

APPLIED SCIENCES AND ENGINEERING

Inhalable myofibroblast targeting nanoparticles for synergistic treatment of pulmonary fibrosis

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Pulmonary fibrosis (PF) is a life-threatening interstitial lung disease, characterized by excessive fibroblast activation and collagen deposition, leading to progressive pulmonary function decline and limited therapeutic efficacy. Here, the inhalable, myofibroblast-targeted, and pH-responsive liposomes (FL-NI) were developed for effective codelivery of nintedanib, a mainstream antifibrotic drug in clinic, and siIL11, a small interfering RNA that silences the key profibrosis cytokine IL-11. Notably, FL-NI achieved a 117.8% increase in pulmonary drug delivery by non-invasive inhalation and a 71.5% increase in delivery specifically to fibroblast activation protein-positive myofibroblasts while reducing nonspecific immune cell and epithelial uptake by 29.8 and 55.8%, respectively. The accurate inhalation codelivery of nintedanib and siIL11 into myofibroblasts achieved synergistic effects, effectively enhanced myofibroblast deactivation, reduced pathological collagen deposition by 50.8%, and promoted epithelial tissue repair. FL-NI remodeled the aberrant immune microenvironment without inducing systemic toxicities. Therefore, this work demonstrated the notable potential for this pluripotent strategy for improving PF outcomes and its promising clinical translation.

INTRODUCTION

Pulmonary fibrosis (PF) is a chronic interstitial lung disease characterized by respiratory challenges and progressive deterioration of lung function, often culminating in an extremely poor prognosis (1). The median survival rate for untreated patients' postdiagnosis ranges between 3 and 5 years (2). Although the precise etiology of PF remains unclear, a range of occupational and external factors, including tobacco smoking (3), severe infections (4), genetic predisposition (5), and environmental exposures (6), has been implicated in its pathogenesis. In addition, PF has emerged as a substantial complication of the COVID-19 pandemic, as evidenced by multiple clinical reports (7). Nearly all patients with severe COVID-19 exhibit some degree of PF, with a notable incidence rate (8). In light of the morbidity and mortality, PF has become a respiratory disease that poses a serious threat to human health.

Now, various inhibitors including nintedanib (NIN), a tyrosine kinase inhibitor (9), and pirfenitone, an inhibitor of various cytokines such as tumor necrosis factor- α (TNF- α), transforming growth factor- β (TGF- β), and platelet-derived growth factor- β (PDGF- β) have been widely used for PF treatment in the clinic (10). Although these have shown some efficiency in alleviating the physiological progression of PF and recovering the pulmonary function, their oral administration often results in gastrointestinal and liver adverse reactions (11, 12). Moreover, the lack of organ and cell specificity of these drugs significantly limits their therapeutic effectiveness

(12–14). Immunomodulatory treatments, such as injections of N-acetyl-L-cysteine and azathioprine, along with corticosteroids, exhibit unsatisfactory efficiency for PF treatment in the clinic (13, 15). Therefore, developing effective therapeutic strategies for PF is of utmost importance.

In the pathological process of PF, damage to alveolar epithelial type II cells initiates pathological epithelial remodeling (16), leading to the recruitment and activation of fibroblasts and abnormal deposition of extracellular matrix (ECM), ultimately resulting in the formation of fibrotic scars and the destruction of lung tissue structure (17). Given the pivotal role of fibroblasts in the progression of PF, inhibiting fibroblast activation and promoting epithelial cell repair are considered as promising therapeutic strategies. Interleukin-11 (IL-11) has been identified as a factor in this process, as it is specifically secreted by fibroblasts (18). In patients with PF, IL-11 levels are elevated in the lungs and fibroblasts, and IL-11 will bind to its heterodimeric receptor subunit IL-11 receptor subunit alpha (IL-11RA) and glycoprotein 130 (gp130) (19), leading to fibroblast activation and increased ECM production by regulating the extracellular signal-regulated kinase (ERK) and AKT signaling pathways (20). In addition, IL-11 can enhance the formation of a profibrotic immune microenvironment through paracrine signaling (21). Therefore, silencing IL-11 expression represents a potential therapeutic strategy for PF.

Small interfering RNA (siRNA) therapies have emerged as a safe and powerful therapeutic approach in various clinical fields, and exogenously synthesized IL-11 siRNA (siIL11) has the ability to silence genes expression in a sequence-specific manner (22, 23). However, because of its susceptibility to degradation, the efficacy of siRNA-mediated therapy highly depends on optimized delivery vectors and appropriate administration routes (24). Liposomes are considered as a mature nonviral nanocarrier for gene therapy, demonstrating significant advancements in clinical application, such as patisiran, a lipid-based siRNA delivery vector successfully developed and approved by Food and Drug Administration (25). To enhance the efficiency of

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drug delivery to the lungs, noninvasive inhalation of nanoparticles allows for preferential accumulation and prolonged residence in the pulmonary environment, making it an ideal strategy for the treatment of PF (26, 27). However, the lung microenvironment consists of various cell types, and nanocarriers are often nonspecifically engulfed by epithelial cells, endothelial cells, immune cells (27, 28), which seriously hinders the antifibrotic effects of inhaled drugs. Therefore, targeting activated fibroblasts for drug delivery could notably improve both the efficiency of drug transport and the therapeutic efficacy against PF.

Recent research has demonstrated that fibroblast surface markers can serve as effective targets for the specific recognition and uptake of nanoparticles by fibroblasts (29, 30), offering a promising approach for precise cell-targeted therapy. Fibroblast activation protein (FAP) is a transmembrane protein specifically expressed in activated fibroblasts and serves as an important biomarker for the diagnosis and treatment of PF (31). Inspired by this, we developed a novel class of liposomes containing FAP-targeted peptides and siIL11 and NIN for noninvasive inhalation therapy. The FAP-targeting peptide enables specific binding to the FAP on activated fibroblasts, thus directing the liposomes with selective targeting ability. Moreover, the polymer poly(2-ethyl-2-oxazoline) (PEOz) was incorporated to facilitate the degradation of liposomes in the acidic conditions of the endosome (32), enhancing their ability to escape endosomal compartments. Once delivered into activated fibroblasts, NIN effectively inhibits signaling pathways mediated by platelet-derived growth factor receptor (PDGFR)/fibroblast growth factor receptor (FGFR), while siIL11 reduces the expression of IL-11. This down-regulation attenuates the IL-11RA/gp130 signaling pathway and decreases the activation of ERK and AKT (Fig. 1). Subsequent studies revealed that intratracheal delivery of NIN and siIL11 coloaded liposomes significantly promoted myofibroblast deactivation, reduced ECM deposition 50.8%, and improved the Ashcroft score from 7.6 ± 0.55 to 2.8 ± 0.45 in a mouse model of PF. Furthermore, the reduction in IL-11 secretion diminishes the profibrotic immune response mediated by its paracrine effects. Thus, the noninvasive inhalable FL-NI presents a promising candidate for clinical translation in the treatment of PF, owing to its remarkable ability to inhibit fibrosis, alleviate chronic inflammation, and remodel the immune microenvironment.

RESULTS

Synthesis and characterization of FL-NI

To synthesize efficient nanoparticles with selective delivery and endosomal escape, blank cationic liposomes were first prepared using the thin-film dispersion technique (33). Given the specific expression of FAP in activated fibroblasts and the pH-sensitive PEOz, we synthesized the FAP-targeting peptide (DRGETGPAC) (fig. S1) and 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE)-PEOz-maleimide (MAL) to create DSPE-PEOz-FAP for liposome modification. The conjugation of FAP-targeting peptide to DSPE-PEOz-FAP was achieved through thiol-MAL chemistry and confirmed by the ^1H nuclear magnetic resonance spectrum (fig. S2) and high-performance liquid chromatography (fig. S3). Next, liposomes were modified with different molar ratios of FAP-targeting peptide (peptides/liposomes) to determine the optimal ratio for efficient siRNA delivery. Specifically, activated mouse embryonic fibroblasts (MEFs) were cocultured with cationic liposomes with different peptide ratios and fluorescein amidite (FAM)-labeled siRNA (FAM-siRNA), followed by

flow cytometry. It was observed that liposomes with peptide ratio above 20% exhibited efficient delivery to MEFs (Fig. 2, A and B). Therefore, 20% FAP-targeting peptides were selected for the synthesis of FL-NI.

The cationic liposomes containing NIN and siIL11 (FL-NI) were subsequently prepared by mixing cationic liposomes [composed of cholesterol, DOTAP (1,2-dioleoyl-3-trimethylammonium-propane), and NIN] with a hydrophilic siRNA core consisting of hyaluronic acid, siIL11, and protamine. The mixture was then fused with the DSPE-PEOz-FAP at a high temperature of 50°C using the postinsertion method. Next, we successfully synthesized L-N (loaded with NIN), L-I (loaded with siIL11), L-NI (loaded with both NIN and siIL11), and FL-NI (loaded with both NIN and siIL11 and surface-modified with FAP-targeting peptide). Dynamic light scattering (DLS) measurements indicated that the particle sizes for L-N, L-I, L-NI, and FL-NI were 181.3 ± 2.1 nm, 175.1 ± 2.0 nm, 176.6 ± 2.3 nm, and 182.0 ± 2.2 nm, respectively (Fig. 2C). The zeta potentials for these formulations were 39.4 ± 0.3 , 38.7 ± 2.8 , 37.8 ± 1.5 , and 29.2 ± 2.1 mV, respectively (Fig. 2D). Ultraviolet-visible absorption spectra of the dual drugs (NIN and siIL11), blank liposomes, and drug-loaded liposomes (L-NI and FL-NI) displayed characteristic absorption peaks at 388 nm (NIN) and 260 nm (siIL11), confirming the encapsulation of both drugs within the liposomes (Fig. 2E). The encapsulation efficiencies of NIN and siIL11 in FL-NI were determined to be $92.1 \pm 0.2\%$ and $92.9 \pm 1.0\%$, respectively (Fig. 2F and fig. S4).

The polydisperse index (Fig. 2G) and transmission electron microscopy (Fig. 2H) of FL-NI demonstrated a concentrated distribution and uniform spherical shape, both of which are favorable for stability (34). The stability of FL-NI in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS, Cellmax, SA112.02, Beijing) was assessed over 7 days. Daily measurements confirmed excellent particle size stability (Fig. 2I). In addition, DLS results revealed nearly no changes in particle size of FL-NI after incubation in DMEM (C3103-0500, VivaCell, Shanghai, China) containing 10% FBS at both pH 7.4 and 6.8 for 24 hours, indicating that FL-NI maintains its structural integrity under mildly acidic conditions, typical of extracellular pH in PF lung tissue (Fig. 2J). Meanwhile, FL-NI was incubated in bronchoalveolar lavage fluid collected from mice with PF for 7 days, showing minimal changes in particle size and demonstrating excellent stability within the PF microenvironment (fig. S5). To assess the protective effect of FL-NI on siRNA, naked siRNA and siRNA encapsulated in FL-NI were incubated with ribonuclease (RNase) for various durations (0, 15, 30, 60, 120, and 240 min). While naked siRNA rapidly degraded upon exposure to RNase for up to 4 hours, FL-NI-encapsulated siRNA remained intact, demonstrating notable protection (Fig. 2K).

