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ORIGINAL RESEARCH

IL-1β Induces a Proinflammatory Fibroblast Microenvironment that Impairs Lung Progenitors' Function

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Abstract

Chronic obstructive pulmonary disease (COPD) is characterized by a persistent inflammatory state in the lungs and defective tissue repair. Although the inflammatory response in patients with COPD is well characterized and known to be exaggerated during exacerbations, its contribution to lung injury and abnormal repair is still unclear. In this study, we aimed to investigate how the inflammatory microenvironment affects the epithelial progenitors and their supporting mesenchymal niche cells involved in tissue repair of the distal lung. We focused on IL-1β, a key inflammatory mediator that is increased during exacerbations of COPD, and used an organoid model of lung epithelial cells and fibroblasts to assess the effect of IL-1β treatment on these cells' transcriptome and secreted factors. Whereas direct treatment of the lung organoids with IL-1β promoted organoid growth, this switched toward inhibition when it was added as fibroblast pretreatment followed by organoid treatment. We then investigated the IL-1β–driven mechanisms in the fibroblasts and found an inflammatory response related to (C-X-C motif) ligand (CXCL) chemokines; we confirmed that these chemokines were responsible for the impaired organoid growth and found that targeting their C-X-C chemokine receptors 1/2 (CXCR1/2) receptors or the IL-1β intracellular signaling reduced the proinflammatory response and restored organoid growth. These data demonstrate that IL-1β alters the fibroblasts' state by promoting a distinct inflammatory response, switching their supportive function on epithelial progenitors toward an inhibitory one in an organoid assay. These results imply that chronic inflammation functions as a shift toward inhibition of repair, thereby contributing to chronic inflammatory diseases like COPD.

Keywords: COPD; organoids; IL-1β; inflammation

Respiratory diseases are a major cause of death and disability worldwide. The main feature linking these diseases is inflammatory lung injury and subsequent impaired tissue repair caused by failure of endogenous repair mechanisms. Among the chronic respiratory diseases, chronic obstructive pulmonary disease (COPD) was the third leading cause of death in 2019 (1). In COPD, persistent inflammation in the peripheral airways and lung parenchyma contributes to small airway remodeling and alveolar wall destruction

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Clinical Relevance

Our data demonstrate that IL-1β alters fibroblasts' state by promoting a distinct inflammatory response, switching their supportive function on epithelial progenitors toward an inhibitory one in an organoid assay. These results imply that chronic inflammation functions as a shift toward inhibition of repair, thereby contributing to chronic inflammatory diseases like chronic obstructive pulmonary disease.

(emphysema), leading to progressive airflow limitation (2). During acute exacerbations of the disease caused by viral and bacterial infections, patients with COPD experience worsening of symptoms, leading to accelerated lung function decline and progressive worsening of the disease (3). Local chronic inflammation in COPD is caused by exposure to inhalation of cigarette smoke, air pollution, or other noxious particles and gases, which activate epithelial cells, macrophages, and other cells in the lung to release chemokines and other chemotactic mediators that recruit circulating neutrophils and other immune cells to the lungs. It has been shown that inflammation is present for years in the lungs of former smokers, suggesting that there are mechanisms that impair the resolution of inflammation even after its cause has been removed (2, 4, 5). Paradoxically, even though acute inflammation is beneficial in resolving infections and in resolving tissue injury, persistent inflammation and impaired resolution of inflammation are known to contribute to chronic lung disease and emphysema development (6). The lung epithelium is capable of regeneration upon injury because of several progenitor populations that are supported by adjacent mesenchymal cells. These cells form stem cell niches in which mesenchymal-derived signaling factors (e.g., fibroblast growth factors [FGFs]; Wingless/Integrated (WNT) signaling) can control the epithelial progenitors' fate (7, 8). In COPD and emphysema, signaling pathways involved in epithelial–mesenchymal cross-talk are altered, contributing to failed tissue repair (9–12).

The current therapeutic strategies for COPD, such as corticosteroids and phosphodiesterase 4 inhibitors, are aimed at reducing chronic inflammation, but they are not effective in many patients. In general, COPD treatments are effective at easing symptoms and reducing severity of exacerbations, but they cannot reverse the course of the disease because they lack the capacity to repair the damaged tissue (13). Taken together, the current lack of efficient therapeutic agents and the complex mechanisms involved in chronic inflammation show us the importance of exploring the failure to resolve inflammation and impaired tissue repair at the molecular level to find new molecular targets and/or therapeutic windows.

In this study, we focused on the distal lung progenitor niche and aimed to investigate the effect of the cytokine IL-1β on the niche microenvironment, considering its role in exacerbations of COPD and also its relation to prorepair signaling mechanisms. We used an organoid model to establish the effect of IL-1β on the niche fibroblasts and investigated their ability to support alveolar progenitor cell function. We then applied transcriptomic analysis to identify the molecular mechanisms affected by IL-1β in the fibroblasts and in the epithelial cells impaired by the fibroblasts' microenvironment to identify potential therapeutic targets to restore endogenous tissue repair during the exacerbation phases of COPD.

Methods

Animal Studies

C57BL/6J mice (both sexes, aged 8–12 wk) were maintained in 12-hour light/dark cycles and had access to food and water *ad libitum* at the Central Animal Facility of the University Medical Center Groningen. All experiments were done in accordance with Directive 2010/63/EU for animal experiments and the national ethical guidelines and upon approval of the experimental procedures by the institutional animal care and use committee of the University of Groningen under the CCD licenses AVD105002015303 and AVD1050020209205.

Cell and Organoid Culture

Human fetal lung fibroblasts (MRC-5; Sigma Aldrich 05081101) were cultured in 6-well plates for treatment with IL-1β (0.01, 0.1, 1 ng/ml) and/or Transforming growth factor beta-activated kinase 1 (TAK-1) inhibitor (5Z)-7-oxozeaenol (500 nM) and subsequently used for the other assays.

The organoid culture system was based on protocols previously published by our group (14, 15). The methods for cell and organoid culture, immunostaining, and resorting are described in the data supplement.

mRNA Isolation and qRT-PCR and RNA Sequencing Analysis

Total RNA was extracted from MRC-5 cells using TRIzol (Life Technologies). Total RNA was extracted from cells resorted from organoids using a NucleoSpin RNA kit (740955; Macherey-Nagel) according to the manufacturer's instructions. The detailed protocols for mRNA isolation and qRT-PCR are available in the data supplement.

For the MRC-5 cells, transcriptome analysis was performed in collaboration with the European Research Institute for the Biology of Ageing (Groningen, The Netherlands). For the epithelial cellular adhesion molecule ($Epcam^+$) cells, RNA sequencing (RNA-seq) was done using the Illumina NovaSeq 6000 sequencer by GenomeScan (https://www.genomescan.nl/). Details of RNA-seq, DESeq2, and fast gene set enrichment analysis (fGSEA) analyses are available in the data supplement.

ELISA

ELISAs for human IL-6 and CXCL8 (R&D Systems; DY206–05 and DY208–05) were performed on supernatant collected from MRC-5 cells treated with IL-1β and their respective nontreated controls according to the manufacturer's instructions. Data were normalized to untreated precision-cut lung slices as a control. A Synergy HTX Multi-Mode Microplate Reader (BioTek) was used to measure luminescence at 450 nm as a readout to assess protein concentration. ELISA measurements for CXCL1, CXCL2, CXCL8, and FGF2 were performed by a MILLIPLEX assay (Millipore) on a Luminex 100 system using Starstation software (Applied Cytometry Systems) according to the manufacturer's instructions. Data were expressed in picograms per milliliter.

