

Alveolar epithelial cell senescence predominates over macrophage senescence in driving pulmonary fibrosis

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a progressive and fatal lung disease in which cellular senescence contributes to disease pathogenesis. However, the relative contributions of senescent macrophages and alveolar epithelial cells (AECs) to fibrosis progression remain unclear. Here, we investigated the cell type-specific roles of senescence in pulmonary fibrosis using p16^{INK4a} (p16)-based gain- and loss-of-function approaches. p16 overexpression induced senescence-associated phenotypes in both THP-1 macrophage-like cells and A549 AECs. Senescent THP-1 cells exhibited enhanced M2-like polarization, whereas senescent A549 cells upregulated epithelial–mesenchymal transition- and fibrosis-associated genes, including *ACTA2* and *CCN2*. Despite these profibrotic phenotypes *in vitro*, macrophage-specific p16 deletion did not affect bleomycin-induced pulmonary fibrosis *in vivo*. In contrast, type II AEC-specific p16 deletion significantly attenuated fibrosis, as demonstrated by reduced soluble collagen levels in bronchoalveolar lavage fluid and decreased collagen deposition. This protective effect was associated with reduced growth differentiation factor 15 (GDF15) levels in bronchoalveolar lavage fluid and decreased *Gdf15* expression in isolated AECs. These findings identify senescent type II AECs, rather than macrophages, as key drivers of pulmonary fibrosis progression and suggest that targeting senescent AEC-derived mediators, including GDF15, may represent a therapeutic strategy for IPF.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, and irreversible interstitial lung disease of unknown etiology, characterized by a poor prognosis and limited therapeutic options. Current antifibrotic therapies attenuate the rate of disease progression; however, they neither reverse established fibrosis nor halt disease progression entirely^{1,2}. These limitations highlight the need to identify key molecular targets of fibrogenesis and develop more effective therapeutic strategies.

Cellular senescence, a stable state of irreversible cell cycle arrest induced by aging and various cellular stressors, has emerged as a key contributor to age-related diseases, including IPF³. Senescent cells accumulate in the aging lung and acquire a senescence-associated secretory phenotype (SASP), characterized by the secretion of proinflammatory cytokines, chemokines, and profibrotic mediators that disrupt tissue homeostasis and promote fibrotic remodeling⁴.

Senolytic therapies that selectively eliminate senescent cells, including dasatinib and quercetin, have recently emerged as promising antifibrotic interventions^{5,6}. Accumulating evidence indicates that cellular senescence occurs across multiple lung cell populations, including macrophages^{7,8}, endothelial cells⁹, alveolar epithelial cells (AECs)^{10,11}, and fibroblasts¹², where it may promote fibrogenesis through complex intercellular signaling within the alveolar microenvironment¹³. Although these studies support a pathogenic role for cellular senescence in pulmonary fibrosis, most have focused on individual cell populations in isolation or distinct experimental conditions⁷. Consequently, the cell type-specific contributions of senescent cells to pulmonary fibrosis remain incompletely understood.

We previously demonstrated that p16^{INK4a} (p16), a key regulator of cellular senescence, is markedly upregulated in both AECs and macrophages in the lungs of mice with bleomycin-induced pulmonary fibrosis¹⁴. These findings indicate that senescence in both cell populations may contribute to fibrotic pathogenesis and suggest that AECs and macrophages may exert distinct cell-type-specific effects during fibrosis progression.

We hypothesized that senescence in macrophages and type II AECs differentially contributes to pulmonary fibrosis. To test this hypothesis, we systematically investigated the cell-type-specific roles of these populations using complementary gain and loss-of-function approaches. Specifically, p16-overexpressing cells were used to assess the direct effects of senescence on profibrotic signaling *in vitro*, whereas conditional knockout mice with selective p16 deletion in macrophages or type II AECs were used to compare the contributions of these cell populations to pulmonary fibrosis *in vivo* under identical bleomycin-induced lung injury conditions. Our findings provide insights into the distinct roles of epithelial and myeloid senescence in pulmonary fibrosis.

Methods

Reagents and antibodies

Recombinant human interferon gamma (IFN γ), interleukin-4 (IL-4) and IL-13 were bought from Peprotech (Rocky Hill, NJ, USA). Lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-p16^{INK4a} (80772), anti-p-RB (Ser807/811) (8516) and anti-COL1A1 (72026) antibodies as well as horseradish peroxidase (HRP) anti-rabbit and anti-mouse IgG antibodies were bought from Cell Signaling Technology (Beverly, MA, USA). Anti-GAPDH (M171-3) was bought from MBL LifeScience (Tokyo, Japan). Anti-CD16/32 (101302), anti-CD45 (103102), anti-CD146 (134702) and anti-F4/80 (123102) were bought from BioLegend (San Diego, CA, USA). Bleomycin was bought from Nippon Kayaku (Tokyo, Japan). Medetomidine hydrochloride and atipamezole hydrochloride were purchased from Zenaoq (Fukushima, Japan). Midazolam was purchased from Maruishi Pharmaceutical (Osaka, Japan), and butorphanol was purchased from Meiji Seika Pharma (Tokyo, Japan).

Cells and senescence induction

Human THP-1 and A549 cells were obtained from RIKEN BRC (Tsukuba, Japan). THP-1 cells were cultured in RPMI-1640 (FUJIFILM Wako, Osaka, Japan), and A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (FUJIFILM Wako), both supplemented with 10% (v/v) fetal bovine serum (FBS) (Biofluids, Fleming Island, FL). THP-1 cells were differentiated into macrophage-like cells using 25 nmol/L PMA. Cellular senescence was induced through stable p16^{INK4a} overexpression. Lentiviral vectors were prepared using pLenti CMV p16 Neo (w111-1) (Addgene #22260; a gift from Dr. Eric Campeau) or the corresponding empty vector, and were transduced as previously described¹⁵. Senescence was confirmed by senescence-associated β -galactosidase (SA- β -gal) activity using a Cellular Senescence Kit (OZ Biosciences, Marseille, France) and by reduced phosphorylation of retinoblastoma (RB) protein. THP-1 cells were treated with (1) LPS (10 ng/mL) and IFN γ (50 ng/mL) or (2) IL-4 (10 ng/mL) and IL-13 (10 ng/mL) for 48 h. Total RNA was subsequently isolated using 500 μ L TRIzol Reagent (Invitrogen, Carlsbad, CA, USA).