Targeted delivery of FL-NI

Given that FAP serves as a specific recognition target for activated fibroblasts (30), we further investigated the enhanced delivery capacity of liposomes mediated by FAP-targeting peptide to activated MEFs. We synthesized liposomes encapsulating FAM-siRNA, creating two variants: FL-siRNA, which contains FAP-targeting peptide, and L-siRNA, which does not. Normal and activated MEFs were respectively treated with L-siRNA, FL-siRNA, or FL-siRNA plus treatment of anti-FAP antibodies with equivalent concentration of siRNA. After 2 hours of cocultivation, flow cytometry was used to quantify the percentage of FAM-positive MEFs (Fig. 3A). The

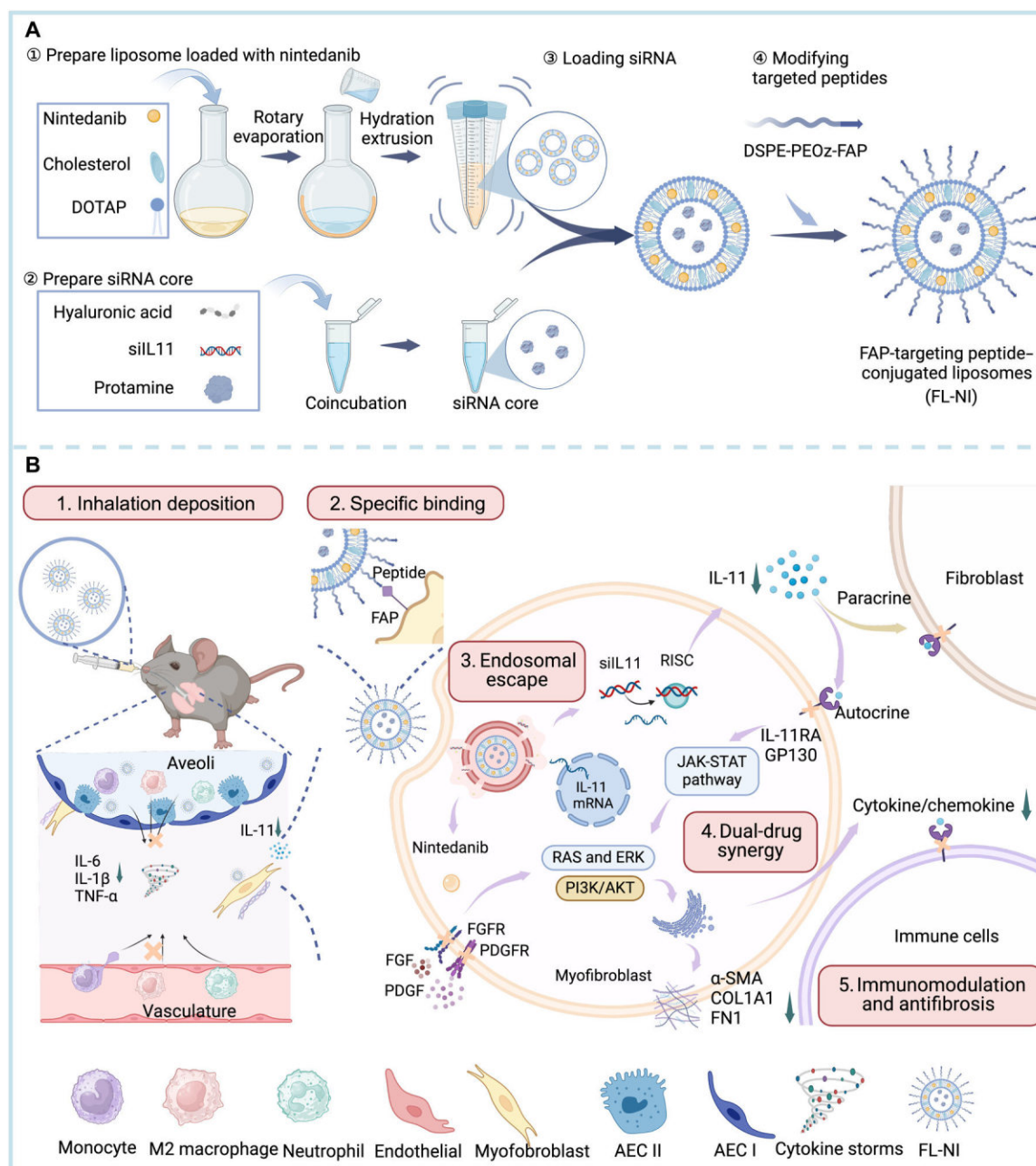


Fig. 1. Inhalation of activated fibroblast-targeted liposomes (FL-NI) for precise codelivery of NIN and siIL11 in PF treatment. (A) Components and construction of the FL-NI. **(B)** Proposed mechanism of FL-NI for PF treatment: 1) Noninvasive inhalation enhances local drug deposition in the lungs; 2) FAP-targeting peptide modification on liposomes improves targeted delivery efficiency to myofibroblasts; 3) pH-responsive PEOz promotes drug release and facilitates endosomal escape; 4) FL-NI codelivers NIN and siIL11 to myofibroblasts, achieving synergistic effects that inhibit fibroblast activation and promote collagen degradation; 5) FL-NI remodels the immune microenvironment of PF. AEC I, type I alveolar epithelial cells; AEC II, type II alveolar epithelial cells; RISC, RNA-induced silencing complex. Created in BioRender. Chen, Q. (2025) <https://BioRender.com/qbup1xu>.

results demonstrated that the FL-siRNA group exhibited significantly higher delivery efficiency compared to L-siRNA, as evidenced by an increased proportion of FAM-positive MEFs. Blocking FAP with antibodies subsequently reduced the siRNA delivery efficiency of FL-siRNA. Conversely, when the same procedure was applied to normal MEFs, the FL-siRNA group showed only a slight increase in FAM-positive MEFs, likely due to minor activation of normal MEFs from unavoidable mechanical stimulation during the experiment.

Moreover, no significant changes were observed in normal MEFs after blocking the FAP protein (Fig. 3B). These findings confirmed that the FAP-targeting peptide significantly enhances the targeting ability of FAM-siRNA to activated fibroblasts.

Simultaneously, activated and normal MEFs were treated with either L-siRNA or FL-siRNA for 2 or 4 hours, followed by analysis using confocal microscopy. Consistent with the flow cytometry results, FL-siRNA exhibited higher FAM fluorescence intensity in

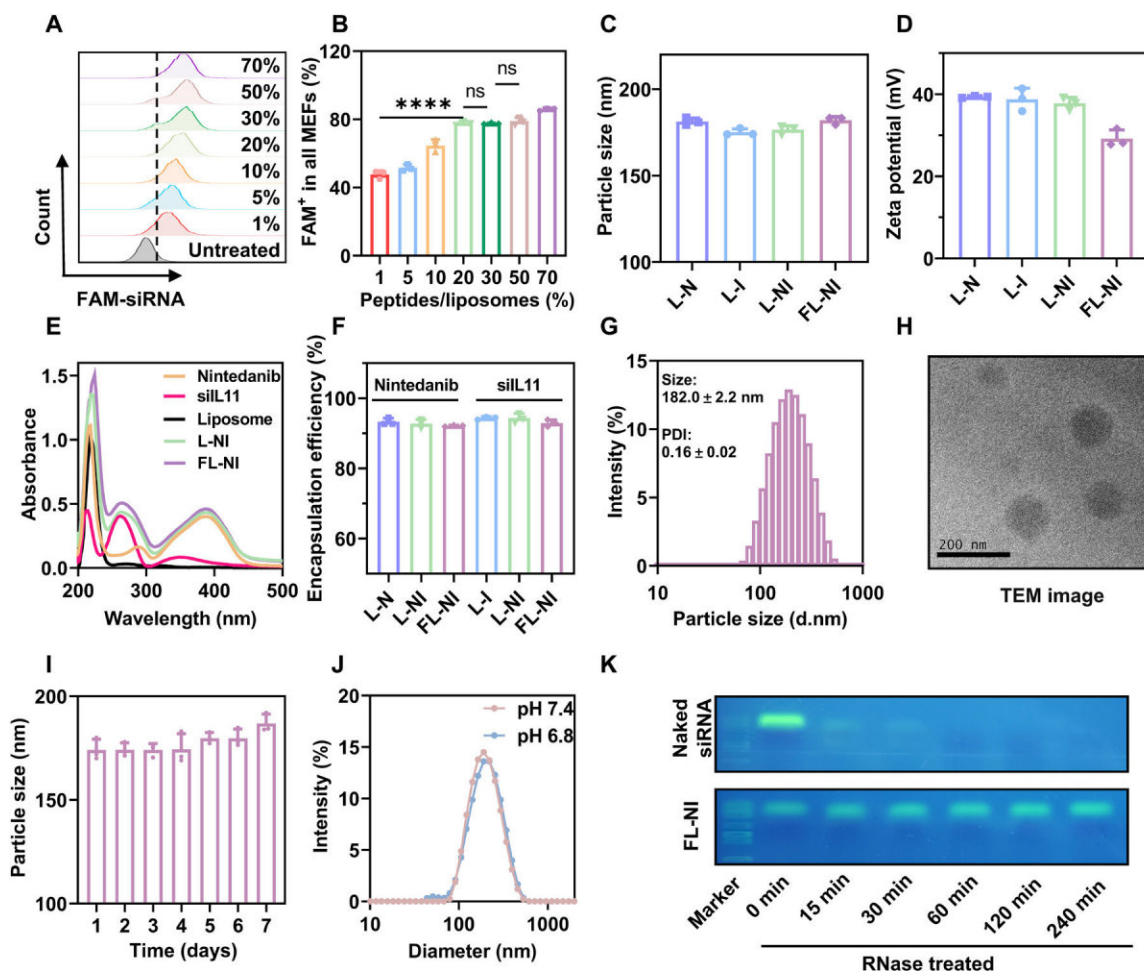


Fig. 2. Characterization of FL-NI. (A and B) Fluorescence intensity of MEFs incubated with preparations of different peptides/liposomes ratios. (C) Average particle sizes and (D) zeta potential of different liposomes determined by DLS. (E) Ultraviolet-visible spectra of NIN, siL11, blank liposome, L-NI, and FL-NI. (F) Drug encapsulation efficiency of NIN and siL11. (G) Particle size distribution of FL-NI. (H) Representative transmission electron microscopy (TEM) micrograph of FL-NI. Scale bar, 200 nm. (I) Particle size of FL-NI in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). (J) Particle size of FL-NI at different pH values. (K) Stability of naked siRNA and siRNA loaded in FL-NI against RNase. All data are presented as means $\pm$ SD ($n = 3$). Statistical significance was calculated via one-way analysis of variance (ANOVA) (B) in GraphPad Prism. **** $P < 0.0001$; not significant (ns), $P > 0.05$.

activated MEFs compared to L-siRNA. In contrast, normal MEFs showed no notable increase in fluorescence when treated with FL-siRNA (Fig. 3C). In addition, coumarin 6, a lipophilic fluorescent probe, was encapsulated in liposomes to create FL-C6 and L-C6 for modeling the intracellular distribution of the hydrophobic drug NIN. It was found that coumarin 6 fluorescence intensity in the FL-C6 group was higher in activated MEFs (fig. S6). Collectively, these results demonstrate the specific targeting and delivery capacity of FL-siRNA to activated fibroblasts, facilitated by the FAP-targeting peptide.

Once internalized by cells, liposomes were typically sequestered in endocytic vesicles through an energy-dependent endocytic mechanism (35), and failure to escape the endosome will hinder drug release and therapeutic effects (36). We then investigate the endosomal escape capacity of liposomes mediated by PEO₂, which reported to intensify the "proton sponge effect" (37). Therefore, we synthesized L_{PEG}-siRNA using DSPE-PEG_{2k} as a control for L-siRNA. MEFs were treated with either L_{PEG}-siRNA or L-siRNA for varying durations,

and endosomes/lysosomes were labeled with LysoTracker Red. Confocal microscopy was then used to monitor the distribution of FAM-siRNA within endosome/lysosome (Fig. 3D). It was found that FAM-siRNA exhibited lower colocalization with endosomes/lysosomes in cells treated with L-siRNA compared to those treated with L_{PEG}-siRNA, as evidenced by fewer colocalized puncta indicated by orange-colored fluorescence, suggesting enhanced endosomal escape (Fig. 3E). In addition, with extended incubation time, there was a gradual decrease in the colocalization of FAM-siRNA within endosomes/lysosomes, further indicating that L-siRNA has an enhanced ability to escape endosomal compartments.