Western Blot

After treatment, lysis buffer with protease inhibitors was added to Medical Research Council cell strain 5 (MRC-5) cells to obtain protein lysate; protein concentration was assessed with a Pierce BCA Protein Assay kit (Thermo Fisher) according to the manufacturer's instruction. Samples were run with Mini-PROTEAN Tetra Cell (Bio-Rad) in

a 10% SDS gel, then transferred on a nitrocellulose membrane, which was incubated overnight with primary antibodies for phospho-p38 and GAPDH and later with secondary antibodies for 2 hours. Protein bands were visualized with Western Lightning Plus-ECL (Perkin Elmer; NEL105001EA) in the G:Box imaging system (Syngene).

Statistical Analysis

All data are presented as mean±SEM unless otherwise stated. For normally distributed data, statistical significance was assessed by Student's *t* test or one-way ANOVA as appropriate. In case normality tests were not passed, statistical significance was determined by Friedman test or Mann-Whitney test. The *P* values indicating statistically significant differences were defined as $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$. Statistical analyses were performed with GraphPad Prism 9.3 software.

Results

IL-1β Switches from a Supportive to a Repressive Organoid Growth Stimulus Depending on Context

Because IL-1β plays a role in acute exacerbations, which happen in the context of a chronic inflammatory microenvironment, we aimed to further investigate its role in the proinflammatory niche microenvironment on lung tissue repair. We used an established organoid model that allowed us to study the interaction between fibroblasts and epithelial progenitors of the alveolar niche in a three-dimensional *in vitro* system. To mimic different inflammatory settings, we tested different treatment approaches (Figure 1A). To simulate the direct effect of inflammation, we added IL-1β (1 ng/ml) directly to the organoid media for as long as 14 days in culture and observed that IL-1β increased the number of organoids compared with the nontreated control (Figure 1B), confirming previous data in the literature pointing toward a prorepair effect of IL-1β (16, 17). A similar effect was also obtained with a shorter treatment (first 4 d in culture) in the organoids (Figure E1A in the data supplement). To mimic a proinflammatory

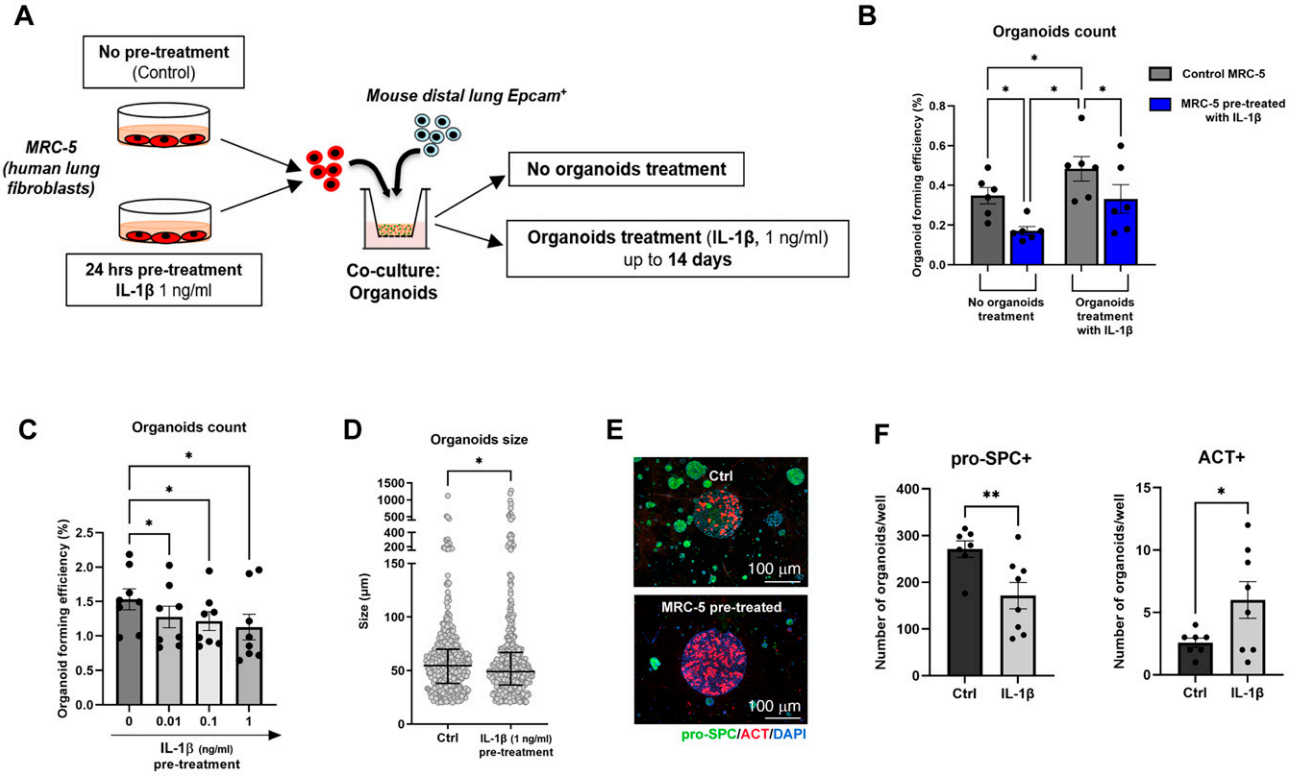


Figure 1. IL-1β shows a dual effect on organoid growth depending on microenvironment. (*A*) Human MRC-5 fibroblast pretreatment and organoid experimental setup in different experimental settings: *1*) no MRC-5 pretreatment, no organoid treatment; *2*) pretreatment of MRC-5 with 1 ng/ml IL-1β, no organoid treatment; *3*) no MRC-5 pretreatment, organoid treatment with 1 ng/ml IL-1β for as long as 14 days; and *4*) pretreatment of MRC-5 with 1 ng/ml IL-1β and organoid treatment with 1 ng/ml IL-1β for as long as 14 days. (*B*) Quantification at day 14 of organoids cultured according to the experimental setups described in *A* (reported in the same order as *A*); results are presented as organoid-forming efficiency obtained from the following calculation: (number of counted organoids/number of seeded epithelial cellular adhesion molecule [Epcam$^+$] cells) × 100 ($n = 6$ mice). (*C*) Quantification at day 14 of organoids derived from IL-1β–pretreated MRC-5 (0.01, 0.1, 1 ng/ml; 24 h) compared with control nontreated MRC-5 ($n = 4$ mice). (*D*) Diameter of organoids derived from 1 ng/ml IL-1β–pretreated MRC-5 compared with control nontreated MRC-5 (data presented as median±interquartile range; $n > 450$ organoids per group obtained from $n = 7$ mice). (*E*) Representative images of immunostaining of organoids derived from 1 ng/ml IL-1β–pretreated MRC-5 or control nontreated MRC-5: blue, DAPI; red, acetylated-α-tubulin (ACT); green, pro-surfactant protein C (SPC). Scale bar, 100 µm. (*F*) Quantification at day 14 of alveolar (SPC$^+$) and airway (ACT$^+$) organoids derived from 1 ng/ml IL-1β–pretreated MRC-5 compared with control nontreated MRC-5 ($n = 7$ mice). *$P < 0.05$; **$P < 0.01$.