Animals and the bleomycin-induced lung fibrosis model

p16^{flox/flox}, LysM-Cre and SPC-Cre transgenic mice were kindly provided by Dr. Gloria H. Su (Columbia University, USA), Dr. Satoru Takahashi (University of Tsukuba, Japan) and Dr. Yuichi Oike (Kumamoto University, Japan), respectively¹⁶. Macrophage-specific (M ϕ -cKO: LysM-Cre; p16^{flox/flox}) and type II AEC-specific p16 knockout (AEC-cKO: SPC-Cre; p16^{flox/flox}) mice were generated by crossing p16^{flox/flox} mice with the respective Cre-driver lines. Littermate p16^{flox/flox} mice without Cre expression (F/F) were used as controls. Eight to ten-week-old mice were

intraperitoneally (i.p.) administered 20 mg/kg bleomycin twice a week for 4 weeks to induce pulmonary fibrosis. In a separate model, mice received a single intratracheal (i.t.) instillation under anesthesia. Mice were euthanized 30 days after the initial i.p. injection or 21 days after i.t. instillation, and lung tissues were collected for analysis. For i.t. instillation, mice were anesthetized by i.p. of a three-drug anesthetic mixture consisting of medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg). After i.t. instillation, atipamezole (0.3 mg/kg) was administered by i.p. to reverse the anesthesia. Mice were then maintained on a heating plate at 37°C until recovery from anesthesia. Male mice were used because they exhibit more consistent and severe bleomycin-induced pulmonary fibrosis than female mice, as reported previously¹⁷. Mice were euthanized by intravenous administration of an overdose of thiopental sodium (200 mg/kg) via the tail vein. Blood was collected from the inferior vena cava under deep anesthesia when blood samples were required. In animals for which blood collection was not required, the inferior vena cava was incised under deep anesthesia to induce exsanguination. All animal experiments were approved by the Kawasaki Medical School Animal Research Committee (approval number 21–083; 21–084; 23–087; 23–088 and 24–097) and were conducted in accordance with institutional guidelines and relevant national regulations.

Sample collection and histological analysis

Bronchoalveolar lavage fluid (BALF) was collected by instilling 1 mL PBS twice, and total cell counts, soluble collagen levels (Sircol Collagen Assay Kit; Biocolor Ltd., Carrickfergus, UK), and protein concentrations (BCA Protein Assay Kit; Nacalai Tesque, Kyoto, Japan) were determined. Lung tissues were fixed in 4% paraformaldehyde and stained with Masson's trichrome to evaluate collagen deposition.

Primary AEC isolation and molecular analysis

Primary AECs were isolated as described previously with minor modifications¹⁸. Lungs were enzymatically digested and filtered to obtain a single-cell suspension. Non-epithelial cells were depleted by magnetic cell sorting-based negative selection using antibodies against CD16/32, CD45, CD146, and F4/80, followed by incubation with goat anti-rat IgG antibody-conjugated magnetic beads, and LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany).

RNA and protein expression analysis

Total RNA was extracted and analyzed by quantitative real-time PCR (qPCR) using the primers listed in Table. Protein expression was assessed using western blotting. Gene and protein expression levels were normalized to GAPDH. Growth differentiation factor 15 (GDF-15) levels in plasma and BALF were measured using a Mouse/Rat GDF-15 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

For animal experiments, each data point represents one individual mouse. For cell culture experiments, each biological replicate was generated from an independent cell culture. Data are presented as mean ± SEM. Two-group and multiple-group comparisons were performed using Student's t-test and two-way analysis of variance (ANOVA), followed by Dunnett's or Tukey's tests, respectively. A $P < 0.05$ was considered statistically significant. Survival was assessed using Kaplan–Meier survival curves and the log-rank (Mantel-Cox) test. Statistical analyses were performed using Prism 8 (GraphPad Software, San Diego, CA, USA).

Results

p16-Induced senescence promotes a profibrotic M2-like phenotype of macrophage *in vitro*

A p16-overexpressing THP-1 macrophage model was established to investigate the effects of macrophage senescence on profibrotic polarization (Fig. 1). qPCR and western blot analyses confirmed significant upregulation of p16 mRNA and protein levels (Fig. 1A and B). This was accompanied by decreased phosphorylated retinoblastoma protein (p-RB), a downstream target of p16, indicating functional cell cycle arrest (Fig. 1B). Furthermore, senescence-associated β -galactosidase (SA- β -gal) activity was markedly elevated in p16-overexpressing cells, confirming the induction of cellular senescence (Fig. 1C).

Following stimulation with LPS + IFN- γ or IL-4/IL-13 to induce M1- and M2-associated responses, respectively, p16-overexpressing macrophages exhibited altered polarization-associated gene expression profiles compared with mock cells. Specifically, p16 overexpression reduced the expression of the M1 marker *TNF*, whereas IL-4/IL-13-induced *ARG1* expression was further enhanced in p16-overexpressing cells (Fig. 1D and E), suggesting a shift toward an M2-like profibrotic phenotype. Additionally, expression of the fibrosis-related genes, *TGFB1* and *FN1*, was increased under basal conditions (Fig. 1F and G). *MMP1* expression remained unchanged under basal conditions, but significantly increased after IL-4/IL-13 stimulation in p16-overexpressing cells compared with mock cells (Fig. 1H).

p16 overexpression in alveolar epithelial cells induces cellular senescence and enhances fibrotic marker expression

A549 cells overexpressing p16 were generated as an *in vitro* model of senescent AECs. Similar to THP-1 cells, p16 mRNA and protein levels were markedly increased, accompanied by reduced p-RB levels and enhanced SA- β -gal activity, confirming senescence induction (Fig. 2A–C). Given that epithelial-mesenchymal transition (EMT) contributes to fibrosis¹⁹, EMT- and fibrosis-associated markers were subsequently examined. The expression of the EMT marker *ACTA2* and fibrosis-related genes (*CCN2* and *FN1*) and the inflammatory chemokine *CCL2* was significantly elevated in p16-overexpressing A549 cells (Fig. 2D–G).

Macrophage-specific p16 deletion does not alter bleomycin-induced pulmonary fibrosis

Next, we assessed the functional role of macrophage senescence *in vivo* employing macrophage-specific p16 conditional knockout (M ϕ -cKO: LysM-Cre; p16^{f/f}) mice. Following i.p. bleomycin administration, both control (F/F) and M ϕ -cKO mice exhibited comparable body weight loss (Fig. 3A). Total BALF cell counts, soluble collagen levels, and total protein concentrations were significantly elevated following bleomycin treatment but did not differ between genotypes (Fig. 3B–D). Similar findings were observed in an independent model induced by i.t. bleomycin administration, in which M ϕ -cKO and control mice displayed comparable survival rates and BALF parameters (Supplementary Fig. 1A–D). Histological analysis using hematoxylin and eosin (H&E) and Masson's trichrome staining revealed comparable levels of inflammatory cell infiltration and collagen deposition between F/F and M ϕ -cKO mice (Fig. 3E and F). Efficient p16 deletion was confirmed in BALF cells collected from M ϕ -cKO mice (Supplementary Fig. 2A). However, the M1/M2 marker alterations observed *in vitro* were not recapitulated *in vivo* (Supplementary Fig. 2B and C). Similarly, although whole lung tissue from M ϕ -cKO mice showed significantly lower p16 mRNA levels compared to F/F mice after bleomycin treatment, no detectable differences were observed in the

expression of fibrotic marker genes such as *Fn1* and *Col1a1*, or in that of the inflammatory chemokine *CCL2* (Supplementary Fig. 2D–G).