Antifibrotic effect of FL-NI in vitro

After confirming that FL-NI facilitates drug delivery into activated fibroblasts via receptor-mediated endocytosis through the FAP-targeting peptide, we processed to evaluate its antifibrotic effects in vitro. First, the cell viability assay was performed, and the results indicated that the cell viability remained 80% after MEFs were incubated

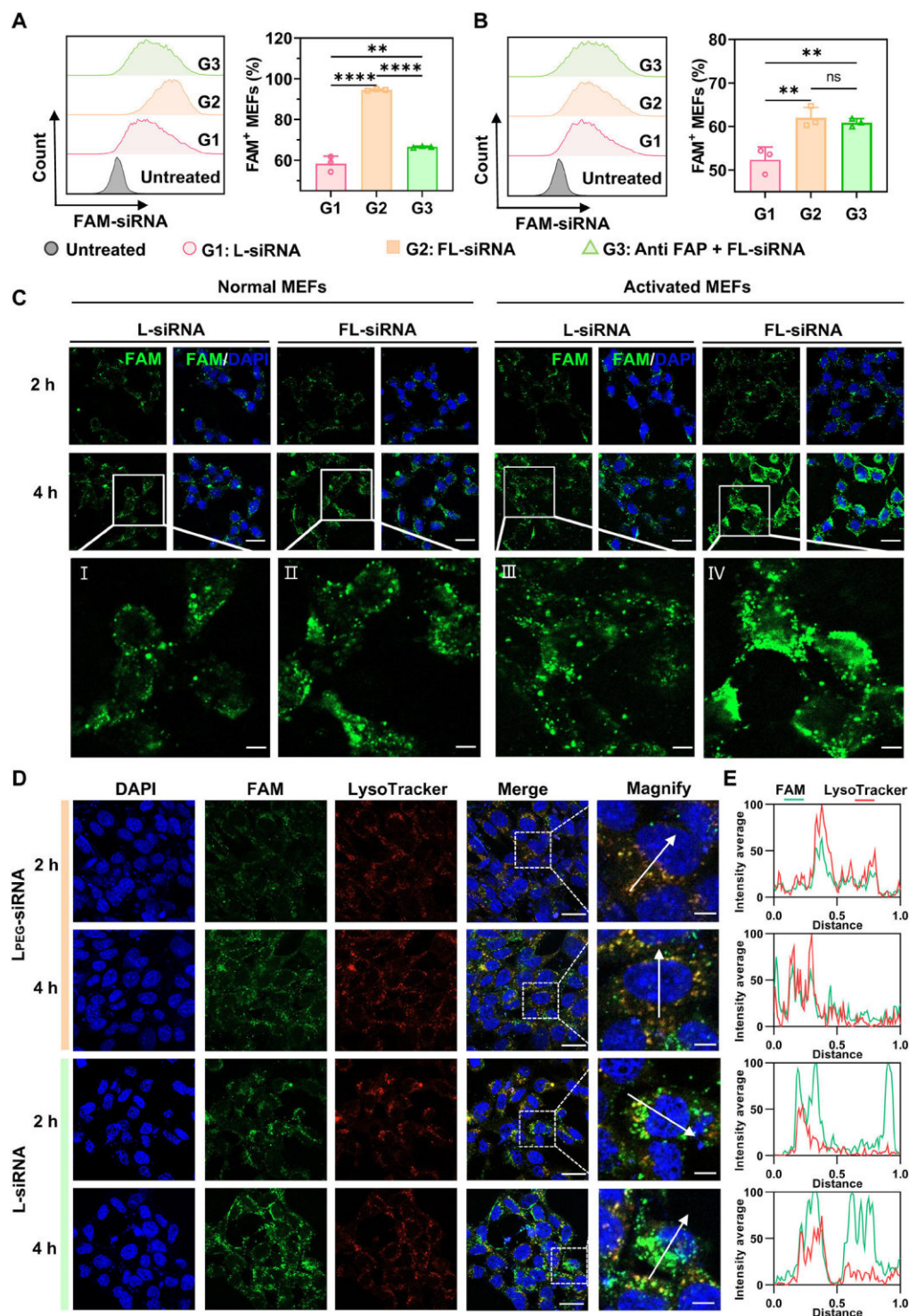


Fig. 3. Targeting and endosomal escape ability of FL-NI in vitro. (A and B) Fluorescence intensity of normal (A) and activated (B) MEFs incubated with L-siRNA (pink line), FL-siRNA (orange line), or FL-siRNA with pretreatment with anti-FAP antibodies (1:500 concentration, 2 hours, 37°C) (green line). Bar graphs indicate the percentage of cells exhibiting fluorescence offset. (C) Fluorescence microscopic images of normal and activated MEFs treated with L-siRNA and FL-siRNA for 2 and 4 hours at 37°C. Scale bars, 20 and 5 μm (low- and high-magnification images, respectively). (D) Confocal laser scanning microscopy images of MEFs treated with LPEG-siRNA or L-siRNA for 2 and 4 hours at 37°C. Endosomes/lysosomes were labeled by LysoTracker Red (red), and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). Scale bars, 20 and 5 μm (low- and high-magnification images, respectively). (E) Magnified images of the region of interest with colocalization analysis. All data are presented as means ± SD (n = 3). Statistical significance was calculated via one-way ANOVA [(A) and (B)] in GraphPad Prism. **P < 0.01; ****P < 0.0001; and not significant, P > 0.05. h, hours.

with different concentrations of FL-NI or L-NI for 24 hours (fig. S7). Subsequently, the activated MEFs after stimulating by TGF- β 1 were coinocubated with L-I, L-N, L-NI, and FL@NI with equal amounts of NIN ($1 \mu\text{mol ml}^{-1}$) and siIL11 (40 pmol ml^{-1}) for 24 hours. Then, we used immunofluorescence staining to examine the expression of α -smooth muscle actin (α -SMA) and collagen I (COL1A1) as they are key markers of PF and indicators of fibroblast activation (10). As expected, MEFs exhibited strong red fluorescence of α -SMA and COL1A1 when treated with TGF- β 1. However, fluorescence associated with both α -SMA (Fig. 4, A and B) and COL1A1 (Fig. 4, C and D) decreased to varying degrees after treatment with different formulations. Notably, only slight fluorescence was detected in the cells treated with dual-drug combination therapy, such as L-NI or FL-NI, with the FL-NI group showing fluorescence levels reduced to near normal. Slight variation in COL1A1 fluorescence was observed between the L-NI and FL-NI treatments, which may be attributed to the diminished impact of endocytosis differences due to prolonged incubation time. In addition, both Western blot (WB) (Fig. 4E) and quantitative polymerase chain reaction (qPCR) (Fig. 4, F to H) results confirmed varying degrees of reduction in protein and mRNA expression levels of α -SMA and COL1A1 among various treatments, with MEFs treated with FL-NI exhibiting the lowest expression levels.

Given the pivotal role of fibroblast migration into fibroblastic foci during PF development (38), we further assessed the inhibitory effect of FL-NI on this process using transwell migration and wound healing assays. MEFs after the same treatment procedure as above were seeds to wells for the migration experiment (fig. S8), and the migration capability of MEFs was quantified by counting migrated cells. The migrated number of MEFs after activated by TGF- β 1 was 160 ± 11 , while the number decreased to 36 ± 7 after treated with the FL-NI, significantly lower than 66 ± 11 of those with L-NI, indicating the superior efficacy of FL-NI in attenuating TGF- β 1-induced activation and migration of MEFs (Fig. 4, I and J). For the wound healing experiments, MEFs subjected to same treatments with the above and the images were captured at 0 and 24 hours post-administration (Fig. 4K), and the relative wound closure area at 24 hours compared to 0 hours was analyzed to evaluate the migration ability (Fig. 4L). When MEFs were activated with TGF- β 1, the wound healing area was $66.02 \pm 5.69\%$. However, following incubation with FL-NI, the wound healing area significantly decreased to $17.97 \pm 3.95\%$, which was notably lower than $29.82 \pm 2.50\%$ observed in the L-NI treatment group. These findings suggest that FL-NI, which codelivers NIN and siIL11 to myofibroblasts, effectively induces their deactivation and inhibits their migration in the context of PF. This outcome demonstrates superior antifibrotic efficacy compared to L-NI, likely due to the enhanced internalization facilitated by the FAP-targeting peptide.

Targeted delivery of FL-NI in fibrotic lungs

To investigate the targeted delivery of FAP-targeting peptide-conjugated liposomes in vivo, the naked siRNA, L-siRNA, and FL-siRNA containing equivalent cyanine 5 (Cy5) siRNA were inhaled into the lungs of mice by a pulmonary spray needle. After 24 hours, major organs were harvested, and the fluorescence distribution and intensity of Cy5 were visualized using an in vivo imaging systems (IVIS) imaging system (Fig. 5A and fig. S9). The results demonstrated a uniform and robust Cy5 fluorescence signal in the lungs of the FL-siRNA group, indicating the superior lung deposition of FL-NI through enhanced uptake by myofibroblasts, compared to the L-siRNA group.

In contrast, naked siRNA, without protective encapsulation, showed significant degradation and elimination, as evidenced by reduced Cy5 fluorescence signals. Moreover, these data highlight the advantages of inhalation administration, as weak Cy5 fluorescence signals were observed in the liver, kidney, heart, and spleen across all three groups (Fig. 5A). Quantitative analysis of fluorescent signals in these organs (Fig. 5B) and calculation of the lung/liver ratio (Fig. 5C) further supported the preferential lung deposition of FL-NI compared to L-NI, which was also corroborated with the immunofluorescence fluorescence imaging of frozen lung sections, where the naked siRNA group exhibited minimal fluorescence signals due to the degradation by nucleases (Fig. 5D). To further explore the reasons behind the superior pulmonary deposition of FL-siRNA, frozen lung sections from the L-siRNA and FL-siRNA groups were subjected for FAP immunofluorescence staining. Confocal microscopy images revealed a higher degree of colocalization of FAP-positive fibroblasts (labeled by AF488) and Cy5-siRNA in the FL-siRNA group, manifesting enhanced targeting specificity of myofibroblasts in vivo compared to the L-siRNA group (Fig. 5E).

To precisely determine the distribution of FL-siRNA among major cell subtypes in the PF microenvironment, we digested the lungs and prepared into single-cell suspensions for flow cytometry analysis. This analysis quantified the proportion of Cy5-positive cells among epithelial cells (CD326⁺), endothelial cells (CD31⁺), immune cells (CD45⁺), and activated fibroblasts (CD45⁺ and FAP⁺) (Fig. 5F), with the flow cytometry gating strategy shown in fig. S10. The results indicated that the FL-siRNA group exhibited a significantly higher proportion of Cy5-positive cells in fibroblasts, accounting for 21.3%, compared to 12.2% in the L-siRNA group (Fig. 5G). Conversely, negligible difference was observed in the proportion of Cy5-positive cells between the FL-siRNA and L-siRNA groups among endothelial cells (Fig. 5H). The proportion of Cy5-positive cells was significantly reduced in both immune cells (Fig. 5I) and epithelial cells (Fig. 5J) in the FL-siRNA group. These findings suggest that following inhalation, FL-siRNA exhibits a higher propensity for uptake by FAP-positive fibroblasts while demonstrating reduced nonspecific entry into epithelial cells and immune cells. This indicates that FAP peptides facilitate targeted delivery in vivo, enhancing the therapeutic potential of FL-siRNA for treating PF.

Antifibrotic effects of FL-NI in vivo

To evaluate the antifibrotic efficacy of FL-NI in vivo, a PF mouse model was established using a single intratracheal injection of bleomycin (BLM) at a dose of 2 USP kg^{-1} , followed by a 7-day progression period. Then, BLM-induced C57BL/6 mice were randomly divided into the following groups: BLM, L-N, L-I, L-NI, and FL-NI, with normal C57BL/6 mice serving as the normal control group. Starting on day 1 postmodeling and continuing until day 14, each group received corresponding formulation with NIN and siIL11 at doses of 1 mg kg^{-1} each time via intratracheal instillation every other day (Fig. 6A). Throughout the experiment, the weights of mice were monitored every 2 days as an indicator of disease burden. Normal mice exhibited gradual weight gain, whereas all mice treated with BLM experienced weight loss. Notably, compared to the BLM group, mice receiving different formulations displayed varying degrees of weight loss attenuation. Among these, the L-NI group was more effective than the L-N and L-I groups in slowing weight loss. Notably, FL-NI-treated mice demonstrated significantly greater