microenvironment, we pretreated the MRC-5 human fibroblasts with IL-1β for 24 hours before organoid culture; after thoroughly washing away the treatment with PBS solution, we then used these pretreated fibroblasts to form organoids, and no other treatment was subsequently added to the organoids. Importantly, we observed a significant reduction in the number of organoids at day 14 (Figures 1B and 1C), and this reduction was dose-dependent, with the greatest effect observed at 1 ng/ml IL-1β (Figure 1C). At this concentration of IL-1β, organoid size was also significantly reduced (Figure 1D). Similarly, a reduction in organoid number was observed in organoids derived from IL-1β (1 ng/ml)–pretreated murine fibroblasts (CCL-206) (Figure E1B). At this concentration of IL-1β, immunostaining analysis (Figure 1E) showed that the numbers of prosurfactant protein C^+ (alveolar-type) organoids were significantly reduced, whereas the number of acetylated α-tubulin$^+$ (airway-type) organoids was enhanced (Figure 1F). Pretreatment of MRC-5 with the proinflammatory cytokine TNF-α, alone or in combination with IL-1β, did not affect organoid growth (Figure E1C). These results suggest that IL-1β negatively affects the fibroblasts' ability to support epithelial progenitor cells in organoid formation, possibly altering the microenvironment, and this may affect alveolar type II (AEC2) progenitors (prosurfactant protein C^+) specifically. Considering the opposite IL-1β results we observed in the acute model, we aimed to investigate if the IL-1β prorepair effect was preserved in a context in which the microenvironment was already altered, as is the case during disease exacerbations. We therefore cultured epithelial progenitors in the organoid model using IL-1β (1 ng/ml)–pretreated MRC-5 and continued adding IL-1β in the organoid culture media every other day for as long as 14 days. Notably, we observed that addition of IL-1β (up to 14 d) in a system in which the niche fails to support epithelial regeneration (because of previous IL-1β fibroblast pretreatment) was also reducing organoid numbers and, in fact, partially prevented the prorepair effect of IL-1β (Figure 1B). This indicates that, although the preexposure impairs the subsequent effect of IL-1β, this inhibition is not complete, implicating that a repair response is still partially possible. However, the difference between IL-1β preexposure only and IL-1β preexposure followed by IL-1β exposure in the organoid assay was not statistically significant.

IL-1β Alters the Transcriptome and Supports a Proinflammatory Microenvironment in Fibroblasts

To understand how IL-1β switches the fibroblasts' microenvironment toward such an inhibitory role, we performed whole transcriptome analysis upon treatment with IL-1β using RNA-seq of the MRC-5 fibroblasts treated with the highest optimal IL-1β concentration (1 ng/ml). The analysis revealed 4,400 genes significantly differentially expressed after IL-1β treatment (adjusted $P < 0.05$) compared with nontreated control fibroblasts, confirming a profound change on the cells' transcriptome (Figure 2D). Among the highest upregulated genes, we found genes coding for several CXCL chemokines including *CXCL8*, *CXCL1*, and *CXCL2*; cytokines like *IL6*, *IL1B*, and *CSF2/3*; and many other genes involved in inflammation (e.g., *PTGS2*, interferon-induced *IFI44*, *OAS1*, and *OAS2*) (Figures 2A and 2E). Additionally, the gene coding for the matrix metalloproteinase (MMP) MMP-12, which is known to play an important role in emphysema, was also highly upregulated (log fold change 10.27), as were other MMP genes (*MMP1*, *MMP3*, *MMP10*, *MMP14*). The top downregulated genes included several genes coding for membrane transporters and ion channels (e.g., *SLC14A1*, *ANO1*, *SCN3A*) (Figures 2B and 2C).

We then performed fGSEA to highlight enrichment of genes involved in specific pathways (Figure 2F and Table E1 in the data supplement). We confirmed a significant upregulation of pathways involved in inflammation, specifically interferon signaling and various interleukins' and chemokines' signaling, which were among the pathways with the highest normalized enrichment scores (Figure 2F). Several pathways related to endoplasmic reticulum function were also enriched and upregulated (e.g., *Calnexin Calreticulin Cycle*). Specifically, some pathways suggest a dysfunctional stress response in the endoplasmic reticulum (e.g., *ATF4 Activates Genes In Response To Endoplasmic Reticulum Stress*, *Unfolded Protein Response* [*UPR*]), as seen with upregulation of specific genes coding for proteins involved in the checkpoint of misfolded proteins (e.g., *SEL1L*, *EIF2AK3*). We also observed upregulation of pathways related to the extracellular matrix, specifically collagen and integrins (e.g., *Collagen Biosynthesis And Modifying Enzymes*, *Integrin Cell Surface Interactions*, *Extracellular Matrix Organization*) and pathways related to cell metabolism (e.g., *Glycolysis*, *Gluconeogenesis*, *Metabolism Of Water Soluble Vitamins And Cofactors*).

Within the downregulated gene signature, ribosomal protein subunit genes (e.g., *RPL5*, *RPL6*) showed the highest enrichment in pathways with high normalized enrichment scores, including *Selenoamino Acid Metabolism*, *Eukaryotic Translation Initiation* and *Elongation*, *rRNA Processing*, and *Nonsense Mediated Decay*. Several genes related to the *Respiratory Electron Transport Chain* pathway were also enriched in the downregulated signature, specifically genes related to the nicotinamide adenine dinucleotide reduced ubiquinone oxidoreductase (e.g., *NDUFAF4*), ATP synthases (e.g., *ATP8B1*), cytochrome oxidase subunits (e.g., *COX5A*), and specific mitochondria-encoded genes (e.g., *MT-COX3*). Together with other downregulated pathways (e.g., *Formation Of ATP By Chemiosmotic Coupling*, *Cristae formation*), this suggests reduced ATP synthesis. The increased glycolysis signature, together with reduced electron transport chain, is indicative of a metabolic switch caused by activation of innate inflammation (18). We then looked specifically into the genes we found altered with qRT-PCR; for WNT signaling, we confirmed downregulated *FRZB* and identified the DNA binding factors *TCF7* and *LEF1*, both downregulated together with their target gene *CCND1*. The gene coding for the β-catenin–independent WNT ligand WNT-5A was significantly upregulated; the genes *WNT5B* and *ROR1* (WNT-5A receptor) were instead downregulated. For FGF signaling, DESeq2 analysis confirmed significant upregulation of *FGF7* and *FGF2* with high statistical significance.

We then performed qRT-PCR on the fibroblasts for genes involved in epithelial-mesenchymal signaling and in the proinflammatory response to analyze the concentration dependency of these changes further. After 24 hours of IL-1β treatment, we observed a significant and concentration-dependent downregulation of the genes coding for the WNT/β-catenin target gene *AXIN2* and the Frizzled-related protein *FRZB* (Figure 2G). Similarly, FGF signaling seemed impaired, with significant upregulation of *FGF7* and *FGF2* (Figure 2H).

A

Gene ID	log2 FoldChange	pvalue	padj
CSF3	12.29526	1.31E-21	3.88E-20
CXCL8	12.21529	4.15E-105	1.01E-102
CXCL1	11.83887	5.06E-29	2.01E-27
CXCL2	11.71425	1.41E-09	1.76E-08
MMP12	10.27963	0.008516	0.029628
CXCL3	9.825549	7.97E-41	4.49E-39
OAS2	9.69931	3.65E-21	1.05E-19
CSF2	9.656961	3.80E-12	6.04E-11
IL1B	9.551529	3.11E-36	1.54E-34
CXCL6	9.449821	9.43E-55	9.26E-53
CXCL5	9.348832	1.09E-42	6.53E-41
OAS1	9.296808	3.33E-19	8.65E-18
C15orf48	9.151217	4.11E-13	7.15E-12
ELF3	9.09565	1.36E-13	2.45E-12
IFI44L	9.076941	5.17E-64	6.27E-62
PTGS2	8.893006	2.23E-308	2.23E-308
CMPK2	8.857114	2.64E-13	4.64E-12
IL1A	8.610325	5.24E-19	1.36E-17
IL6	8.405933	2.23E-308	2.23E-308
C3	8.331389	1.15E-47	8.80E-46