Type II alveolar epithelial cell-specific p16 deletion attenuates pulmonary fibrosis

Type II AEC-specific p16 conditional knockout (AEC-cKO: SPC-Cre; p16^{f/f}) mice were used to evaluate the contribution of epithelial p16 expression to bleomycin-induced pulmonary fibrosis. Following bleomycin administration, AEC-cKO mice exhibited significantly reduced total cell counts, soluble collagen levels, and total protein concentrations in BALF compared to F/F controls (Fig. 4B–D), despite exhibiting similar weight loss (Fig. 4A). These protective effects were further validated in a separate i.t. bleomycin instillation model, in which AEC-cKO mice exhibited significantly improved survival rates and attenuated BALF parameters compared with control mice (Supplementary Fig. 1E–H). Histological analysis via H&E and Masson's trichrome staining revealed significantly diminished inflammatory cell infiltration and reduced collagen deposition in the lungs of AEC-cKO mice (Fig. 4E and F). The expression of fibrosis-related genes was subsequently assessed in whole-lung tissue. *Acta2* was significantly upregulated in F/F mice following i.p. bleomycin administration, whereas only a trend toward increased expression was observed in AEC-cKO mice (Fig. 5A). *Col1a1* expression was also significantly induced by bleomycin treatment, and this increase was attenuated in AEC-cKO mice (Fig. 5B). *Fn1* and *Ccn2* expression levels did not differ significantly between genotypes in bleomycin-treated lungs (Fig. 5C and D). Western blot analysis further confirmed that COL1A1 protein expression was significantly lower in bleomycin-treated AEC-cKO mice compared with F/F controls (Fig. 5E and F).

Deletion of p16 in primary alveolar epithelial cells attenuate profibrotic gene induction

Primary AECs isolated from mice were analyzed to assess the effects of p16 deletion on fibrosis-related gene expression. Bleomycin administration significantly increased *Acta2* mRNA expression in AECs from F/F mice, whereas no significant increase was observed in AECs from AEC-cKO mice (Fig. 6A). Furthermore, the expression of key profibrotic markers, *Col1a1*, *Fn1*, and *Ccn2*, was significantly reduced or showed a clear trend toward reduction in bleomycin-treated AEC-cKO-derived cells compared with that in those from bleomycin-treated F/F mice (Fig. 6B–D).

AEC-specific p16 knockout reduces growth differentiation factor 15 (GDF15) expression during fibrosis

Growth differentiation factor 15 (GDF15) has been implicated in pulmonary fibrosis, and senescent AECs have been proposed as a potential source of GDF15¹⁴. Therefore, GDF15 expression was assessed in the bleomycin-induced fibrosis model to determine whether p16 deletion in AECs affects GDF15 production during fibrosis. Plasma GDF15 levels did not significantly differ between genotypes (Fig. 7A); however, BALF GDF15 concentrations were significantly lower in AEC-cKO mice than those in F/F control mice (Fig. 7B). Consistently, *Gdf15* mRNA expression in isolated type II AECs was also reduced in AEC-cKO mice (Fig. 7C). Previous studies demonstrated that GDF15 neutralization attenuates bleomycin-induced pulmonary fibrosis, accompanied by reduced *Col3a1* expression²⁰. Consistent with this observation, *Col3a1* expression was also significantly decreased in AEC-cKO mice (Fig. 7D).

Discussion

In the present study, we systematically investigated the contribution of senescent type II AECs and macrophages to pulmonary fibrosis using both *in vitro* and *in vivo* approaches. Our findings highlight a pivotal role for senescent AECs in driving fibrosis progression. While *in vitro* experiments demonstrated that p16-induced senescence in both macrophages and epithelial cells enhanced fibrogenic gene expression, only type II AEC-specific p16 deletion in mice significantly attenuated bleomycin-induced fibrosis. These findings suggest that although p16-induced senescence promotes profibrotic responses in both cell types *in vitro*, senescence in type II AEC has a more prominent role in promoting fibrosis progression *in vivo*, whereas the contribution of macrophage senescence appears to be limited in this model.

p16 was used as a key molecular tool to manipulate senescent cells. p16 is a key regulator of cellular senescence that inhibits cyclin-dependent kinases 4 and 6 (CDK4/6), thereby maintaining RB protein in a dephosphorylated state and preventing G1-to-S phase cell-cycle progression²¹. This activity underlies the establishment of a stable growth arrest and the acquisition of the SASP, characterized by the release of profibrotic mediators, including Transforming Growth Factor- β (TGF- β), IL-6, and GDF15^{22,23}. Consistent with our previous findings that p16-positive cells accumulate in fibrotic lungs¹⁴, we demonstrated here that p16 overexpression in both THP-1 macrophages and A549 cells led to reduced p-RB levels and enhanced SA- β -gal activity. Together, these findings support the use of p16 as both a reliable marker and a useful genetic tool for investigating senescence-associated phenotypes.

The role of macrophages in fibrosis is complex and is frequently discussed in terms of proinflammatory (M1) or profibrotic/anti-inflammatory (M2) phenotypes²⁴. While our *in vitro* data showed that p16-induced senescence in THP-1 cells promoted M2-associated markers, our *in vivo* study using macrophage-specific p16 conditional knockout (M ϕ -cKO) mice revealed no significant impact on fibrosis severity. Despite efficient p16 deletion in BALF cells, the expression of M1/M2 markers such as *Tnf* and *Arg1* remained unchanged *in vivo*. Although some reports suggest that inhibiting macrophage senescence can alleviate fibrosis in specific contexts^{8,25}, our findings indicate that in the bleomycin-induced lung fibrosis model, macrophage senescence is unlikely to be the major determinant of fibrotic progression. This limited *in vivo* effect may reflect the highly plastic nature of macrophages and the influence of multiple microenvironmental cues within the injured lung. These findings suggest that the contribution of macrophage senescence to fibrosis may be context- and model-dependent, rather than universally pathogenic.

Conversely, our results demonstrate that senescence in type II AECs plays a central role in pulmonary fibrosis. Type II AECs are essential for lung homeostasis and repair, and their senescence contributes to the severity of lung injury and impaired regeneration^{26,27}. In p16-overexpressing A549 cells, we observed increased expression of the EMT-related and fibrotic genes, *ACTA2*, *CCN2*, and *FN1*. Notably, single-cell transcriptomic studies have identified senescent AECs as a major source of fibrogenic SASP factors in patients with IPF²⁸. Furthermore, recent single-cell transcriptomic analyses of human lung aging have demonstrated increased transcriptional dysregulation and cellular heterogeneity in epithelial populations, including type II AECs, suggesting that aging-associated epithelial dysfunction may predispose these cells to senescence and enhanced SASP production²⁹. Consistent with these observations, selective deletion of p16 in type II AECs attenuated bleomycin-induced fibrosis, supporting a key contribution of epithelial senescence to fibrotic progression and highlighting its potential as a therapeutic target.

GDF15 is a stress-responsive cytokine induced by aging, mitochondrial dysfunction, inflammation, tissue injury, and cellular senescence³⁰. Recent studies have identified GDF15 as a component of the SASP that regulates tissue microenvironments and fibroblast activation²³. Previous studies, including our own, demonstrated that GDF15

promotes extracellular matrix production and pulmonary fibrosis^{14,20,31}. Consistent with these findings, we observed reduced BALF GDF15 levels and decreased *Gdf15* mRNA expression in isolated AEC from AEC-cKO-Bleo mice, accompanied by lower Col1a1 and Col3a1 expression. These findings suggest that GDF15 may mediate, at least in part, the profibrotic effects of senescent AECs during pulmonary fibrosis.

