotic effects during the course of IPF.

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