B

Gene ID	log2 FoldChange	pvalue	padj
SLC14A1	-6.89865	4.00E-24	1.35E-22
ANO1	-4.48405	1.19E-18	3.02E-17
TNNT2	-3.78847	2.76E-05	0.00018
SIGLEC15	-3.77291	0.000438	0.002205
PLCXD2	-3.76782	1.36E-05	9.49E-05
FGL2	-3.64927	5.36E-86	1.03E-83
MYEOV	-3.1231	0.00037	0.001896
GRPR	-2.90952	4.84E-10	6.30E-09
SCN3A	-2.88932	1.33E-05	9.28E-05
AL109918.1	-2.85132	1.68E-20	4.65E-19
OXTR	-2.71023	4.22E-16	9.06E-15
LUZP2	-2.66123	2.53E-07	2.37E-06
RCAN2	-2.65088	7.60E-05	0.000452
GDF5	-2.64907	4.20E-68	5.74E-66
SLC7A14	-2.64588	5.97E-09	6.91E-08
NPTX1	-2.53782	3.27E-53	3.02E-51
SLC16A12	-2.4836	9.44E-05	0.00055
FAM46B	-2.45961	3.23E-05	0.000207
FMO2	-2.44158	8.65E-05	0.000509
TAF9B	-2.42424	4.97E-14	9.26E-13

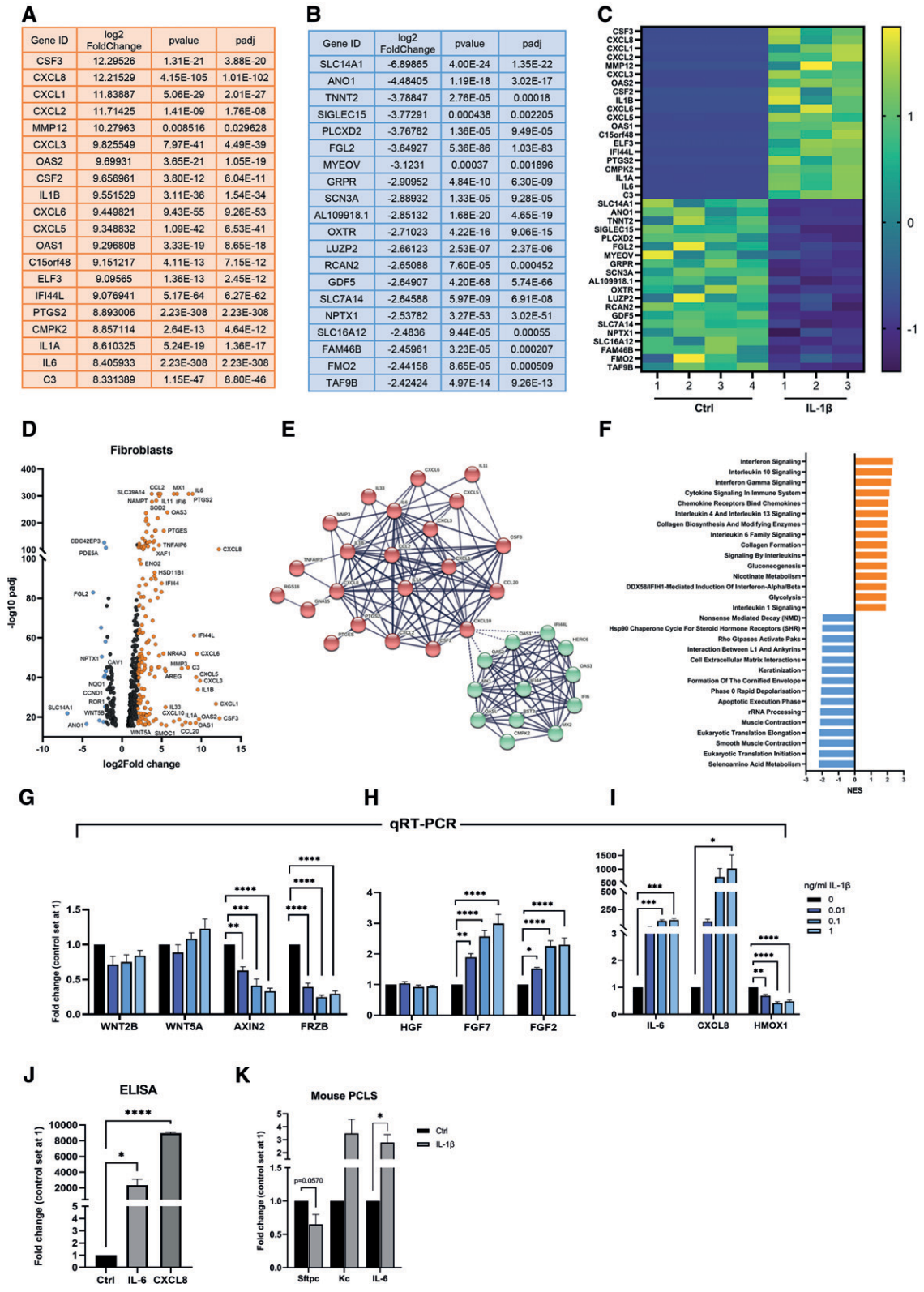


Figure 2. RNA sequencing (RNA-seq) of IL-1β–treated fibroblasts shows a proinflammatory signature. (*A–C*) MRC-5 fibroblasts were treated for 24 hours with 1 ng/ml IL-1β and subjected to RNA-seq. Data were normalized and analyzed for differential gene expression using DESeq2 analysis (adjusted $P < 0.05$); control, $n = 4$; IL-1β: $n = 3$. (*A*) Top 20 upregulated genes by log2fold change. (*B*) Top 20 downregulated genes by log2fold change. (*C*) Heat map for the top differentially expressed genes, ordered by log2fold change and represented as *z*-score of each sample.

The oxidative stress–related gene *HMOX1* was significantly downregulated by IL-1β (Figure 2I). We also saw significant upregulation of genes that code for the proinflammatory cytokine IL-6 and chemokine CXCL8 (Figure 2I), which was also confirmed at protein level with ELISA, whereby CXCL8 secretion in IL-1β (1 ng/ml)–treated fibroblasts reached a mean 8,000-fold increase compared with control fibroblasts (Figure 2J). We also evaluated the effects of IL-1β in precision-cut lung slices as an independent confirmation and found reductions in *Sftpc* expression concomitant with increased expression of *Cxcl8* and *Il6*, similar to what we had observed in the organoid and fibroblast cultures (Figure 2K). Collectively, these results show that IL-1β treatment of fibroblasts caused altered epithelial-mesenchymal signaling and a significant proinflammatory response in the fibroblast niche.

Targeting TAK-1 Signaling and CXCR1/2 Receptors Hampers the Inflammatory Response and Restores Support of Organoid Growth

After discovering the alterations in the fibroblasts through the RNA-seq analysis, we wanted to identify the intracellular signaling pathway activated by IL-1β in the treated fibroblasts to interfere with the downstream signaling as a potential therapeutic target. TAK-1 is a major intracellular effector of IL-1β signaling, which relays signals to downstream kinases such as p38 Mitogen Activated Protein Kinase (MAPK) (19). Accordingly, Western blot analysis of protein lysates from MRC-5 treated with IL-1β at concentrations between 0.001 and 1 ng/ml showed a dose-dependent activation by phosphorylation of p-p38 (Figure 3A). Similarly, we confirmed activation of p-p38 colocalized with the mesenchymal marker vimentin in an *ex vivo* model of murine lung slices treated with IL-1β (1 ng/ml; Figure 3B). Therefore, we sought to block IL-1β signaling by treating the MRC-5 with 1 ng/ml IL-1β concomitantly with a TAK-1 inhibitor ([5Z]-7-oxozeaenol), as IL-1β is known to signal through TAK-1 upstream of p38 (19). We observed significant restoration of gene expression of genes like *IL6*, *CXCL8*, and *FGF7* that were altered in the fibroblasts after IL-1β treatment (Figure 3C). The gene expression of the highly upregulated CXCL chemokines according to the RNA-seq analysis (*CXCL1/2/3*) and the interferon-induced gene *OAS1* was also restored upon TAK-1 inhibition (Figure 3D). Subsequently, we used fibroblasts pretreated with 1 ng/ml IL-1β and/or the TAK-1 inhibitor to form organoids. Blocking of the TAK-1 intracellular signaling was able to restore the organoid number, essentially reversing the IL-1β impairment of organoid growth (Figure 3E). Collectively, these results show that blocking TAK-1 signaling can hamper the inflammatory response and effectively restores the fibroblasts' support for epithelial progenitors.