While senolytic drugs, such as dasatinib and quercetin, show promise, they lack cell-type specificity and may cause off-target effects by eliminating senescent cells that play beneficial roles in tissue repair⁶. Our findings suggest that targeting senescent AEC-derived mediators, such as GDF15, may represent a more targeted and therapeutic strategy for pulmonary fibrosis, while potentially avoiding some limitations associated with global senescent cell elimination.

This study has several limitations. Although p16 is a widely used marker and regulator of cellular senescence, senescence is controlled by multiple interconnected pathways, including the p53-p21 axis, persistent DNA damage responses, epigenetic remodeling, and inflammatory SASP signaling. Therefore, modulation of p16 alone may not fully suppress senescence-associated profibrotic programs. In addition, A549 cells were used as a surrogate model of type II AECs and may not fully recapitulate the biology of primary alveolar epithelial cells. While GDF15 expression was reduced in AEC-cKO mice, the present study does not establish a direct causal role for GDF15 in mediating the antifibrotic effects of p16 deletion. Furthermore, other SASP factors may also contribute to the development and progression of pulmonary fibrosis. Finally, the relevance of epithelial senescence and GDF15 signaling to human IPF requires further investigation. Nevertheless, our findings suggest that the pathogenic impact of senescence in pulmonary fibrosis depends not only on the presence of senescent cells but also on their cellular origin. Our findings demonstrate that p16-induced senescence in type II AECs plays a central role in the progression of bleomycin-induced pulmonary fibrosis. The reduced GDF15 expression observed following AEC-specific p16 deletion suggests that AEC senescence and its downstream mediators may represent promising therapeutic targets for IPF.

Declarations

Competing interests

The authors have no competing interests declare.

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Author Contribution

Conceptualization, Y.T. and Y.O.; Methodology, Y.T., K.Kitakaze and Y.O.; Investigation, Y.T., R.M., Y.N., K.M., K.U., S.H., K.Kai, A.S. and R.S.; Resources, K.Kitakaze; Formal analysis, Y.T.; Data curation, Y.T.; Writing—original draft, Y.T. and Y.O.; Writing—review and editing, Y.T., K.Kitakaze, K.T., Y.I., Y.S. and Y.O.; Visualization, Y.T.; Supervision, Y.O.; Project

administration, Y.T. and Y.O.; Funding acquisition, Y.T. and Y.O. All authors reviewed and approved the manuscript for publication.

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Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Table - Primers used for qPCR

Species	Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
Human	ACTA2	GTGTTGCCCCTGAAGAGCAT	GCTGGGACATTGAAAGTCTCA	109
	ARG1	TGGACAGACTAGGAATTGGCA	CCAGTCCGTCAACATCAAACT	102
	CCL2	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT	190
	CCN2	ACCGACTGGAAGACACGTTTG	CCAGGTCAGCTTCGCAAGG	195
	CDKN2A	GTGGACCTGGCTGAGGAG	CTTTCAATCGGGGATGTCTG	132
	FN1	CGGTGGCTGTCAGTCAAAG	AAACCTCGGCTTCCTCCATAA	130
	MMP1	AAAATTACACGCCAGATTTGCC	GGTGTGACATTACTCCAGAGTTG	82
	TGFB1	GGCCAGATCCTGTCCAAGC	GTGGGTTTCCACCATTAGCAC	201
	TNF	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG	220
Mouse	Acta2	GACGCTGAAGTATCCGATAGAACACG	CACCATCTCCAGAGTCCAGCACAAT	281
	Arg1	CTCCAAGCCAAAGTCCTTAGAG	GGAGCTGTCATTAGGGACATCA	184
	Ccl2	TAAAAACCTGGATCGGAACCAAA	GCATTAGCTTCAGATTTACGGGT	120
	Ccn2	GGGCCTCTTCTGCGATTTC	ATCCAGGCAAGTGCATTGGTA	151
	Cdkn2a	AACTCTTTCGGTCGTACCCC	TCCTCGCAGTTCGAATCTG	98
	Col1a1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG	103
	Col3a1	GCCTTCTACACCTGCTCCTG	CCAGGGTCACCATTCTCCC	102
	Fn1	TCTGGGAAATGGAAAAGGGGAATGG	CACTGAAGCAGGTTTCCTCGGTTGT	267
	Gdf15	GAGCTACGGGGTCGCTTC	GGGACCCCAATCTCACCT	130
	Tnf	CCTGTAGCCACGTCGTAG	GGGAGTAGACAAGGTACAACCC	148