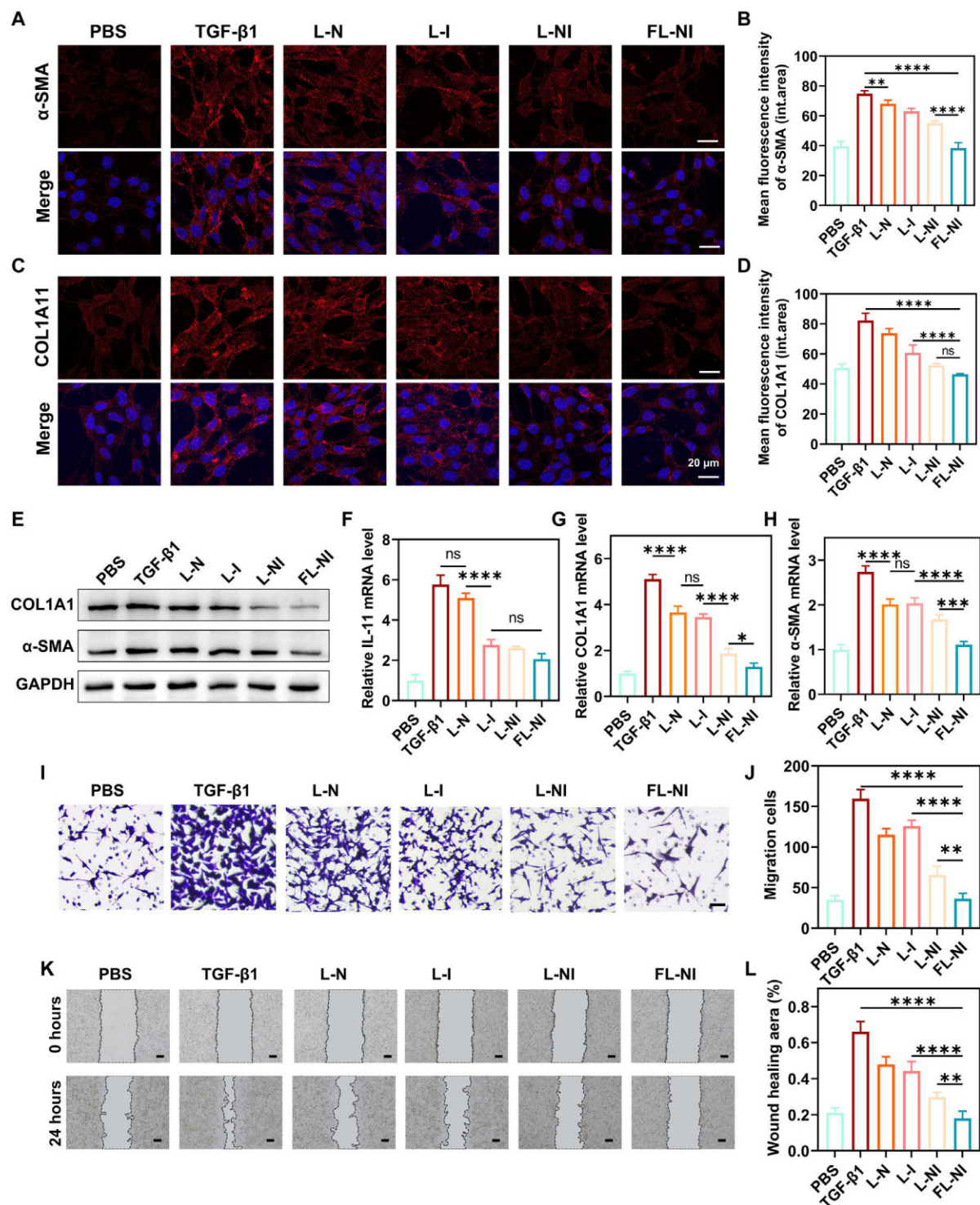


Fig. 4. The activation and migration of fibroblast inhibited by FL-NI in vitro. (A and B) Immunofluorescence analysis of α-SMA expression in fibroblasts. Scale bars, 20 μm. Data are presented as means ± SD (n = 5). (C and D) Immunofluorescence analysis of COL1A1 expression in fibroblasts. Scale bars, 20 μm. Data are presented as means ± SD (n = 5). (E) WB analysis of COL1A1 and α-SMA in fibroblasts treated by PBS, L-N, L-I, L-NI, and FL-NI. (F to H) qPCR analysis of IL-11, COL1A1, and α-SMA in fibroblasts treated by PBS, L-N, L-I, L-NI, and FL-NI. Data are presented as means ± SD (n = 3). (I) Representative images and (J) statistical analysis of migration cell counts from the transwell migration assay in fibroblasts with different treatments. Scale bar, 50 μm. Data are presented as means ± SD (n = 4). (K) Representative images and (L) statistical analysis on wound healing area of fibroblast with different treatments. Scale bars, 200 μm. Data are presented as means ± SD (n = 3). Statistical significance was calculated via one-way ANOVA in GraphPad Prism. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; not significant, $P > 0.05$. GAPDH, glyceraldehyde phosphate dehydrogenase.

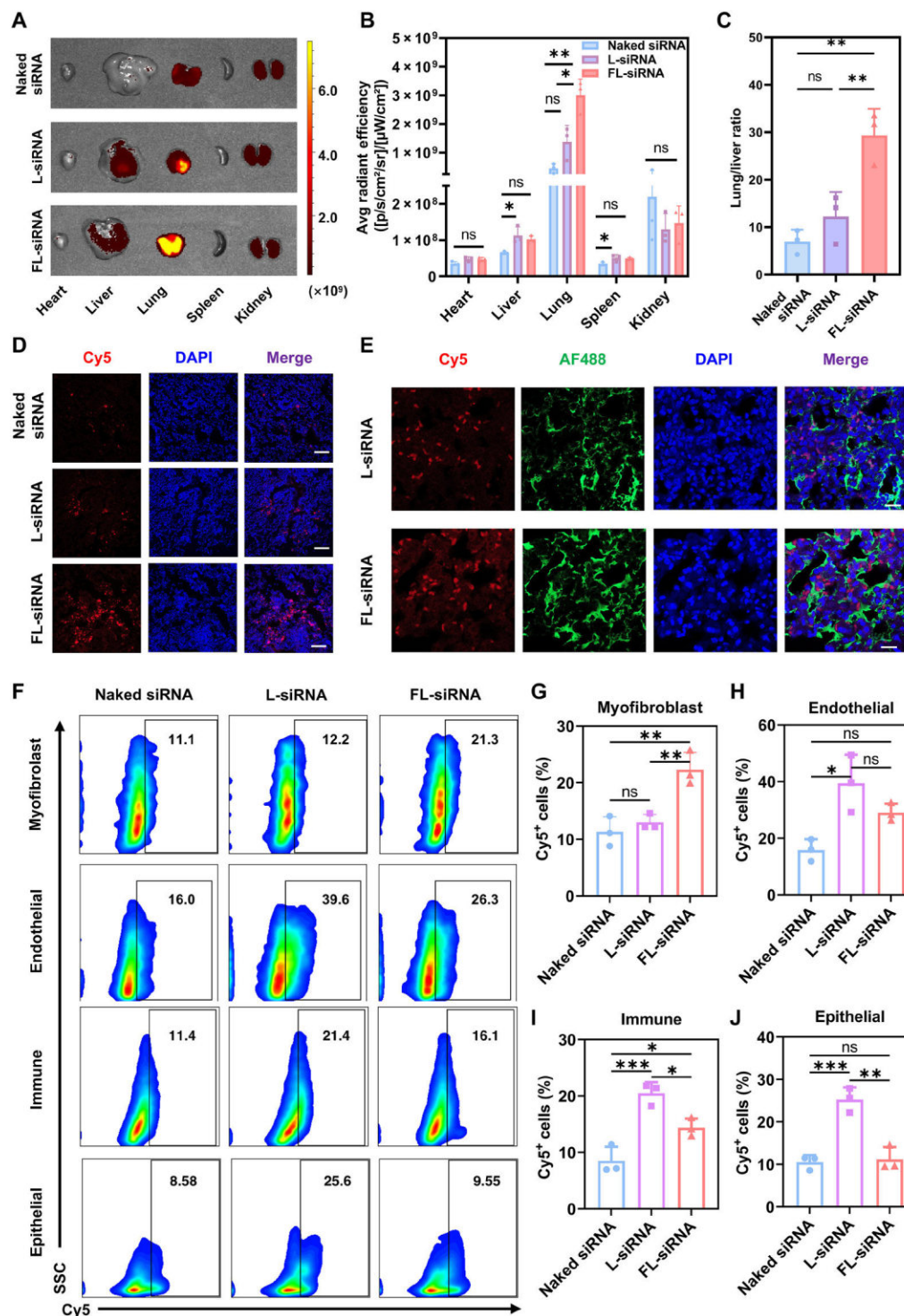


Fig. 5. Targeted delivery of FL-NI in vivo. (A) IVIS images of the heart, liver, lungs, spleen, and kidneys, 24 hours after inhalation of naked Cy5-siRNA, L-siRNA, and FL-siRNA. (B) Fluorescence intensity of the heart, liver, lung, spleen, and kidney, expressed as average radiant efficiency units. (C) Fluorescence intensity ratio of the lung to liver. (D) Confocal microscopy image of Cy5-siRNA distribution in the lung sections in naked siRNA, L-siRNA, and FL-siRNA groups. Scale bars, 100 μ m. (E) Immunofluorescence distribution analysis of FAP⁺ myfibroblasts and Cy5-siRNA in the lung sections in L-siRNA and FL-siRNA groups. Scale bars, 20 μ m. (F) Representative flow cytometry diagram showed the distribution of Cy5-siRNA in the lungs after inhalation of the different preparations. (G to J) Statistical data showed the proportion of Cy5-positive cells in (G) myofibroblasts, (H) endothelial cells, (I) immune cells, and (J) epithelial cells after inhalation of the different preparations. All data are presented as means $\pm$ SD ($n = 3$). Significant difference was calculated via one-way ANOVA [(B), (C), and (G) to (J)] in GraphPad Prism. AF488, Alexa Fluor 488; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; not significant, $P > 0.05$.

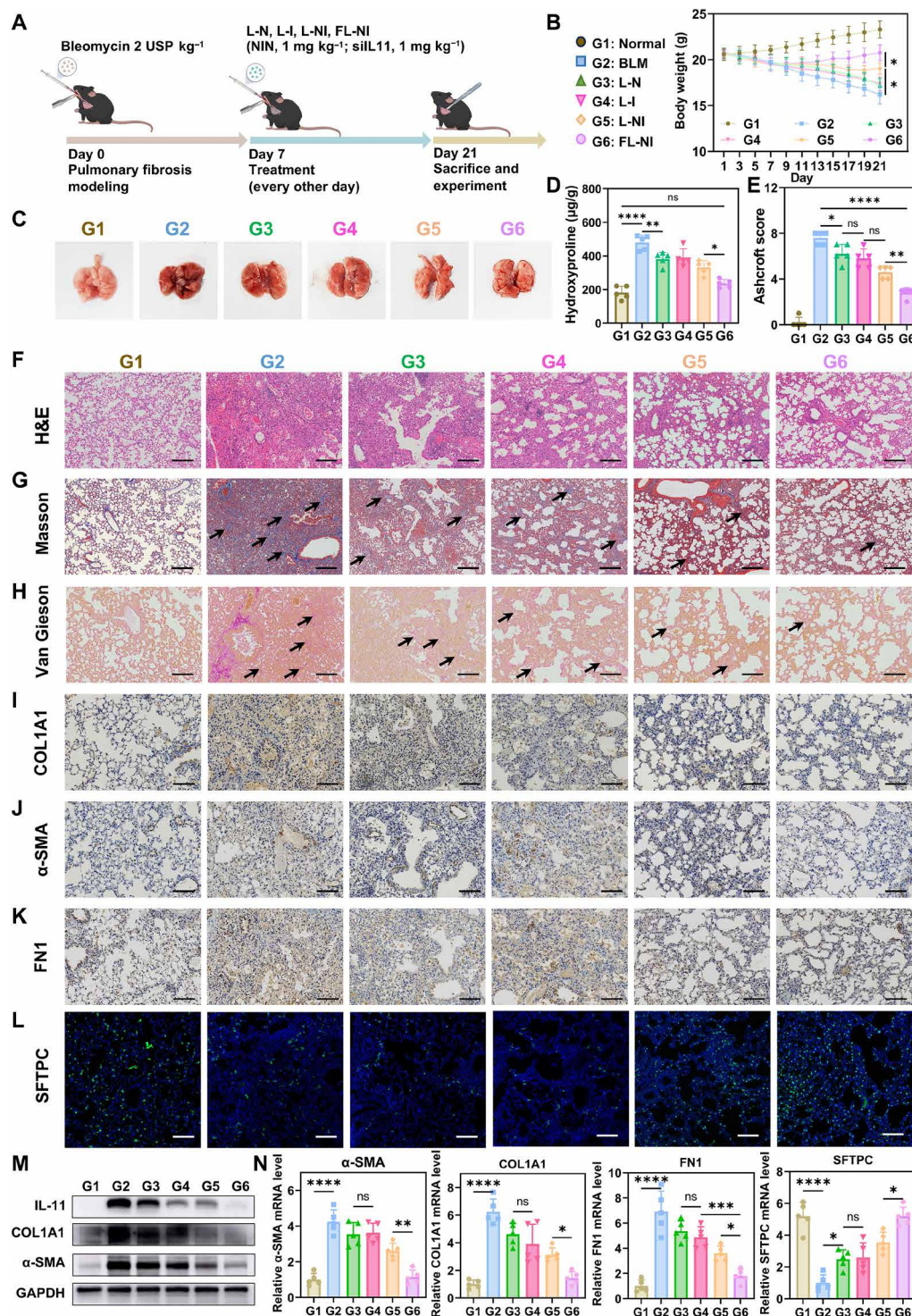


Fig. 6. FL-NI alleviates BLM-induced PF in vivo. (A) Timeline for modeling PF and inhalation treatment. (B) Body weights of mice in different groups. G1: normal; G2: BLM; G3: L-N; G4: L-I; G5: L-NI; G6: FL-NI. (C) Morphologic images of lungs after mice were euthanized at the end of treatment period. (D) Quantitative analysis of hydroxyproline content in mouse lung tissues after different treatments. (E) Ashcroft scores of fibrotic lung sections. (F) H&E staining of lung sections from different groups. The collagen fibers are stained blue in (G) Masson staining and red in (H) Van Gieson staining of lung sections from different groups. (I to K) Immunohistochemistry staining of COL1A1 (I), α-SMA (J), and FN1 (K) of lung sections from different groups. (L) Immunofluorescence analysis of SFTPC of lung sections from different groups. (M) WB analysis of IL-11, COL1A1, and α-SMA expression of lung sections from different groups. (N) qPCR analysis of α-SMA, COL1A1, FN1, and SFTPC expression of lung sections from different groups. All data are means ± SD ($n = 5$). Significant difference was calculated via Student's t test (B) and one-way ANOVA [(D), (E), and (N)] in GraphPad Prism. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; not significant, $P > 0.05$.

alleviation of weight loss compared to all other groups, likely due to the enhanced *in vivo* targeting capability of FAP (Fig. 6B).