Next, considering the significant proinflammatory response, which seems to be highly dependent on CXCL chemokines, we tested the hypothesis that these chemokines are mainly responsible for the impaired organoid formation we observed from IL-1β pretreatment. We therefore first confirmed the quantitative PCR data with ELISA data and showed that IL-1β induced a strong increase in the release of CXCL1, CXCL2, CXCL8, and FGF2 from the fibroblasts, which was inhibited by concomitant treatment with TAK-1 inhibitor (Figure 3F). We next treated organoids with the two highest expressed chemokines from the RNA-seq analysis, CXCL1 and CXCL8, added directly to the organoid culture for as long as 14 days and saw a significant reduction in organoid number (Figure 3G). Following up on these results, we aimed to block these chemokines' signaling (acting mainly through the CXCR1/2 receptors) to see if this was able to reverse the IL-1β effect. We pretreated fibroblasts with IL-1β and used them in organoid culture as previously described and subsequently treated the organoid culture with the CXCR1/2 receptor antagonist reparixin. Reparixin treatment did not affect the release of CXCL1, CXCL2, CXCL8, and FGF2 by the fibroblasts (Figure 3F), indicating that any effects on organoid growth are a result of interference with their subsequent effects on epithelial cells. After treatment for as long as 14 days, reparixin was able to restore the number of organoids derived from IL-1β–pretreated fibroblasts (Figure 3H). These results confirm that the harmful effect on organoid growth is caused by CXCL chemokines derived from the IL-1β–pretreated MRC-5 and show that blocking the CXCL/CXCR1/2–dependent inflammation represents a potential therapeutic target to restore this defect. Notably, the expression of these CXCL chemokines and CXCR2 receptors was found to increase in epithelial cells of mice following cigarette smoke exposure (Figure E1G). *In vivo* treatment with reparixin normalized the expression of CXCL1 and CXCL2 in epithelial cells obtained from cigarette smoke–exposed mice (Figure E1H).

Effect of the Fibroblasts' Altered State on the Epithelial Cells' Transcriptome

Finally, we investigated how the proinflammatory microenvironment created by fibroblasts affected the epithelial cells in the organoid system. To this end, we dissociated the organoids after 7 days in organoid culture with IL-1β (1 ng/ml)–pretreated MRC-5 and collected the $Epcam^+$ population for qRT-PCR (Figure 4A). We initially assessed expression of several target genes for FGF receptor, IL6, and CXCL8 signaling and oxidative stress markers, but found only significant downregulation of the gene coding for *Nrf2*, involved in the antioxidant response (Figure 4B). We then investigated the expression of specific epithelial marker genes, proliferation markers, and WNT/β-catenin target genes, but did not find any significant variation other than a reduction in *Muc5ac* expression (Figure E1E). Subsequently, we used RNA-seq analysis for a comprehensive view of the transcriptome. From a paired analysis, we identified 21 differentially

Figure 2. (*Continued*). (*D*) Volcano plot of differentially expressed genes obtained from DESeq2 analysis (adjusted $P < 0.0001$; orange, genes with log2fold change >2; blue, genes with log2fold change <−2). (*E*) StringDB network analysis of the top 50 upregulated genes (network by high confidence, 0.700). (*F*) Fast gene set enrichment analysis of the top enriched pathways reported by NES, top 15 up- and downregulated pathways. Full list of pathways shown in Table E1. (*G–I*) qRT-PCR of MRC-5 after 24 hours of treatment with IL-1β (0, 0.01, 0.1, 1 ng/ml) for WNT signaling–related genes (*G*), growth factors (*H*), and proinflammatory cytokines, oxidative stress (*I*). ($n = 4$). Data were normalized to the geometric mean of RPL13A and β2-microglobulin were used as housekeeping genes. (*J*) ELISA for proteins IL-6 and CXCL8 secreted by MRC-5 treated with 1 ng/ml IL-1β for 24 hours ($n = 3–4$). (*K*) qRT-PCR for alveolar type II marker Sftpc and inflammatory mediators Kc (murine Cxcl1) and IL-6 in mouse precision-cut lung slices (PCLSs) treated with 1 ng/ml IL-1β (24 h treatment; $n = 4$). Data were normalized to untreated PCLSs as a control. NES = normalized enrichment score. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

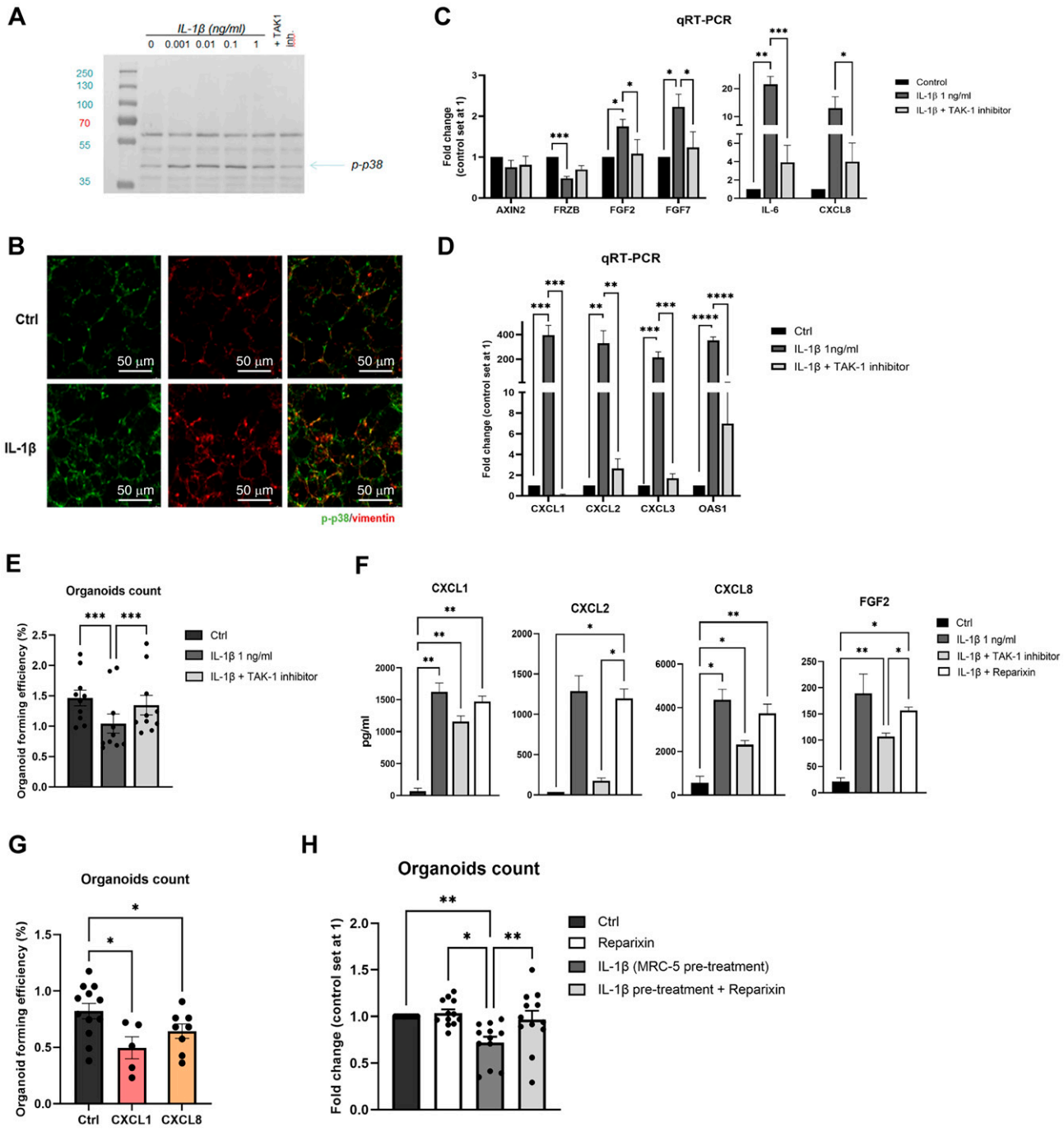
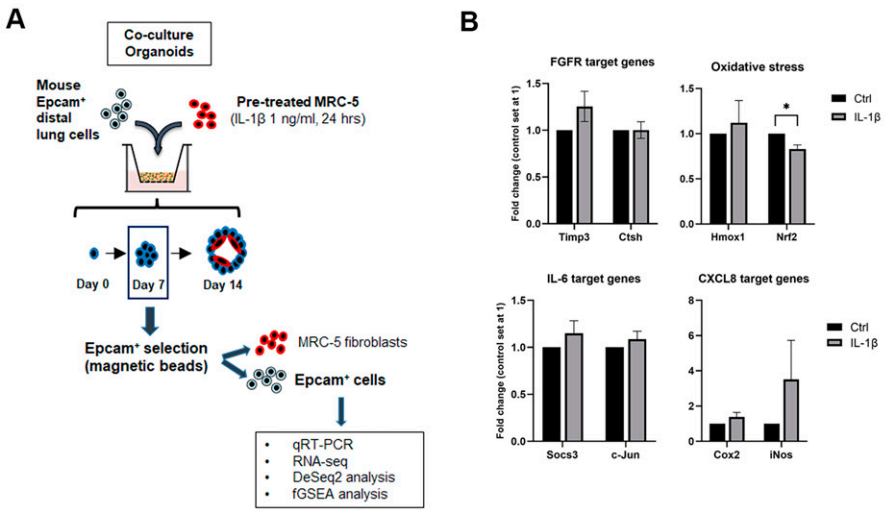