Figures

Figure 1

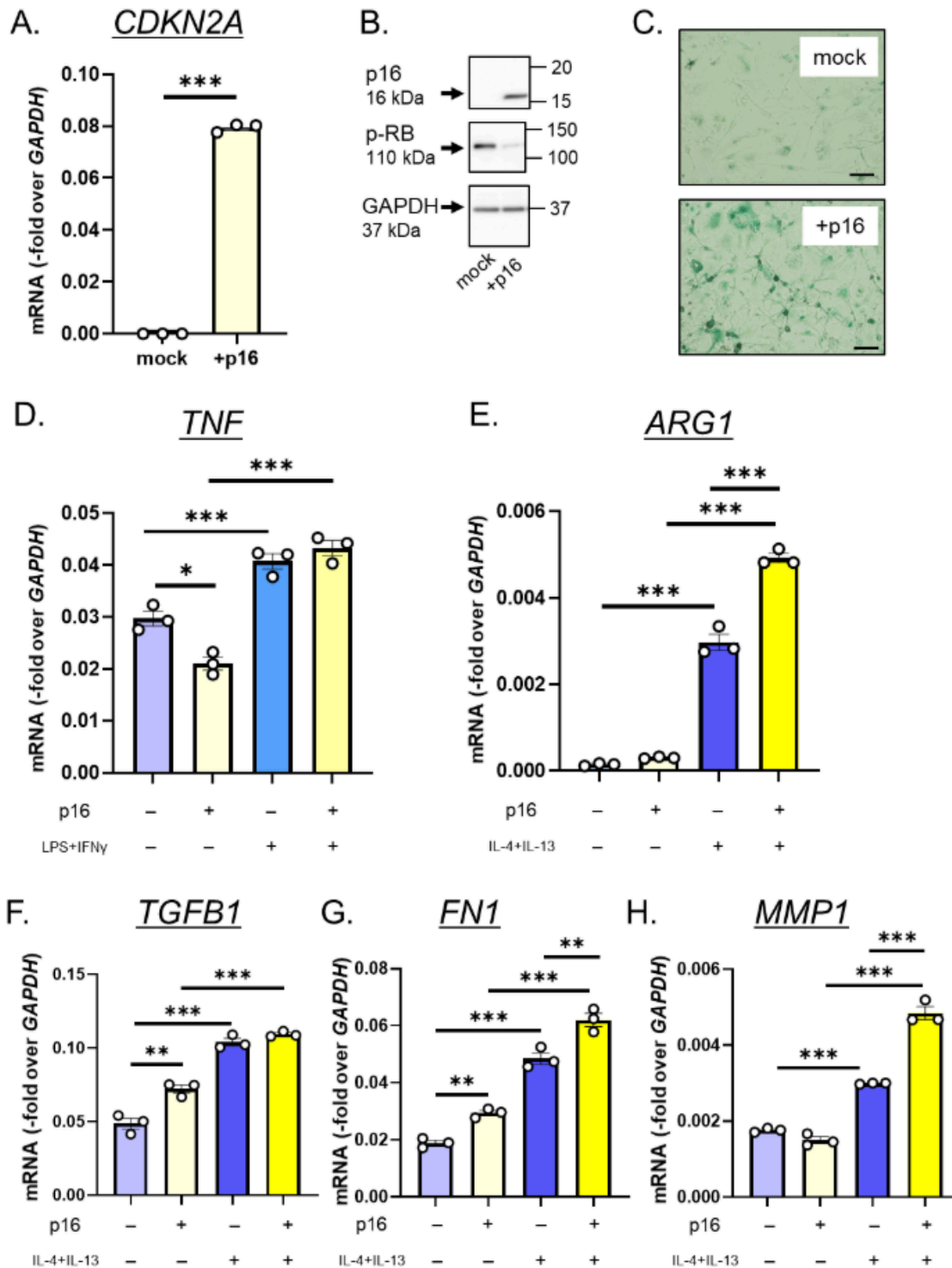


Figure 1

p16 overexpression modulates macrophage polarization and fibrotic gene expression

(A) mRNA expression levels of *CDKN2A* (p16). (B) Representative immunoblot showing p16 and p-RB expression in control (mock) and p16-overexpressing cells (+p16). *GAPDH* was used as a loading control. (C) Representative images of SA- β -gal staining (green). Scale bars, 100 μ m. (D–H) mRNA expression levels of *TNF*, *ARG1*, *TGFB1*, *FN1*,

and *MMP1* under the indicated stimulation conditions. Data are normalized to *GAPDH* and shown as mean $\pm$ SEM (n = 3). Statistical significance was determined by appropriate tests (*p < 0.05, **p < 0.01, ***p < 0.001).

Figure 2

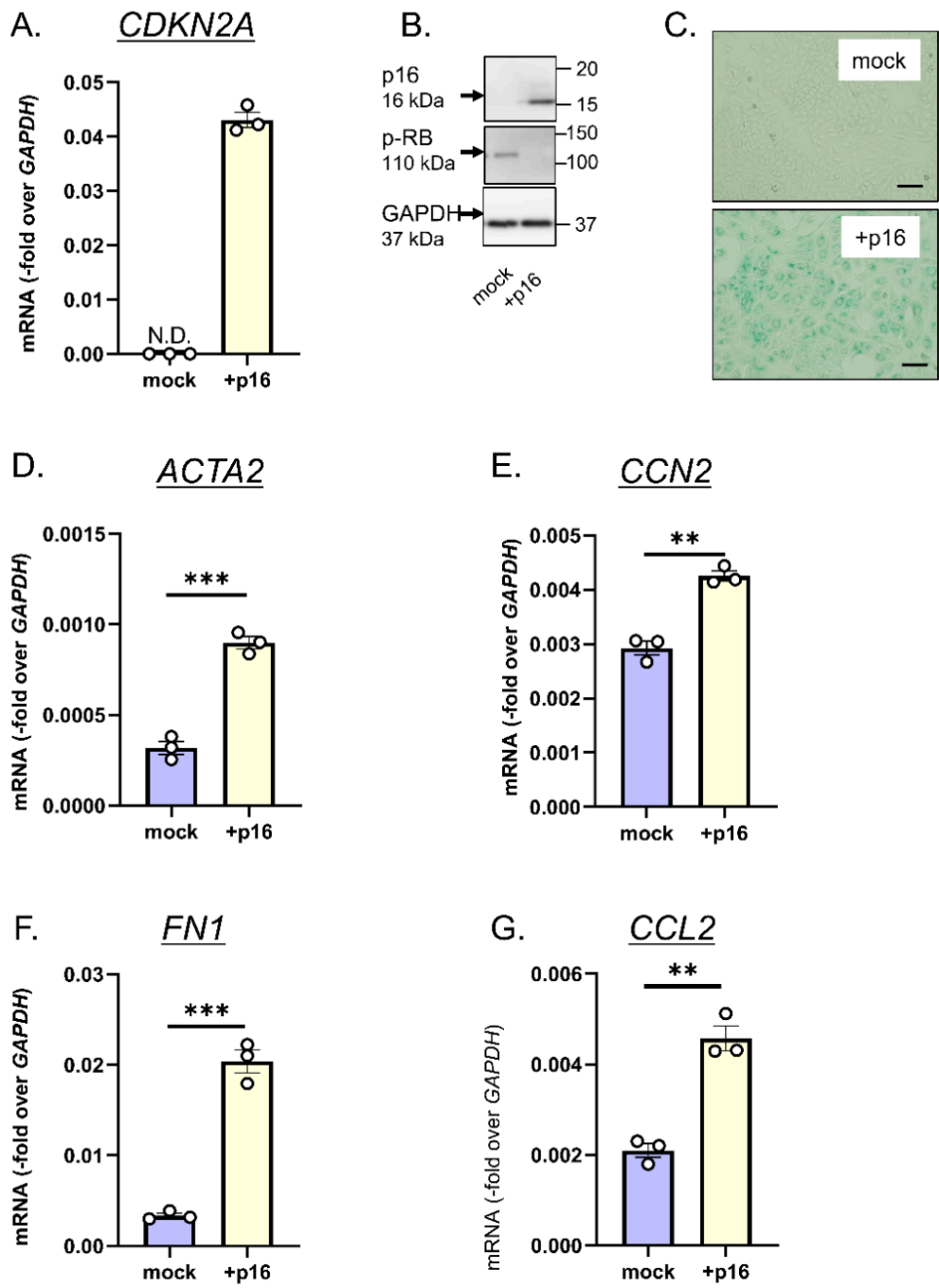


Figure 2

p16 induces fibrotic and inflammatory gene expression in A549 cells

(A) *CDKN2A* expression confirming p16 overexpression. (B) Representative immunoblot showing p16 and p-RB expression in mock and p16-overexpressing cells (+p16). *GAPDH* was used as a loading control. (C) Representative images of SA- β -gal activity (green). Scale bars, 100 μ m. (D–G) Relative mRNA expression of fibrotic markers (*ACTA2*, *CCN2*, and *FN1*) and inflammatory chemokine *CCL2* in mock and p16-overexpressing cells. Data are normalized to *GAPDH* and presented as mean $\pm$ SEM (n = 3). N.D., not detected.