At the end of treatment, the mice were euthanized for different analysis. As exhibited in Fig. 6C, mice treated with BLM alone exhibited hemorrhagic necrosis in the lung, while the FL-NI-treated group demonstrated substantial restoration of lung morphology. Quantitative analysis of hydroxyproline levels (Fig. 6D) revealed that the FL-NI group exhibited the most effective remodeling of pathological collagen accumulation in the lungs. To visualize alveolar structure damage caused by PF, we performed hematoxylin and eosin (H&E) staining of lung sections and Ashcroft scoring. The Ashcroft score of BLM-induced fibrosis was 7.6 ± 0.6 , but it was significantly reduced to 2.8 ± 0.5 following FL-NI inhalation, which was significantly lower than that in the L-NI group (Fig. 6E). H&E staining showed severe disruption of alveolar structure in BLM-induced fibrosis model, characterized by extensive fibrotic foci, thickened alveolar walls, and infiltration of inflammatory cells. The FL-NI-treated group exhibited marked reduction in necrotic areas, with near-complete restoration to normal alveolar architecture (Fig. 6F and fig. S11). Given that excessive pathological collagen accumulates in the pulmonary interstitium of fibrotic tissue, leading to parenchymal defects and respiratory impairment, Masson's trichrome staining was used to differentiate collagen distribution in the lung (Fig. 6G). The FL-NI group showed greater reduction in interstitial collagen deposition compared to the L-NI group, indicating that FL-NI has an optimal antifibrotic effect. Similarly, Van Gieson staining confirmed the same results (Fig. 6H). Collectively, these findings demonstrated the potent efficacy of FL-NI in reducing collagen protein and fiber deposition in BLM-induced PF.

In PF, the up-regulation of α -SMA and excessive pathological collagen secretion are key contributors to disease progression (10). Immunohistochemical staining was carried out to evaluate the expression levels of COL1A1, α -SMA, and fibronectin 1 (FN1) in fibrotic lungs. It was found that FL-NI treatment substantially normalized the expression of COL1A1 (Fig. 6I), α -SMA (Fig. 6J), and FN1 (Fig. 6K), indicating effective inhibition of activated fibroblasts in the lung interstitium and elimination of the ECM. Given the strong association between PF and epithelial injury (39), we further assessed the repair and functional remodeling of the epithelium by immunofluorescence staining of surfactant protein C (SFTPC). Obviously, the increased expression of SFTPC was observed in the FL-NI treatment group, suggesting its reparative effect on the epithelium. These findings were corroborated by WB, which showed significant up-regulation of IL-11, COL1A1, and α -SMA in the BLM group, with varying degrees of down-regulation following FL-NI treatment, neatly restoring them to normal levels (Fig. 6M). The qPCR results further confirmed the decreased expression of COL1A1, α -SMA, and FN1, alongside increased SFTPC levels after FL-NI treatment (Fig. 6N). Considering that fibrosis in young mice usually spontaneously reverse 4 weeks after a single intratracheal instillation of BLM, despite this model being the most widely used for studying the pathogenesis and therapeutic effects of PF, we used both the repetitive BLM-induced PF model and the aged mice PF model to access the antifibrosis effects of FL-NI. In the repetitive BLM model, the treatment process followed the experimental timeline outlined in the flow chart (fig. S12A). It was found that FL-NI treatment extended survival (fig. S12B) and significantly alleviated fibrosis progression in mice subjected to repetitive BLM treatment (fig. S12C). In addition, H&E and Masson staining revealed substantial

restoration of alveolar structure disruption and a marked reduction in collagen deposition in the FL-NI-treated mice (fig. S12D). Notably, the Ashcroft score for the FL-NI group was reduced to 1.8 ± 1.5 (fig. S12E), in contrast to the higher score observed in the BLM group. Consistently, in the aged mice, following the administration protocol outlined in fig. S13A, mice inhaled with FL-NI exhibited significantly greater alleviation of weight loss (fig. S13B), hemorrhagic necrosis (fig. S13C), alveolar structure disruption, and collagen deposition (fig. S13D) compared to the BLM group. The Ashcroft score in the FL-NI-treated group was notably lower, at 2.6 ± 1.1 (fig. S13E), compared to the BLM group. These results collectively highlight the exceptional antifibrotic efficacy of FL-NI. Moreover, FL-NI and other formulations showed no additional toxicity to major organs such as the liver and kidneys. Quantification of alanine aminotransferase and aspartate aminotransferase levels in mouse serum indicated negligible hepatotoxicity (fig. S14). Similarly, H&E staining of the heart, liver, spleen, and kidneys revealed nearly no histopathological changes in these organs (fig. S15). Therefore, FL-NI effectively inhibits excessive fibroblast activation and ECM deposition while promoting epithelial repair *in vivo*, without causing side effects to other major organs.

The mechanism of FL-NI for antifibrotic treatment

To elucidate the underlying mechanism by which FL-NI ameliorates PF, we performed bulk RNA sequencing (RNA-seq) analysis on lung tissues from the normal, BLM, and FL-NI groups (every group contains five samples) (Fig. 7A). Transcriptome sequencing revealed significant gene expression changes: 413 genes were down-regulated, and 1715 mRNAs were up-regulated in the BLM group compared to the normal group, while 608 genes were down-regulated and 840 genes were up-regulated in the FL-NI group compared to the BLM group (Fig. 7B). Notably, FL-NI treatment effectively restored the expression of fibrosis-related molecules. Specifically, it down-regulated the expression of ECM components, growth factors and their receptors, chemokines and their receptors, matrix metalloproteinases, and other fibrosis-associated regulatory proteins while up-regulating surfactant proteins (Fig. 7C). To further identify the pathways modulated by FL-NI, we intersected the mRNAs up-regulated in the BLM group and down-regulated in the FL-NI group, identifying 451 differentially expressed mRNAs for functional enrichment analysis (Fig. 7D). The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis indicated that FL-NI treatment significantly inhibited several vital signaling pathways, including cytokine-cytokine receptor interaction, chemokine, Janus kinase/signal transducer and activator of the transcription (JAK-STAT), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PI3K-AKT), nuclear factor κ B, and Ras signaling pathways (highlighted in orange) (Fig. 7E). In addition, Gene Ontology (GO) analysis revealed that FL-NI regulated the process related to cell chemotaxis, immune response, and inflammatory response (highlighted in green). Notably, the inflammatory responses regulated by FL-NI appear to be associated with cytokine signaling pathways involving TNF, IL-6, and IL-1, as visualized in an enriched chord diagram (highlighted in blue) (Fig. 7, F and G). To validate these findings, we measured the levels of TNF- α (Fig. 7H), IL-6 (Fig. 7I), and IL-1 β (Fig. 7J) in lung tissue homogenate supernatants using enzyme-linked immunosorbent assay (ELISA). The results showed that cytokine levels were significantly reduced in the FL-NI group. Therefore, siIL11 and NIN inhibit the activation of IL-11RA and PDGFR/FGFR signaling pathways, respectively, which, in

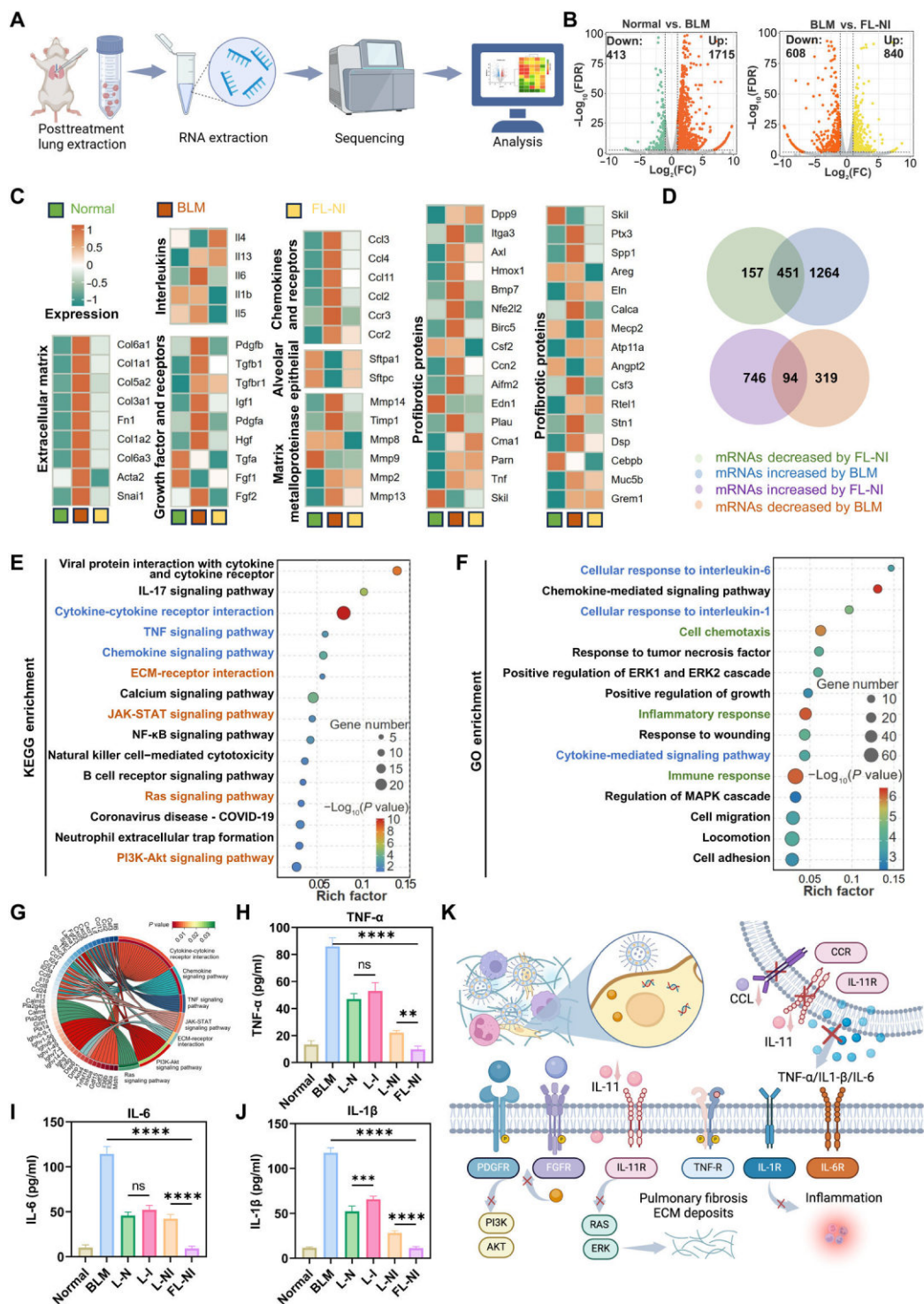


Fig. 7. Antifibrotic mechanism of FL-NI. (A) RNA-seq analysis of the lung tissue of mice from different groups. (B) Volcano plots of differentially expressed genes comparing normal versus BLM and BLM versus FL-NI (the latter versus the former). (C) Heatmap illustrating the average expression of fibrosis-related genes in lung tissue of mice from different groups. (D) Venn diagram showing the intersection of genes regulated by BLM and FL-NI. (E) KEGG enrichment analysis of 451 mRNAs that are simultaneously down-regulated by FL-NI and up-regulated by BLM. (F) GO enrichment analysis revealing biological pathways enriched for 451 mRNAs. (G) Chord diagram showing the enrichment of KEGG pathways and the related genes. (H to J) The levels of TNF-α (H), IL-6 (I), and IL-1β (J) in lung tissue homogenates measured by ELISA. Data are means ± SD (n = 5). (K) Diagram illustrating the dual-drug synergistic mechanism for antifibrotic treatment. Significant difference was calculated via one-way ANOVA [(H) to (J)] in GraphPad Prism. **P < 0.01; ***P < 0.001; ****P < 0.0001; not significant, P > 0.05. FDR, false discovery rate; FC, fold change; NF-κB, nuclear factor κB; MAPK, mitogen-activated protein kinase.

turn, suppress downstream PI3K-AKT and Ras signaling pathways to alleviate fibrosis. In addition, reducing IL-11 and associated chemokine levels helps attenuate the recruitment of inflammatory cells and decreases the expression of TNF- α , IL-1 β , and IL-6, thereby suppressing the inflammatory response. As a result, FL-NI demonstrates great potential for effectively regulating the pathological microenvironment in the mesenchyme and remodeling the parenchyma in PF treatment (Fig. 7K).