Figure 3. Targeting of IL-1β intracellular signaling and inflammatory response restores IL-1β effect. (*A*) Western blot of MRC-5 treated with IL-1β and/or TAK-1 inhibitor shows IL-1β intracellular signaling through activation (phosphorylation) of p38. (*B*) Staining shows colocalization of p-p38 and vimentin in mouse PCLSs treated with 1 ng/ml IL-1β (1 h treatment). Scale bars, 50 μm. (*C*) qRT-PCR of MRC-5 after 24 hours of treatment with 1 ng/ml IL-1β and (5Z)-7-oxozeaenol (TAK-1 inhibitor) for WNT-related genes, cytokines, and fibroblast growth factors (FGFs) ($n = 6$). Data were normalized to the geometric mean of RPL13A and β2-microglobulin were used as housekeeping genes. (*D*) qRT-PCR of MRC-5 after 24 hours of treatment with 1 ng/ml IL-1β and (5Z)-7-oxozeaenol (TAK-1 inhibitor) for the top upregulated proinflammatory genes ($n = 3$–5). Data were normalized to the geometric mean of RPL13A and β2-microglobulin were used as housekeeping genes. (*E*) Quantification at day 14 of organoids derived from MRC-5 pretreated with 1 ng/ml IL-1β alone and in the presence of TAK-1 inhibitor (organoid-forming efficiency; $n = 5$ mice). (*F*) ELISA for secreted chemokines CXCL1, CXCL2, CXCL8 and growth factor FGF2 from MRC-5 fibroblasts after 24 hours of treatment with IL-1β 1 ng/ml with the addition of TAK-1 inhibitor or reparixin ($n = 3$). (*G*) Quantification at day 14 of organoids treated for as long as 14 days with CXCL1 and CXCL8 ($n = 4$–6 mice). (*H*) Quantification at day 14 of organoids derived from MRC-5 pretreated with 1 ng/ml IL-1β and then treated for as long as 14 days with CXCR1/2 receptor antagonist reparixin (100 nM) ($n = 12$ mice). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.



C

Gene ID	log2 FoldChange	pvalue	padj
Tgm1	-0.79	1.00E-10	1.49E-06
Tm4sf1	0.60	1.21E-08	8.98E-05
Cd300lb	-2.37	2.80E-08	1.39E-04
Rgs5	-0.85	6.08E-08	2.26E-04
Krt79	-2.53	1.59E-07	4.71E-04
Rhof	-0.98	2.48E-07	6.14E-04
Cxcl2	1.29	4.87E-07	1.03E-03
Anxa10	-0.59	2.41E-06	4.48E-03
H2-Eb2	1.99	2.72E-06	4.49E-03
Frmpd1	-1.46	6.15E-06	9.15E-03
Smoc2	3.35	8.22E-06	1.11E-02
Pigr	0.71	1.01E-05	1.25E-02
Hc	0.52	2.83E-05	3.24E-02
Gm7887	0.60	3.20E-05	3.39E-02
Thsd4	-0.48	3.47E-05	3.43E-02
Bicc1	0.70	3.77E-05	3.50E-02
Lmo7	-0.47	4.39E-05	3.84E-02
D130019J16Rik	3.85	5.17E-05	4.10E-02
Cxcl1	0.84	5.24E-05	4.10E-02
Prkcdbp	-0.86	5.63E-05	4.18E-02
Myh7	-1.17	6.82E-05	4.83E-02