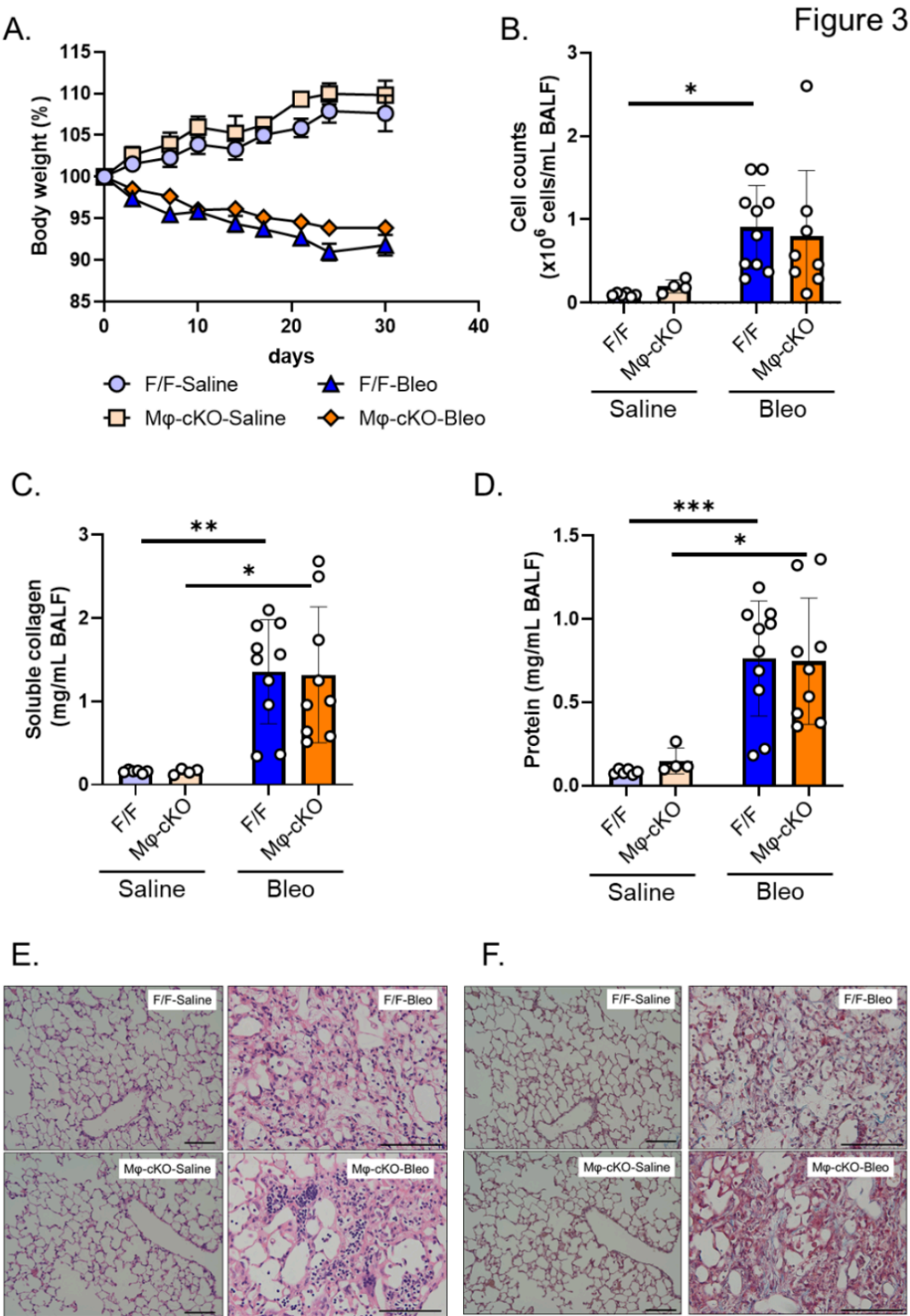


Figure 3

Macrophage-specific deletion does not affect bleomycin-induced lung injury

(A) Body weight changes over time following i.p. administration of saline or bleomycin (Bleo) in control (F/F) and macrophage-specific knockout (M ϕ -cKO) mice. (B) Total cell counts in BALF. (C) Soluble collagen levels in BALF. (D) Total protein concentration in BALF. Data are shown as mean $\pm$ SEM (n = 4–10). (E, F) Representative images of H&E (E) and Masson's trichrome (F) staining of lung tissues (scale bars, 100 μ m).