To further investigate the role of FL-NI in immune response and cell chemotaxis in PF, immune-related signaling pathway enrichment analysis was conducted using the RNA-seq data. The analysis revealed that FL-NI treatment significantly influenced neutrophil, monocyte, and macrophage chemotaxis (Fig. 8A). Furthermore, using the ImmuCellAI-mouse (Immune Cell Abundance Identifier for mouse) (40), we evaluated immune cell infiltration in PF lung tissues. The results showed a significant increase in the abundance of infiltrated immune cells including macrophages, neutrophils, and monocytes in the BLM group, which was restored to normal levels following FL-NI treatment (Fig. 8B and fig. S16). To validate these findings, flow cytometry analysis was performed. C57/BL6 mice were treated according to the protocol described in Fig. 6A, and single-cell suspensions were prepared from lung lobes for analysis (Fig. 8C). The results demonstrated that FL-NI treatment significantly reduced the percentage of monocytes in fibrotic lungs (Fig. 8D). Similarly, the proportion of neutrophils or granulocytic myeloid-derived suppressor cells (MDSCs) (CD11b⁺Ly6G⁺Ly6C⁻) was also obviously decreased in the FL-NI group, while no significant changes were observed in the proportion of monocytic MDSCs (CD11b⁺Ly6G⁻Ly6C⁺) (Fig. 8E and fig. S17). Moreover, macrophages, known to induce fibroblast differentiation into myofibroblasts to promote PF progression (41), were significantly reduced following FL-NI treatment (Fig. 8F). Further analysis divided pulmonary macrophages into CD11c⁺ [resident alveolar macrophages (AM)] and CD11b⁺ (interstitial macrophages or recruited AM). CD11b⁺ macrophages, which are implicated in fibrosis development and the promotion of a profibrotic phenotype (42), were significantly elevated in PF lungs. However, their levels returned to normal following FL-NI treatment (Fig. 8G). Conversely, there was a notable trend in CD11c⁺ resident AM (fig. S17). Furthermore, the M2 macrophages in CD11b⁺ macrophages are reported to regulate tissue damage repair by secreting profibrotic cytokines (41). Consistently, the results showed that the proportion of M2 macrophages significantly increased in the BLM group and decreased in all treatment groups, especially returning to normal in the FL-NI group (Fig. 8H). Collectively, these findings suggest that inhalation therapy with FL-NI can effectively remodel the normal immune microenvironment and protect against BLM-induced PF.

DISCUSSION

In this work, we embarked on an innovative “one-stone-three-birds” strategy by developing a multifunctional nanoplatform (FL-NI) designed to achieve efficient and step-by-step drug delivery across three key dimensions: organ deposition, cellular endocytosis, and intracellular release. This strategy was tailored to enhance the targeted delivery and release of a combination of siLL11 and NIN, ultimately facilitating a synergistic therapeutic effect to improve treatment efficacy. Numerous therapeutic strategies have been proposed for PF; however, drug delivery remains inefficient due to the reliance on either intravenous or inhalation administration without cellular

targeting (43–45). Nanoparticles often undergo nonspecific uptake by normal cells, seriously limiting the efficacy of antifibrotic treatments following inhalation. Therefore, combining inhaled delivery with myofibroblast targeting offers a promising strategy for enhanced drug delivery and improved therapeutic outcomes. Specifically, our findings demonstrated that inhalation administration of FL-NI significantly enhanced lung deposition, achieving a 117.8% increase compared to the deposition observed with L-NI. This was followed by a precise interaction between FAP-targeting peptides and FAP on the surface of activated fibroblasts, resulting in a 71.5% increase in myofibroblast targeting compared to L-NI. This was accompanied by 29.8 and 55.8% reduction in nonspecific immune cell and epithelial uptake, respectively. Furthermore, FL-NI effectively circumvented the acidic lysosomal environment through its pH-responsive PEOz components, enabling controlled drug release. Subsequent *in vivo* assessments revealed that FL-NI inhalation exhibited strong antifibrosis and immune-regulatory efficacy. Specifically, it significantly reduced collagen deposition by 50.8%, bringing it back to normal levels, and reduced the Ashcroft score from 7.6 ± 0.55 to 2.8 ± 0.45 . In addition, FL-NI treatment inhibited fibroblast activation and structural damage associated with PF. Besides the single BLM instillation model, in progressive PF models that closer to PF clinical pathology, such as repetitive instillation BLM and aged mouse model (46, 47), FL-NI still demonstrated excellent antifibrotic capacity, indicating a strong clinical value. FL-NI inhalation also normalized the profibrotic microenvironment, showing significant reductions in inflammatory factors, such as IL-1 β , IL-6, and TNF- α , as well as in immune cell infiltration, including M2 macrophages and granulocytic MDSCs, indicating remarkable restoration of tissue homeostasis. Mechanistic investigations, including RNA-seq, revealed that the enhanced antifibrotic efficacy of FL-NI was derived from the synergistic effects of siLL11 and NIN. These components effectively inhibited key signaling pathways involved in fibrosis, including JAK-STAT, PI3K-AKT, and ERK pathways. Moreover, evaluations of physicochemical properties and safety of FL-NI demonstrated its long-term colloidal stability and high biocompatibility after inhalation in mice, without any safety concerns. In conclusion, the multifunctional nanoplatform developed in this work represents a significant advancement in drug delivery precision, providing a highly effective therapeutic strategy for PF, and it not only demonstrates substantial therapeutic potential but also offers insights for the future development of noninvasive precision therapy.

MATERIALS AND METHODS

Materials

The compounds DOTAP and cholesterol were acquired from Weihua Biotechnology Co. Ltd. (Guangzhou, China). The liposome size extruder was obtained from Avanti Polar Lipid Inc. (Alabaster, AL, USA), while polycarbonate membranes were sourced from Merck Millipore (Billerica, MA, USA). The NIN, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide], protamine sulfate, and hyaluronic acid were acquired from Sigma-Aldrich (St. Louis, MO). The FAP-targeting peptide (DRGETGPAC) was obtained from Jiangsu Jitai Technology Co. Ltd. DSPE-PEO_{2k}-MAL was provided by Weihua Biotechnology Co. Ltd. (Guangzhou, China), and DSPE-PEO_{2k}-FAP was synthesized by combining the FAP-targeting peptide with DSPE-PEO_{2k}-MAL based on the previous established protocol (33). The serum-free cell cryopreservation solution (FL10020L) and

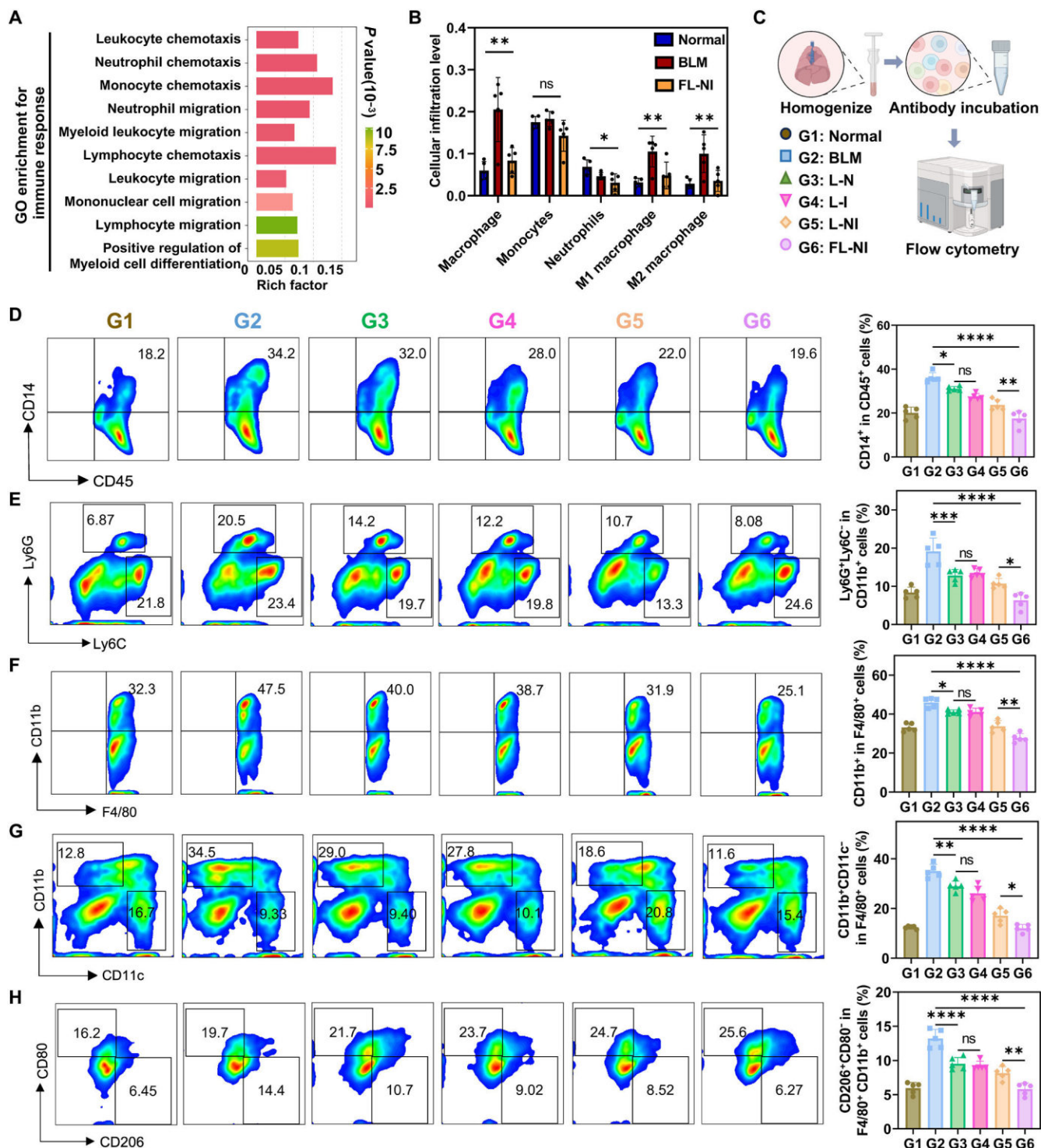


Fig. 8. Immune microenvironment of PF remodeled by FL-NI. (A) GO enrichment analysis results for immunomodulatory biological pathways. (B) Analysis of immune cell infiltration in different groups of mice. (C) Schematic representation of immuno-evaluation of lung tissues of mice from different groups. (D to H) Representative flow cytometry plots and percentages of CD14⁺ cells in CD45⁺ cells (D), Ly6G⁺Ly6C⁻ and Ly6C⁺Ly6G⁻ cells in CD11b⁺ cells (E), CD11b⁺ cells in F4/80⁺ cells (F), CD11b⁺CD11c⁻ and CD11c⁺CD11b⁻ cells in F4/80⁺ cells (G), and CD206⁺CD80⁻ cells in F4/80⁺CD11b⁺ cells (H) in mouse lungs after various treatments. Data are means ± SD (n = 5). Significant difference was calculated via one-way ANOVA [(H) to (J)] in GraphPad Prism. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; not significant, P > 0.05.

trypsin-EDTA digestion solution (FL20011) were purchased from Bio-land Biotechnology Co. Ltd. (Hangzhou, China).