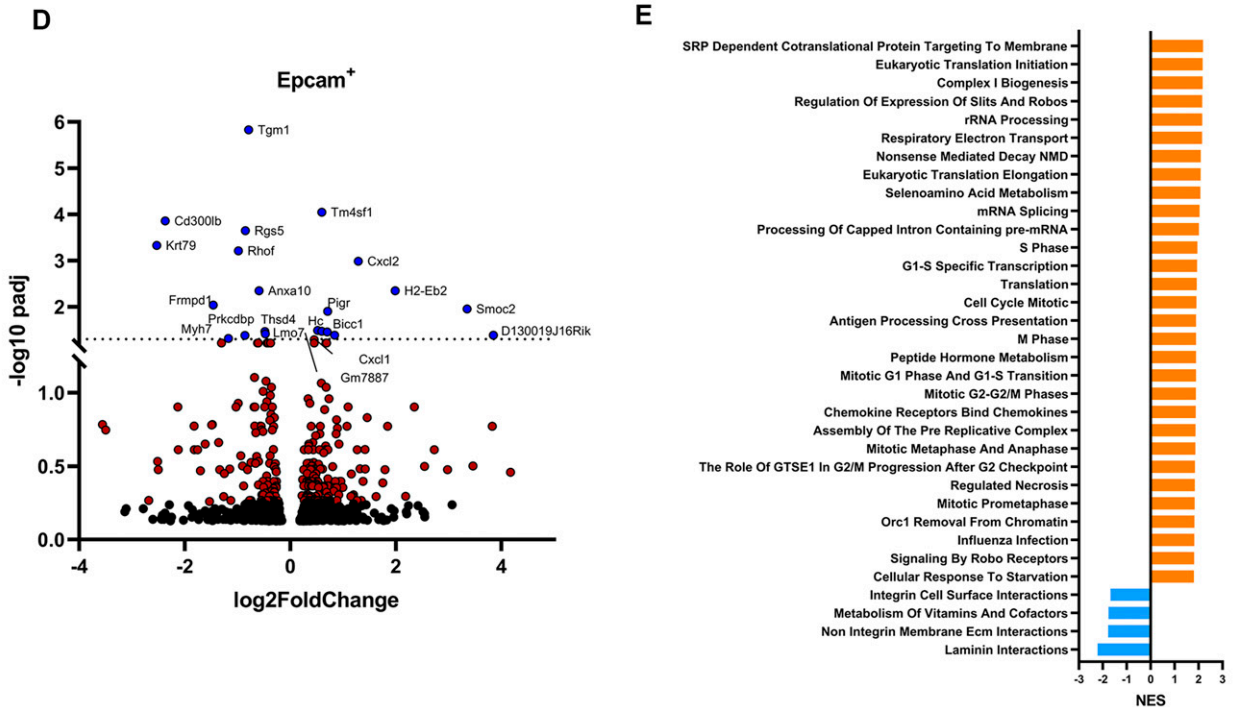


Figure 4. RNA-seq of Epcam$^+$ cells after 7 days in organoid culture with IL-1β–pretreated MRC-5. (*A*) Scheme of resorting of Epcam$^+$ cells after 7 days in organoid culture with IL-1β–pretreated MRC-5 fibroblasts ($n = 4$ samples per condition; total $n = 8$). (*B*) qRT-PCR of resorted Epcam$^+$ cells. Data were normalized to the geometric mean of RPL13A and β2-microglobulin were used as housekeeping genes. (*C*) Differentially expressed genes in resorted Epcam$^+$ cells after 7 days in organoid culture and subjected to RNA-seq and DESeq2 analysis (adjusted $P < 0.05$). (*D*) Volcano plot of differentially expressed genes obtained from DESeq2 analysis (blue: genes with adjusted $P < 0.05$). (*E*) fGSEA analysis of the top enriched pathway reported by NES; the top 30 upregulated and all the downregulated pathways are reported; the full list is provided in Table E2. fGSEA = fast gene set enrichment analysis. *$P < 0.05$.

expressed genes (statistical significance: adjusted $P < 0.05$) in the Epcam$^+$ population from the organoids derived from the IL-1β–pretreated compared with nontreated fibroblasts ($n = 4$ samples per condition; Figures 4C and 4D). In this gene list, we found upregulation of several genes related to inflammation, namely the genes coding for two CXCL chemokines (*Cxcl1* and *Cxcl2*); T cell–stimulating immunoglobulin superfamily members *Cd300lb*; *H2-Eb2*, which codes for a major histocompatibility complex class II molecule; and *Hc*, which codes for the complement protein C5 and has been shown before to be produced by alveolar type II cells (20). Additionally, we found genes that mark specific epithelial populations: the

upregulated *Tm4 sf1* gene, which codes for a transmembrane protein and was identified as a marker for a WNT-responsive alveolar epithelial progenitor subpopulation within the human alveolar type 2 cell population (21); and *Lmo7*, an early marker for alveolar type I cells, which was found downregulated. When looking at specific epithelial subpopulations' cell markers in the differentially expressed gene list, we observed downregulation of the alveolar type I marker *Aqp5* and upregulation of basal cell markers like *Krt5* and *Krt17*, albeit only significant by *P* value.

We performed fGSEA analysis to find specific gene enrichment. Among the pathways with the highest positive normalized enrichment scores, we found signatures related to eukaryotic translation and several pathways linked to the respiratory transport chain (e.g., *Complex I biogenesis*, *The Citric Acid* [*TCA*] *Cycle And Respiratory Electron Transport*). Furthermore, pathways related to most phases of the cell cycle were also upregulated, but so were the *Regulated necrosis* and *Programmed Cell Death* pathways. We found upregulated pathways related to chemokines and cellular response to viruses and the *Cellular response to hypoxia* pathway, including the gene coding for Hypoxia-Inducible Factor (HIF1α); HIF1α-mediated metabolic changes and response to hypoxia have been described to affect the fate of different progenitor cells during alveolar regeneration (16, 22). Several pathways related to the extracellular matrix were found enriched with a negative normalized enrichment score. Specifically, we found the pathways *Integrin Cell Surface Interactions*, *Non-Integrin Membrane ECM Interactions*, and *Laminin Interactions* (Figure 4E). This enrichment of downregulated genes, particularly collagen, integrin-α, and laminin genes (e.g., *Col1a1*, *Col4a1*, *Itga2*, *Itga3*, *Lama5*, *Lamc2*), suggests alterations in cell adhesion and cell-matrix interactions (Table E2 in the data supplement). Some of these α-integrins were shown to modulate repair in monolayer cultures of human airway epithelial cells on collagen-IV after acute mechanical injury (23). In conclusion, Epcam$^+$ cells exposed to the inflammatory microenvironment showed alteration of genes mainly involved in inflammation, cellular metabolism, and cell adhesion; these processes may contribute to failed epithelial repair in the organoid model.

Discussion

In the present study, we aimed to investigate the effect of IL-1β on the distal lung niche fibroblasts and epithelial progenitor cells using a lung organoid model. By using different experimental settings, we show that IL-1β can switch between supporting and antagonizing tissue repair depending on inflammatory context. Previous studies have investigated the effect of the IL-1β–derived inflammatory microenvironment in the alveolar progenitor niche. Interestingly, they showed that, in a context of acute inflammation, IL-1β had a prorepair effect. For example, when added directly into the organoid culture, IL-1β promoted organoid growth by acting on AEC2 cells (SFTPC$^+$) through an NF-κB axis, increasing their proliferation but maintaining their differentiation capacity. This prorepair effect of IL-1β on AEC2 was also evident in mice after influenza injury (17). A similar prorepair effect on the growth of organoids derived from SFTPC$^+$ AEC2 cells was achieved by Choi and colleagues, who also identified subtypes of AEC2 cells that are primed by IL-1β to become damage-associated transient progenitors, which will eventually differentiate into mature AEC1 cells. However, they showed that chronic exposure to IL-1β caused accumulation of damage-associated transient progenitors, which failed to terminally differentiate into mature AEC1 cells (16).

When we added IL-1β directly to our organoid culture, we also saw a supportive role for IL-1β, which led to a significant increase in organoid numbers, and this was the opposite of the effect of fibroblast pretreatment. However, when we added IL-1β treatment to our organoids in the presence of an already proinflammatory microenvironment, the effect of IL-1β switched to a repressive one by failing to increase organoid numbers. Collectively, these results show that IL-1β may exert disparate effects on epithelial progenitors depending on the context of acute or chronic inflammation.

It would be of great interest and potential therapeutic value to understand the causes of this switch. In the IL-1β–pretreated fibroblasts, we found a specific signature of several upregulated CXCL chemokines using several independent assays, including RNA-seq, quantitative PCR, and ELISA, and confirmed that this inflammatory microenvironment alone was capable of impairing organoid formation using recombinant CXCL chemokine ligands. Most of these chemokines (e.g., CXCL8, CXCL1) are specific neutrophil attractants known to be increased in the sputum of patients with COPD (2), and they act on the CXCR1/2 receptors, which have been studied as a therapeutic target to halt inflammation in lung injury. For example, CXCR2-knockout mice were protected against cigarette smoke–induced lung inflammation and injury (24) and CXCR2 therapeutic inhibition with reparixin attenuated lung injury in mice, in both cases as a result of reduced neutrophil recruitment (25). We used the same CXCR1/2 inhibitor and found that blockage of the CXCL chemokines' signaling was beneficial also in our epithelial cell/fibroblast organoid model to restore organoid numbers; this indicates that targeting these receptors may be effective not only in reducing neutrophil infiltration and inflammation, but also in aiding lung epithelium repair.