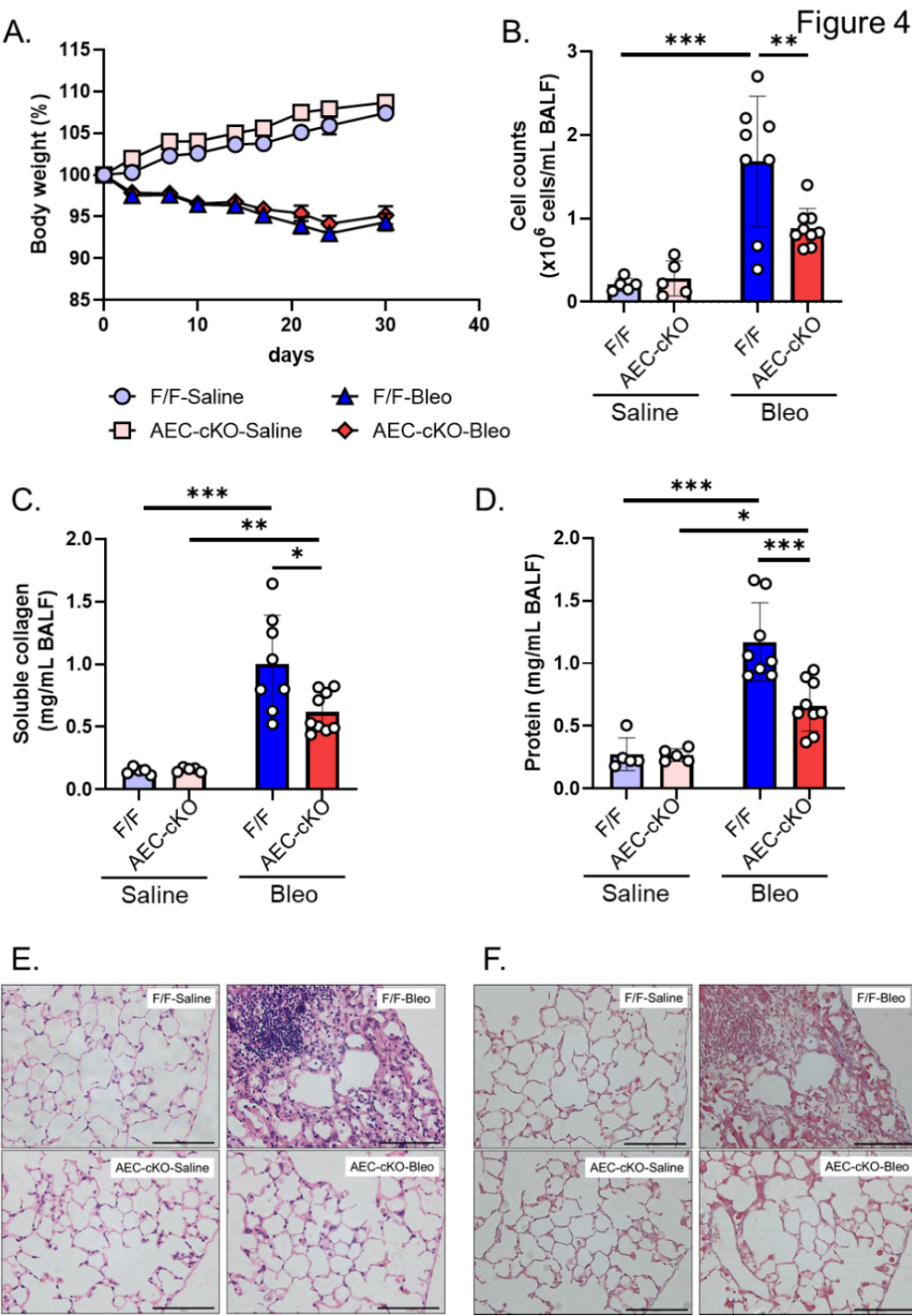


Figure 4

Epithelial cell-specific deletion attenuates lung injury responses

(A) Body weight changes over time following i.p. administration of saline or bleomycin (Bleo) in control (F/F) and type II alveolar epithelial cell-specific knockout (AEC-cKO) mice. (B) Total cell counts in BALF. (C) Soluble collagen levels in BALF. (D) Total protein concentration in BALF. Data are shown as mean $\pm$ SEM (n = 5–9). Statistical significance was determined by appropriate tests (*p < 0.05, **p < 0.01, ***p < 0.001). (E, F) Representative images of H&E (E) and Masson's trichrome (F) staining of lung tissues (scale bars, 100 μ m).

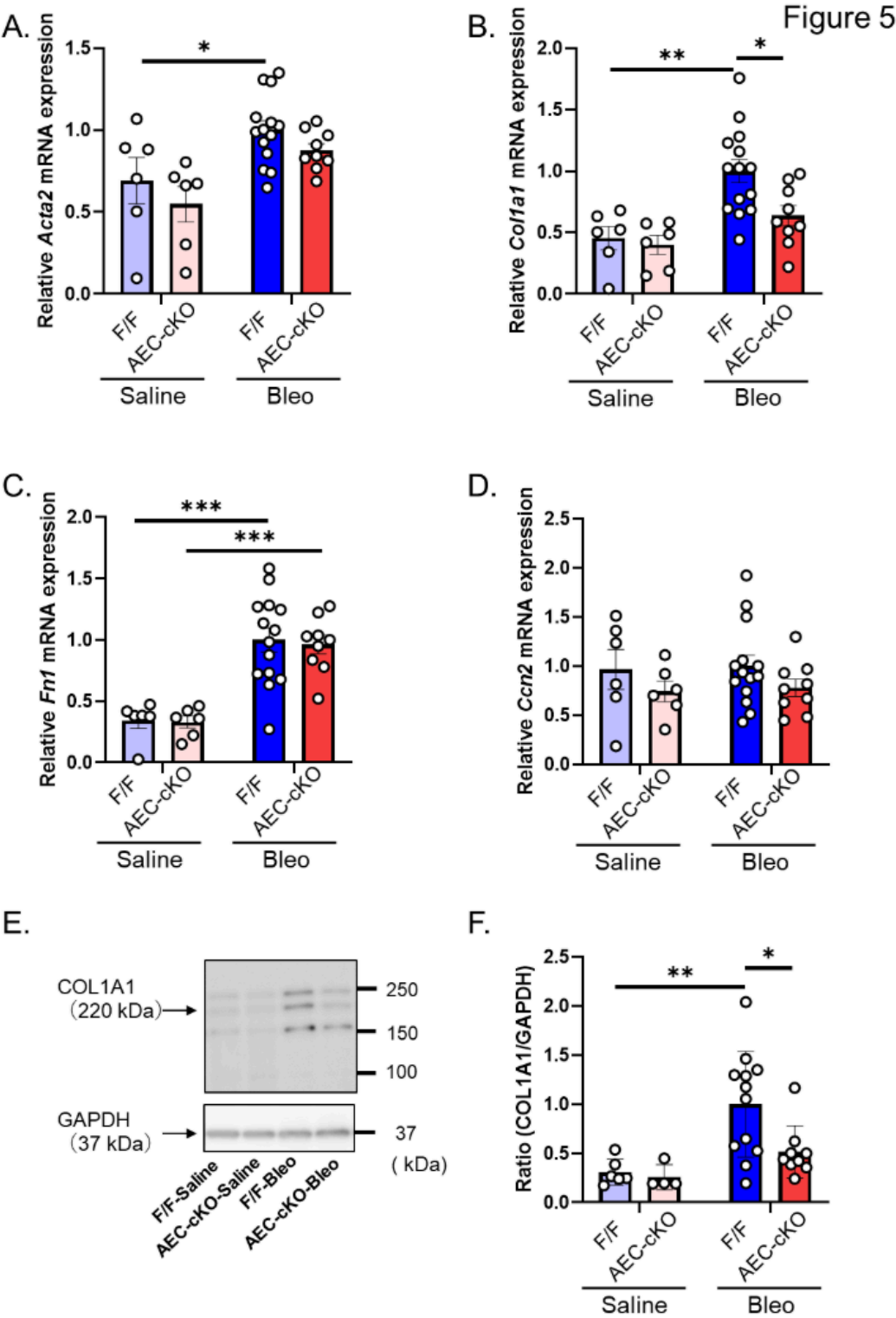


Figure 5

mRNA and protein expression of fibrotic markers in whole lung tissue from mice

(A–D) Relative mRNA expression of fibrotic markers (*Acta2*, *Col1a1*, *Fn1*, and *Ccn2*). mRNA levels were normalized to *Gapdh* and are presented as the ratio to the F/F-Bleo group. (E) Representative immunoblot of COL1A1 expression. (F) Quantification of COL1A1 protein normalized to GAPDH. Data are presented as mean $\pm$ SEM (n = 4–14). Statistical significance was determined by appropriate tests (*p < 0.05, **p < 0.01, ***p < 0.001).