Synthesis of FL-NI

FL-NI were prepared according to a stepwise self-assembly process described in the previous established protocols (33). First, using a hydration-extrusion method, the DOTAP/cholesterol/NIN lipid film was hydrated with diethyl pyrocarbonate-treated water and followed by extrusion through 200- and 400-nm polycarbonate membranes (Millipore, Billerica, MA) to form cationic liposomes. For the siIL11 core formation, solution A (containing siRNA and hemagglutinin) and solution B (containing protamine) were allowed for 10 min. The resulting siIL11 core solution was then incubated with the prepared liposomes for 10 min to encapsulate siIL11. Following this, DSPE-PEOz-FAP or DSPE-PEOz_{2k} was coincubated with the mixture for 1 hour at 50°C to form liposomes modified with or without the FAP targeting peptide. Free NIN and unencapsulated siIL11 were removed by ultrafiltration centrifugation using a 100-kDa MWCO filter (Millipore, USA). For comparison, other liposomal formulations containing NIN (L-N), siIL11 (L-I), or both (L-NI) were synthesized following the same procedure.

Characterization of FL-NI

The particle size, zeta potential, and the morphology of the nanoparticles were determined using Zetasizer Nano ZS (Malvern Instrument) and transmission electron microscopy (FEI Tecnai 20). The free NIN and siRNA unencapsulated in liposomes were removed by ultrafiltration centrifugation. The microplate reader (Bio-Rad) was used to detect the free and total amount of NIN and siRNA to calculate the encapsulation efficiency according to the following equations; encapsulation efficiency (EE) % = $(W_{\text{Total}} - W_{\text{Free}})/W_{\text{Total}} \times 100\%$. Furthermore, the stability of the nanoparticles was assessed by measuring particle size at predetermined time points in DMEM containing 10% FBS.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay was used to evaluate the resistance of siRNA encapsulated in FL-NI to nuclease degradation. Briefly, naked siRNA and FL-NI (containing an equivalent amount of siRNA) were coincubated with RNase (10 $\mu\text{g ml}^{-1}$) for 15, 30, 60, 120, and 240 min. After incubation, 2% sodium heparin solution was added to the FL-NI group to extract siRNA, followed by mixing with 6 $\times$ loading buffer (No. K1161, APExBIO, Houston, USA). Equal volumes of naked siRNA and FL-NI samples were then loaded into gel electrophoresis wells, and electrophoresis was performed at 80 V for 12 min. The results were photographed under ultraviolet irradiation.

Assessment of specific binding ability of FL-siRNA

MEFs (0.8×10^5 cells per well) were seeded onto 24-well plates containing coverslips (1170000, SAINING Biotechnology) for overnight at 37°C. Then, these fibroblasts were activated by incubation with TGF- β 1 (10 ng ml^{-1}) for 24 hours. The activated and normal MEFs were washed with phosphate-buffered saline (PBS) and then treated with L-siRNA and FL-siRNA for 2 and 4 hours at 37°C. The cells were then fixed with 4% paraformaldehyde for 1 hour, followed by nuclei staining with 4',6-diamidino-2-phenylindole. Cells were mounted on coverslips with an antifade reagent. FAM fluorescence was subsequently visualized using confocal fluorescence microscopy.

Meanwhile, for flow cytometry analysis, MEFs (1.5×10^5 cells per well) were seeded into 12-well plates overnight and then activated by TGF- β 1 (10 ng ml^{-1}) for 24 hours. Both activated and normal MEFs were treated with L-siRNA or FL-siRNA containing an equivalent amount of FAM-siRNA for 2 hours at 37°C. To block cell surface ligands targeted by FAP-targeting peptides, cells were pre-treated with 1:500 anti-FAP antibodies for 2 hours before the treatment with FL-siRNA. After incubation, cells were harvested by trypsinization, resuspended in 300 μl of PBS, and analyzed for FAM fluorescence (siRNA internalization) using flow cytometry.

Antifibrosis treatment in vitro

MEFs were seeded in six-well plates until they reached 90% confluence. MEFs were incubated with TGF- β 1 (10 ng ml^{-1}) for 24 hours for activation. After activation, the following treatments were applied to the MEFs for another 24 hours: L-N, L-I, L-NI, FL-NI, or an equivalent volume of PBS. Normal MEFs treated with PBS served as the positive control. Following incubation, the treated cells were subjected to WB with precast gel (GenScript, M00664) and secondary antibodies (Signalway Antibody, L3032), qPCR, and wound healing assays for assessing the antifibrosis ability of different treatments.

Antifibrotic effects of FL-NI in vivo

The PF models were established by receiving a single BLM (2 USP kg^{-1}) intratracheal injection for 7 days. BLM-induced C57BL/6 mice were randomly divided into the following groups: BLM, L-N, L-I, L-NI, and FL-NI, with normal C57BL/6 mice serving as the normal control group ($n = 5$). Each group received a corresponding formulation with the dose of NIN at 1 mg kg^{-1} and siIL11 at 1 mg kg^{-1} using a microsprayer aerosolizer every other day for 2 weeks. During the experiment, body weight was recorded every 2 days. The mice were euthanized for different analysis 2 days after the last treatment. All animal experiments were conducted in accordance with the relevant laws and guidelines and were approved by the Institutional Animal Care and Use Committee of Soochow University (no. SUDA20250304A06).

Content of hydroxyproline and cytokines in lungs

The content of hydroxyproline in fibrotic lungs was evaluated by a hydroxyproline detection kit. The lung homogenized supernatants were used to measure IL-6 (Beijing Solarbio Science and Technology Co. Ltd., SEKM-0007), IL-1 β (Beijing Solarbio Science and Technology Co. Ltd., SEKM-0002), and TNF- α (Beijing Solarbio Science and Technology Co. Ltd., SEKM-0034) by ELISA according to the manufacturer's instructions.

RNA-seq and data analysis

To explore the mechanism behind the antifibrotic efficacy of FL-NI, lung tissues from normal mice, PF mice, and PF mice treated with FL-NI were analyzed by RNA-seq on the Illumina HiSeq platform (GENEWIZ Biotech Inc.). Bioinformatic analysis of RNA-seq was performed using tools available at <https://omicshare.com/tools/>. The fibrosis-related molecules listed and analyzed are derived from the Molecular Signatures Database (48). The abundance of cellular infiltration in the lung microenvironment was evaluated on the basis of RNA-seq data from the mice with PF. Immune infiltration analysis was conducted using ImmuCellAI-mouse (40), a tool designed to estimate the abundance of 36 different immune cell types from gene expression datasets. These immune cells are categorized

into seven major types: B cell, natural killer cell, monocytes, macrophages, T cells, dendritic cells, and granulocytes.

In vivo immunological evaluation

To investigate the immune regulation effects of FL-NI in vivo, mice in all groups were euthanized 2 weeks after treatment. The lungs were euthanized from each mouse to prepare single-cell suspensions. These suspensions were treated with red blood cell (RBC) lysis buffer (ZUNYAN, NanJing, China, ZYFB006) to eliminate RBC, following by washing and blocking with anti-CD16/CD32 (BioLegend, 101303) to prevent Fc receptor binding. The cells were then stained with fluorophore-labeled antibodies as follows. To analyze monocyte, the cells were stained with anti-CD14–fluorescein isothiocyanate (FITC; BioLegend, 123308) and anti-CD45–phycoerythrin (PE; BioLegend, 147712). To analyze AMs and interstitial macrophages, the cells were stained with anti-Percp-F4/80 (BioLegend, 123126), anti-CD11b-FITC (BioLegend, 101206), and anti-CD11c-allophycocyanin (APC; BioLegend, 117310). To analyze M2 macrophages, the cells were stained with anti-Percp-F4/80 (BioLegend, 123126), anti-CD11b-FITC (BioLegend, 101206), anti-CD80-PE (BioLegend, 104708), and anti-CD206-APC (BioLegend, 141708). To evaluate monocytic MDSCs and granulocytic MDSCs, the cells were stained with anti-Percp-CD45 (BioLegend, 103130), anti-CD11b-FITC (BioLegend, 101206), anti-Ly6G-PE (BioLegend, 127608), and anti-Ly6C-APC (BioLegend, 128016). Subsequently, these cells were analyzed by flow cytometry (FongCyte).

Statistics and reproducibility

Data are presented as means $\pm$ SD. Student's *t* test or one-way analysis of variance (ANOVA) followed by the Tukey test was applied for comparison between two groups or among multiple groups using GraphPad Prism 9.5.1, respectively. *P* < 0.05 was considered to be statistically significant. Each experiment is repeated at least three times independently with similar results, and the representative dataset is presented.

Supplementary Materials

This PDF file includes:

Supplementary Text
Figs. S1 to S17

REFERENCES AND NOTES

- G. Raghu, M. Remy-Jardin, L. Richeldi, C. C. Thomson, Y. Inoue, T. Johkoh, M. Kreuter, D. A. Lynch, T. M. Maher, F. J. Martinez, M. Molina-Molina, J. L. Myers, A. G. Nicholson, C. J. Ryerson, M. E. Strek, L. K. Troy, M. Wijsenbeek, M. J. Mammen, T. Hossain, B. D. Bissell, D. D. Herman, S. M. Hon, F. Kheir, Y. H. Khor, M. Macrea, K. M. Antoniou, D. Bours, I. Buendia-Roldan, F. Caro, B. Crestani, L. Ho, J. Morisset, A. L. Olson, A. Podolanczuk, V. Poletti, M. Selman, T. Ewing, S. Jones, S. L. Knight, M. Ghazipura, K. C. Wilson, Idiopathic pulmonary fibrosis (an update) and progressive pulmonary fibrosis in adults: An official ATS/ERS/JRS/ALAT clinical practice guideline. *Am. J. Respir. Crit. Care Med.* **205**, E18–E47 (2022).
- A. J. Podolanczuk, C. C. Thomson, M. Remy-Jardin, L. Richeldi, F. J. Martinez, M. Kolb, G. Raghu, Idiopathic pulmonary fibrosis: State of the art for 2023. *Eur. Respir. J.* **61**, 2200957 (2023).
- V. Bellou, L. Belbasis, E. Evangelou, Tobacco smoking and risk for pulmonary fibrosis: A prospective cohort study from the UK Biobank. *Chest* **160**, 983–993 (2021).
- W. J. Huang, X. X. Tang, Virus infection induced pulmonary fibrosis. *J. Transl. Med.* **19**, 496 (2021).
- R. Borie, C. Kannengiesser, K. Antoniou, F. Bonella, B. Crestani, A. Fabre, A. Froidure, L. Galvin, M. Griesse, J. C. Grutters, M. Molina-Molina, V. Poletti, A. Prasse, E. Renzoni, J. van der Smagt, C. H. M. van Moorsel, European Respiratory Society statement on familial pulmonary fibrosis. *Eur. Respir. J.* **61**, 2201383 (2023).
- M. B. Rice, W. Li, J. Schwartz, Q. Di, I. Kloog, P. Koutrakis, D. R. Gold, R. W. Hallowell, C. Zhang, G. O'Connor, G. R. Washko, G. M. Hunninghake, M. A. Mittleman, Ambient air pollution exposure and risk and progression of interstitial lung abnormalities: The Framingham Heart Study. *Thorax* **74**, 1063–1069 (2019).
- P. M. George, A. U. Wells, R. G. Jenkins, Pulmonary fibrosis and COVID-19: The potential role for antifibrotic therapy. *Lancet Respir. Med.* **8**, 807–815 (2020).
- S. J. Singh, M. M. Baldwin, E. Daynes, R. A. Evans, N. J. Greening, R. G. Jenkins, N. I. Lone, H. McAuley, P. Mehta, J. Newman, P. Novotny, D. J. F. Smith, S. Stanel, M. Toshner, C. E. Brightling, Respiratory sequelae of COVID-19: Pulmonary and extrapulmonary origins, and approaches to clinical care and rehabilitation. *Lancet Respir. Med.* **11**, 709–725 (2023).
- J. Shen, L. Chen, X. Lv, N. Liu, Y. Miao, Q. Zhang, Z. Xiao, M. Li, Y. Yang, Z. Liu, Q. Chen, Emerging co-assembled and sustained released natural medicinal nanoparticles for multitarget therapy of choroidal neovascularization. *Adv. Mater.* **36**, e2314095 (2024).
- P. Spagnolo, J. A. Kropski, M. G. Jones, J. S. Lee, G. Rossi, T. Karamitsakos, T. M. Maher, A. Tzouveleakis, C. J. Ryerson, Idiopathic pulmonary fibrosis: Disease mechanisms and drug development. *Pharmacol. Ther.* **222**, 107798 (2021).
- J. A. Galli, A. Pandya, M. Vega-Olivo, C. Dass, H. Zhao, G. J. Criner, Pirfenidone and nintedanib for pulmonary fibrosis in clinical practice: Tolerability and adverse drug reactions. *Respirology* **22**, 1171–1178 (2017).
- C. Vancheri, M. Kreuter, L. Richeldi, C. J. Ryerson, D. Valeyre, J. C. Grutters, S. Wiebe, W. Stansen, M. Quaresma, S. Stowasser, W. A. Wuyts, INJOURNEY Trial Investigators, Nintedanib with add-on pirfenidone in idiopathic pulmonary fibrosis. Results of the INJOURNEY Trial. *Am. J. Respir. Crit. Care Med.* **197**, 356–363 (2018).
- T. Pitre, J. Mah, W. Helmecki, M. F. Khalid, S. Cui, M. Zhang, R. Husnudinov, J. Su, L. Banfield, B. Guy, J. Coyne, C. Scallan, M. R. Kolb, A. Jones, D. Zeraatkar, Medical treatments for idiopathic pulmonary fibrosis: A systematic review and network meta-analysis. *Thorax* **77**, 1243–1250 (2022).
- M. Zheng, W. Zhu, F. Gao, Y. Zhuo, M. Zheng, G. Wu, C. Feng, Novel inhalation therapy in pulmonary fibrosis: Principles, applications and prospects. *J. Nanobiotechnology* **22**, 136 (2024).
- S. Sakamoto, K. Kataoka, Y. Kondoh, M. Kato, M. Okamoto, H. Mukae, M. Bando, T. Suda, K. Yatera, Y. Tanino, T. Kishaba, N. Hattori, Y. Taguchi, T. Saito, Y. Nishioka, K. Kuwano, K. Kishi, N. Inase, S. Sasaki, H. Takizawa, T. Johkoh, F. Sakai, S. Homma, Diffuse Lung Diseases Research Group of the Ministry of Health, Labour and Welfare, Japan, Pirfenidone plus inhaled N-acetylcysteine for idiopathic pulmonary fibrosis: A randomised trial. *Eur. Respir. J.* **57**, 2000348 (2021).
- B. J. Moss, S. W. Ryter, I. O. Rosas, Pathogenic mechanisms underlying idiopathic pulmonary fibrosis. *Annu. Rev. Pathol.* **17**, 515–546 (2022).
- T. Koudstaal, M. Funke-Chambour, M. Kreuter, P. L. Molyneaux, M. S. Wijsenbeek, Pulmonary fibrosis: From pathogenesis to clinical decision-making. *Trends Mol. Med.* **29**, 1076–1087 (2023).
- B. Ng, J. Dong, G. D'Agostino, S. Viswanathan, A. A. Widjaja, W. W. Lim, N. S. J. Ko, J. Tan, S. P. Chothani, B. Huang, C. Xie, C. J. Pua, A. M. Chacko, N. Guimaraes-Camboa, S. M. Evans, A. J. Byrne, T. M. Maher, J. Liang, D. Jiang, P. W. Noble, S. Schafer, S. A. Cook, Interleukin-11 is a therapeutic target in idiopathic pulmonary fibrosis. *Sci. Transl. Med.* **11**, eaaw1237 (2019).
- M. Murakami, D. Kamimura, T. Hirano, Pleiotropy and specificity: Insights from the interleukin 6 family of cytokines. *Immunity* **50**, 812–831 (2019).
- D. A. Knight, M. Ernst, G. P. Anderson, Y. P. Moodley, S. E. Mutsaers, The role of gp130/IL-6 cytokines in the development of pulmonary fibrosis: Critical determinants of disease susceptibility and progression? *Pharmacol. Ther.* **99**, 327–338 (2003).
- S. A. Cook, S. Schafer, Hiding in plain sight: Interleukin-11 emerges as a master regulator of fibrosis, tissue integrity, and stromal inflammation. *Annu. Rev. Med.* **71**, 263–276 (2020).
- Y. Zhu, L. Zhu, X. Wang, H. Jin, RNA-based therapeutics: An overview and prospectus. *Cell Death Dis.* **13**, 644 (2022).
- A. M. Yu, Y. H. Choi, M. J. Tu, RNA drugs and RNA targets for small molecules: Principles, progress, and challenges. *Pharmacol. Rev.* **72**, 862–898 (2020).
- J. A. Kulkarni, D. Witzigmann, S. Chen, P. R. Cullis, R. van der Meel, Lipid nanoparticle technology for clinical translation of siRNA therapeutics. *Acc. Chem. Res.* **52**, 2435–2444 (2019).
- S. M. Hoy, Patisiran: First global approval. *Drugs* **78**, 1625–1631 (2018).
- Q. Wan, X. Zhang, D. Zhou, R. Xie, Y. Cai, K. Zhang, X. Sun, Inhaled nano-based therapeutics for pulmonary fibrosis: Recent advances and future prospects. *J. Nanobiotechnology* **21**, 215 (2023).
- R. Diwan, H. N. Bhatt, E. Beaven, M. Nurunnabi, Emerging delivery approaches for targeted pulmonary fibrosis treatment. *Adv. Drug Deliv. Rev.* **204**, 115147 (2024).
- S. Dong, H. Fang, J. Zhu, Z. Wu, Y. Liu, J. Zhu, B. Ma, Q. Chen, Y. Yang, Inhalable siRNA targeting IL-11 nanoparticles significantly inhibit bleomycin-induced pulmonary fibrosis. *ACS Nano* **19**, 2742–2758 (2025).
- N. Zeng, X. Guan, X. Liu, H. Shi, N. Li, R. Yang, Y. Zhou, Fibroblast activation protein-sensitive polymeric nanobeacon for early diagnosis of renal fibrosis. *Biosens. Bioelectron.* **253**, 116144 (2024).
- P. Yang, Q. Luo, X. Wang, Q. Fang, Z. Fu, J. Li, Y. Lai, X. Chen, X. Xu, X. Peng, K. Hu, X. Nie, S. Liu, J. Zhang, J. Li, C. Shen, Y. Gu, J. Liu, J. Chen, N. Zhong, J. Su, Comprehensive analysis

- of fibroblast activation protein expression in interstitial lung diseases. *Am. J. Respir. Crit. Care Med.* **207**, 160–172 (2023).
31. Y. Mori, V. Kramer, E. Novruzov, E. Mamlins, M. Rohrich, R. Fernandez, H. Amaral, C. Soza-Ried, B. Monje, E. Sabbagh, M. Florenzano, F. L. Giesel, A. Undurraga, Initial results with [^{18}F]FAPi-74 PET/CT in idiopathic pulmonary fibrosis. *Eur. J. Nucl. Med. Mol. Imaging* **51**, 1605–1611 (2024).
 32. A. Sanchez, D. Loughrey, E. S. Echeverri, S. G. Huayameres, A. Radmand, K. Paunovska, M. Hatit, K. E. Tiegreen, P. J. Santangelo, J. E. Dahlman, Substituting poly(ethylene glycol) lipids with poly(2-ethyl-2-oxazoline) lipids improves lipid nanoparticle repeat dosing. *Adv. Healthc. Mater.* **13**, e2304033 (2024).
 33. Y. Wang, W. T. Song, M. Y. Hu, S. An, L. G. Xu, J. J. Li, K. A. Kinghorn, R. H. Liu, L. Huang, Nanoparticle-mediated HMGA1 silencing promotes lymphocyte infiltration and boosts checkpoint blockade immunotherapy for cancer. *Adv. Funct. Mater.* **28**, (2018).
 34. L. Chen, Z. Kang, J. Shen, R. Zhao, Y. Miao, L. Zhang, Z. Zheng, Z. Zhang, N. Liu, C. Wang, H. Fang, J. Zhou, Y. Wang, Z. Liu, Y. Yang, Q. Chen, An emerging antibacterial nanovaccine for enhanced chemotherapy by selectively eliminating tumor-colonizing bacteria. *Sci. Bull.* **69**, 2565–2579 (2024).
 35. J. Zhao, W. Zhu, Y. Mao, X. Li, G. Ling, C. Luo, P. Zhang, Unignored intracellular journey and biomedical applications of extracellular vesicles. *Adv. Drug Deliv. Rev.* **212**, 115388 (2024).
 36. Y. Miao, L. Niu, X. Lv, Q. Zhang, Z. Xiao, Z. Ji, L. Chen, Y. Liu, N. Liu, J. Zhu, Y. Yang, Q. Chen, A minimalist pathogen-like sugar nanovaccine for enhanced cancer immunotherapy. *Adv. Mater.* **36**, e2410715 (2024).
 37. Y. Zhao, Y. Zhou, D. Wang, Y. Gao, J. Li, S. Ma, L. Zhao, C. Zhang, Y. Liu, X. Li, pH-responsive polymeric micelles based on poly(2-ethyl-2-oxazoline)-poly(D,L-lactide) for tumor-targeting and controlled delivery of doxorubicin and P-glycoprotein inhibitor. *Acta Biomater.* **17**, 182–192 (2015).
 38. I. E. Fernandez, O. Eickelberg, New cellular and molecular mechanisms of lung injury and fibrosis in idiopathic pulmonary fibrosis. *Lancet* **380**, 680–688 (2012).
 39. Y. Enomoto, H. Katsura, T. Fujimura, A. Ogata, S. Baba, A. Yamaoka, M. Kihara, T. Abe, O. Nishimura, M. Kadota, D. Hazama, Y. Tanaka, Y. Maniwa, T. Nagano, M. Morimoto, Autocrine TGF- β -positive feedback in profibrotic AT2-lineage cells plays a crucial role in non-inflammatory lung fibrogenesis. *Nat. Commun.* **14**, 4956 (2023).
 40. Y. R. Miao, M. Xia, M. Luo, T. Luo, M. Yang, A. Y. Guo, ImmuCellAI-mouse: A tool for comprehensive prediction of mouse immune cell abundance and immune microenvironment depiction. *Bioinformatics* **38**, 785–791 (2022).
 41. T. A. Wynn, K. M. Vannella, Macrophages in tissue repair, regeneration, and fibrosis. *Immunity* **44**, 450–462 (2016).
 42. L. Meziani, M. Mondini, B. Petit, A. Boissonnas, V. Thomas de Montpreville, O. Mercier, M. C. Vozenin, E. Deutsch, CSF1R inhibition prevents radiation pulmonary fibrosis by depletion of interstitial macrophages. *Eur. Respir. J.* **51**, 1702120 (2018).
 43. M. Y. Yang, Y. J. Lin, M. M. Han, Y. Y. Bi, X. Y. He, L. Xing, J. H. Jeong, T. J. Zhou, H. L. Jiang, Pathological collagen targeting and penetrating liposomes for idiopathic pulmonary fibrosis therapy. *J. Control. Release* **351**, 623–637 (2022).
 44. C. Li, X. Feng, S. Li, X. He, Z. Luo, X. Cheng, J. Yao, J. Xiao, X. Wang, D. Wen, D. Liu, Y. Li, H. Zhou, L. Ma, T. Lin, X. Cai, Y. Lin, L. Guo, M. Yang, Tetrahedral DNA loaded siCCR2 restrains M1 macrophage polarization to ameliorate pulmonary fibrosis in chemoradiation-induced murine model. *Mol. Ther.* **32**, 766–782 (2024).
 45. X. Bai, G. Zhao, Q. Chen, Z. Li, M. Gao, W. Ho, X. Xu, X. Q. Zhang, Inhaled siRNA nanoparticles targeting IL11 inhibit lung fibrosis and improve pulmonary function post-bleomycin challenge. *Sci. Adv.* **8**, eabn7162 (2022).
 46. E. F. Redente, B. P. Black, D. S. Backos, A. N. Bahadur, S. M. Humphries, D. A. Lynch, R. M. Tuder, R. L. Zemans, D. W. H. Riches, Persistent, progressive pulmonary fibrosis and epithelial remodeling in mice. *Am. J. Respir. Cell Mol. Biol.* **64**, 669–676 (2021).
 47. A. L. Mora, M. Rojas, A. Pardo, M. Selman, Emerging therapies for idiopathic pulmonary fibrosis, a progressive age-related disease. *Nat. Rev. Drug Discov.* **16**, 810 (2017).
 48. A. S. Castanza, J. M. Recla, D. Eby, H. Thorvaldsdottir, C. J. Bult, J. P. Mesirov, Extending support for mouse data in the Molecular Signatures Database (MSigDB). *Nat. Methods* **20**, 1619–1620 (2023).

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