When we looked at the specific molecular mechanisms altered in the fibroblasts, some of these results were in line with previous studies in mice in which production of the neutrophil attractant CXCL chemokines CXCL1 and CXCL2, and of MMP-9 and MMP-12, were also increased by chronic production of IL-1β in adult mice (26). We identified that IL-1β acted on our fibroblasts via a TAK-1/p38 signaling axis and proved that, by blocking the intracellular signaling with a TAK-1 inhibitor, we were able to revert the inflammatory microenvironment and restore organoid growth. This signaling axis has been shown before to be involved in the production of IL6 and CXCL8 by MRC-5 fibroblasts when exposed to WNT-5B, acting through Frizzled2 receptor (27). WNT signaling plays an important role in supporting the progenitor cells in the niche (28, 29); IL-1β caused increased expression of the gene for the β-catenin–independent ligand WNT-5A, which has been shown to be upregulated in COPD fibroblasts and to impair alveolar epithelial cell repair *in vitro* and *in vivo* (10).

The transcriptome of the Epcam$^+$ cells exposed for 7 days to the CXCL/CXCR-dependent proinflammatory niche in the organoids revealed a small signature of differentially expressed genes, indicating limited, though present, persistent transcriptional effects of the IL-1β microenvironment, even after 1 week of

washout. Among them, we identified upregulated *Tm4 sf1*, which is a marker of a human WNT-responsive alveolar epithelial progenitor lineage within the AEC2 cells. These cells act as a major facultative progenitor cell in the human distal lung (21, 30). On the contrary, reduced gene expression of the early AEC1 marker *Lmo7* was found. Collectively, this suggests that differentiation is impaired by chronic IL-1β exposure, leading to accumulation of immature progenitors or a slower differentiation progress. We also did not find a specific FGF or WNT signaling target response, as expected from the altered signaling from the fibroblasts, although this may be explained by the time course of these responses; for example, WNT/β-catenin signaling is required in only the initial phase of organoid formation (31). Additionally, an interesting signature shows enrichment of genes involved in Slit/Robo signaling, including the upregulated receptor genes *ROBO1* and *ROBO3*; this pathway signals through Rho GTPases and is involved in cell proliferation, and it was described to be a potential player in alveolar regeneration (32). Notably, although the expression of these genes was altered after 7 days of organoid culture, we did not find changes in CXCL1 and CXCL2 gene expression in the fibroblast or the epithelial cell following IL-1β pretreatment on day 14 (Figure E1D). This result indicates that the effects of early IL-1β/CXCL chemokine exposure are transient and gradually lost with washout. However, this is still reflected in the number of organoids on day 14 because organoid number on day 14 is a determinant of signaling events early in the organoid culture (31). Accordingly, the IL-1β/chemokine exposure early in the organoid assay may have affected organoid number on day 14, whereas proinflammatory gene expression at that time may have normalized.

The implication of the present work is that a persistently inflamed microenvironment switches the prorepair effect of IL-1β to a repressive effect, which may be relevant during exacerbations of COPD driven by bacterial or viral infections. In support, we find that CXCL chemokines and CXCR2 receptors are increased following cigarette smoke exposure in isolated epithelial cells, which is in line with a recently published study that shows increased expression of CXCL chemokines in a subpopulation of alveolar type II cells specifically in patients with COPD (33). Such a background of chronic chemokine-driven inflammation may thus impact the repair response of epithelial progenitors to a subsequent acute inflammatory event such as an exacerbation. Certainly, exacerbations are a complex disease state that requires more complex models, and we need to acknowledge this as a limitation to our study. However, our results could give more insight into the study of acute versus chronic inflammation in progressive lung disease. Whereas acute inflammation associated with infections contributes to resolution of the infection and to restoration of lung structure and function in otherwise healthy individuals, in patients with COPD, such infections are associated with incomplete recovery, adding to lung function loss. The current knowledge on the contribution of inflammation to tissue destruction or repair is still contrasting. For example, it is known that neutrophils can accumulate in the alveolar sacs and release several mediators, including proteases, MMPs, and reactive oxygen species, which play key roles in the alveolar tissue destruction in COPD (34), but they can also have a role in promoting alveolar epithelium repair (35). T and B lymphocyte cells that infiltrate the lung form tertiary lymphoid structures (e.g., Inducible Bronchus-Associated Lymphoid Tissues (iBALT)) through lymphotoxin β-receptor signaling; these were recently described to be crucial to impaired alveolar regeneration (36). On the contrary, several studies described beneficial effects of inflammatory mediators on lung repair, such as IL-6 and IL-1β, which stimulated activation and differentiation of several lung progenitors *in vitro* and in mouse models (16, 17, 37, 38).

IL-1β has been correlated with inflammation derived from cigarette smoke in healthy smokers (39) and in cigarette smoke–exposed mice and has been found upregulated in induced sputum of patients with COPD (40). Additionally, IL-1 signaling can directly contribute to airway remodeling (41). IL-1β gene expression was found upregulated in the lungs of patients with COPD already in the early stage of the disease and at steady state (Figure E1I) (42, 43); furthermore, during acute exacerbations, IL-1β was found upregulated in serum of patients with COPD, correlating with several clinical parameters associated with COPD, including forced expiratory volume in 1 second percentage predicted and neutrophil percentage (44). Another study identified sputum IL-1β, together with serum CXCL10 and peripheral eosinophils, as specific biomarkers of bacteria-, virus-, or eosinophil-associated exacerbations of COPD (45). Acute exacerbations of COPD contribute to progressive lung injury, lung function decline, and worsening of symptoms, and therefore represent a potential therapeutic window to halt the progress of the disease (3, 46).

Considering its role in different diseases involving inflammation, several therapeutic approaches to target IL-1β have been studied. Although some of them were successful in *in vivo* models or in other inflammatory diseases, they have not been successful in clinical trials in patients with COPD thus far. Examples include canakinumab, an anti–IL-1β–specific antibody (ClinicalTrials.gov identifier NCT00581945), and MEDI8968, a monoclonal antibody that targets IL-1 receptor 1 (47). Anakinra, an IL-1 receptor antagonist that was approved in the treatment of rheumatoid arthritis and coronavirus disease (COVID-19), was successful in reducing IL-1β–induced neutrophilic inflammation in a mouse model of influenza-induced exacerbations (48). Clinical trials with CXCR1/2 inhibitors have also been unsuccessful so far. For example, treatment with the CXCR2 inhibitor danirixin was associated with more exacerbations and a higher frequency of pneumonia-related events (49). However, considering the potential benefit of such drugs on tissue repair, it would be of interest in future clinical trials to assess their effect not only on neutrophilic inflammation but also on lung repair.

Despite the contrasting data in the literature, IL-1β and inflammatory mediators still represent viable targets to halt inflammation in acute exacerbations of COPD. In general, considering the importance of recruitment of inflammatory cells by IL-1β and the other chemokines in the initial inflammatory phases, following these therapeutic strategies must be carefully considered in a way that does not hamper inflammation completely. Further studies into the mechanisms involved in prorepair signaling versus impaired repair could help to identify better molecular targets and/or time-specific windows of

treatment during acute exacerbations to restore endogenous lung repair in COPD.

In conclusion, we show that IL-1β causes fibroblasts' inflammatory microenvironment, which impairs the fibroblasts' ability to support epithelial cells in lung tissue repair. Although it has been shown that IL-1β has a prorepair effect in acute inflammation settings *in vitro* and in animal models, we show that this response switches to a repressive one *in vitro* on a background of inflammation. Further insights are needed to explore IL-1β interactions with other inflammatory cells to understand the role of IL-1β in acute versus chronic inflammation in more detail. We propose that IL-1β signaling and its inflammatory response could be valid targets to restore lung repair in chronic lung disease. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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