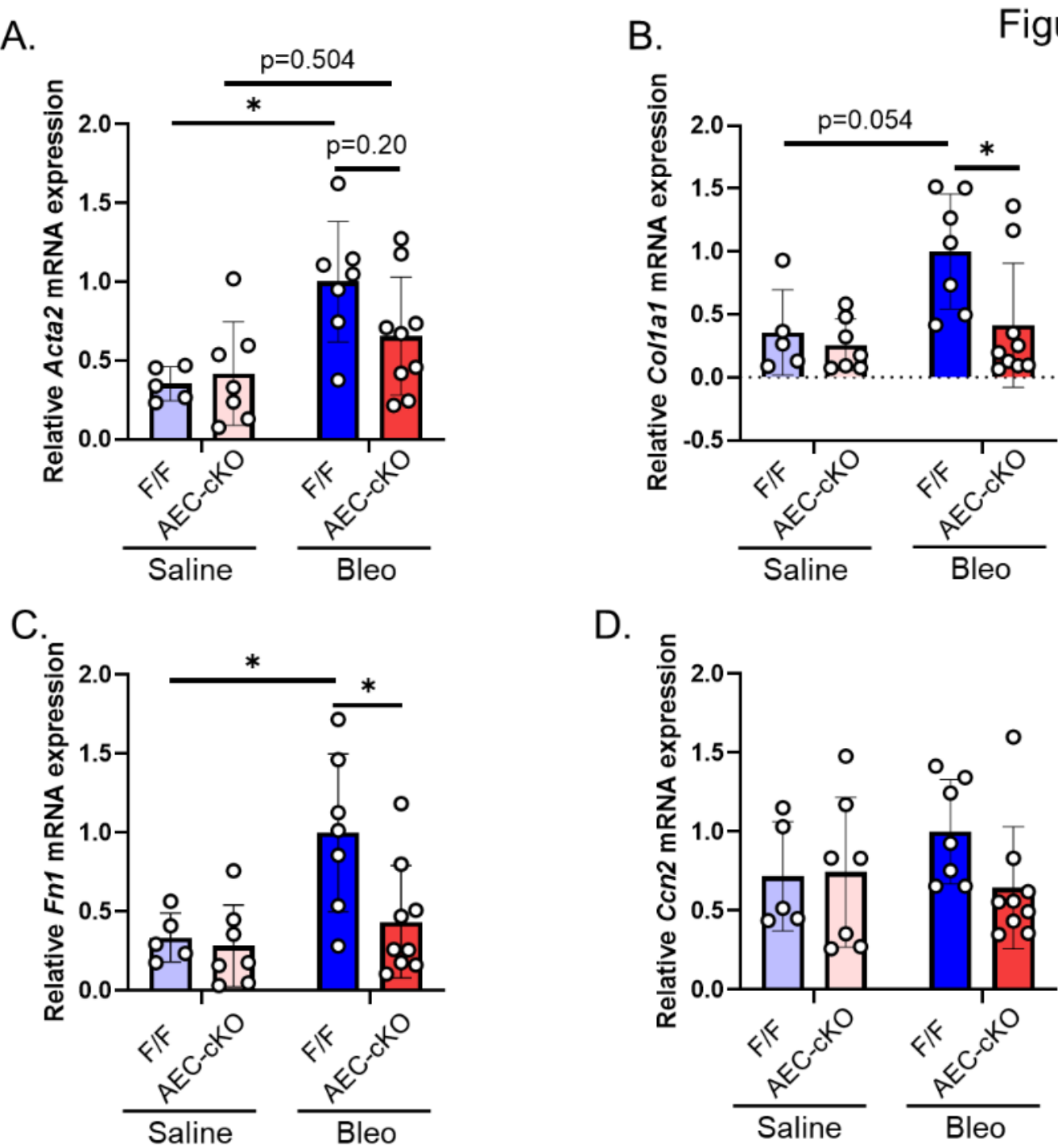


Figure 6

Figure 6

mRNA expression of fibrotic markers in isolated alveolar epithelial cells from mice

(A–D) Relative mRNA expression of *Acta2*, *Col1a1*, *Fn1*, and *Ccn2*. mRNA levels were normalized to *Gapdh* and are presented as the ratio to the F/F-Bleo group. Some comparisons did not reach statistical significance (p values indicated). Data are shown as mean $\pm$ SEM.

Figure 7

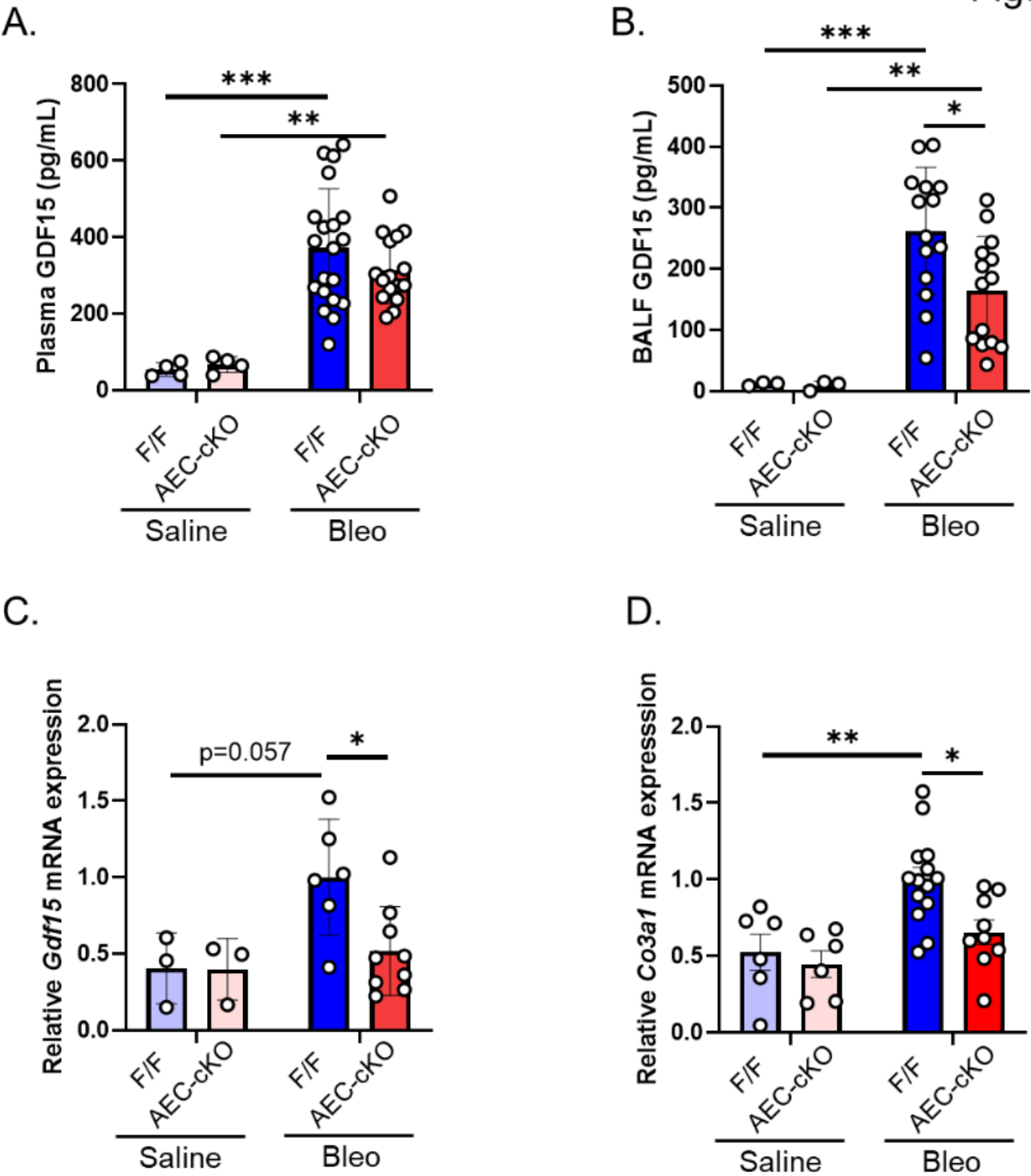


Figure 7

GDF15 is associated with fibrotic response

(A, B) GDF15 protein concentrations in plasma (A) and BALF (B) from saline- and bleomycin-treated mice. (C) Relative *Gdf15* mRNA expression in isolated AECs. (D) Relative *Col3a1* mRNA expression in whole lung tissues. mRNA levels were normalized to *Gapdh* and are presented as the ratio to the F/F-Bleo group. Data are presented as mean $\pm$ SEM (n = 3–20). Statistical significance was determined by appropriate tests (*p < 0.05, **p < 0.01, ***p < 0.001).

Supplementary Files

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