nature portfolio

Peer Review File

Targeted immunotherapy rescues pulmonary fibrosis by reducing activated fibroblasts and regulating alveolar cell profile

Corresponding Author: Professor Yunlong Huo

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, Yan and co-workers reported mRNA-LNP mediated FAP-CART transfection in the spleen to treat FAP-based disease in a preclinical IPF mice model. The authors used liver-directed benchmark MC3 LNP to incorporate β-sitosterol and further conjugate with CD5 antibody for targeted therapy. All of these design parameters have already published before. After IV injection with this design, the authors demonstrated delivery of FLuc mRNA to the spleen, and FAP-CAR mRNA delivery to the splenic T cell. In the lung fibrosis model, FAPCART eliminated overactivated fibroblast, decrease ECM stiffness, and restore epithelial cell polarization for therapeutic treatment. Even this study provides some interesting application of FAPCART for lung fibrosis therapy, however, the low novelty of current delivery platform, lack of enough data demonstration, and logic/writing stuff, making it not suitable for publication in its current form.

1. What's this experiment for in Figure 2G? It is not clear why the authors detect cytokine release for co-culturing FAPCART and 3T3 cell.
2. How did the author get this copy number of mRNA in T cells?
3. Figure 2I only showed the co-localization of LNP and T cell in the spleen. It is difficult to know if LNP can transfect T cell in the spleen. And it is also wired that why the authors did not show the co-localization of LNP and other splenic cells, like macrophages, DC, and B cells.
4. What is the meaning of RBC in Figure 3c? I am not sure, but why the authors only give limited information to understand their paper.
5. Did the FAPCAR therapy change the macrophage polarization? how does M1 /M2 look like before and after the treatment?
6. There is no evidence about the infiltration of FAPCART cell into the fibrotic tissue? how FAPCART entrapped into the fibrotic lung? what is the ratio of FAPCART can deliver from the spleen to the lung? how does the fibrotic lung look like after FAPCAR treatment? This is the most important part for FAPCART to recover lung damage, these detailed experiments should be added from how FAPCART infiltrated lung, and the efficacy to remove FAP.
7. The total pathway (Figure 4G) should be demonstrated clearly by further experiments, rather than a schematic image.
8. Can the author provide more comparison experiment about the efficacy of FAPCART in treatment lung fibrosis? Some commercial benchmark should be added to show the advantage of FAPCART therapy in this study.
9. The authors need to provide detailed information about the MC3 mediated T cell transfection. Based on our understanding, even using CD5 antibody for modification, ~80% transfection of T cell is still very high to be reached.

Reviewer #2

(Remarks to the Author)
Summary:

In this manuscript, Yan et al. describe a modified LNP-mRNA system for generation of FAPCAR-T cells to target FAP-expressing cells in a mouse bleomycin model. Authors showed that FAPCAR-T cells reversed fibrosis markers in a single experimental lung fibrosis model. They subjected the whole lungs from the intervention approach to proteomic profiling,

which showed a decrease in fibrotic ECM proteins and an increase in epithelial cell polarization marker.
The topic of this study is very timely and of interest, it follows upon recent papers using the same approach for cardiac fibrosis (e.g. Rurik et al Science 2022) by applying FAPCAR-T cells as a potential new therapeutic approach. Here, the authors used the same approach but investigate the effect on a experimental lung fibrosis model. While this approach is novel in the lung, the overall approach is not very original and there are several shortcomings in the assessment of lung fibrosis as well as the lack of mechanistic insight above what is known, which dampen the enthusiasm for this study. In addition, data demonstrating the potential human relevance for IPF patients is missing. The manuscripts barely acknowledge relevant literature in the field (both for LNPs as well as lung fibrosis). Overall, the manuscript seems immature and a rushed without providing the rigorous investigation that this potentially important new therapeutic approach requires.

Major points:
1. This manuscript from Yan et al. builds completely on the work by Rurik et al (2022) Science. 2022 Jan 7; 375(6576): 91–96. (reference 12), and most parts of figure 1 and 2 are incremental. Also limits overall originality in the approach. FAPCAR-T cells (reference 12) were previously used in cardiac injury model in mouse. Although FAPCAR-T cell targeting FAP expressing fibroblasts is not novel, its use in bleomycin model is novel and potentially pathbreaking. A thorough analysis of fibrosis-clearing pathways using molecular and computational methods is beneficial to the field of lung fibrosis. Authors characterized the FAPCAR-T cells similar to what was done in reference 12 but the lung fibrosis part of the manuscript is lacking proper analysis. The authors refer to the bleomycin model as an “IPFF model, which is not correct and well known and discussed in the lung community. Moreover, they analysis of lung fibrosis is limited and not well described. It misses lung function, hydroxy proline assessment an additional fibrosis marker readout, all well accepted gold standard for lung fibrosis assessment (PMID: 28459387).
2. In addition, the single bleomycin models has some caveats as it is self-resolving and the authors would need to confirm they findings in a repetitive bleomycin model or using old mice.
3. They are using a preventive approach, which is less informative and a therapeutic approach should be included.
There is further a complete control arm missing with giving CAR T cells in the healthy lung which needs to be included for rigorous and robust study design.
4. Overall, given these limitation, the authors arrived at several bold conclusions that are not backed sufficiently.
5. The proteomic profiling is an elegant addition to the study, however, there is very limited analysis of these data, especially to known new pathways and cell types that are relevant in lung fibrosis (such as ADI cells). Further corroboration and mechanistic insight is needed to advance the potential impact of this study compared to what is known already. In detail: A lot is inferred from proteomics data/KEGG pathway analysis with bold assertions. Statements such as “EMT markers were reduced’ or ‘epithelial cell polarization’ or ‘slowed down AT2 to AT1 differentiation” require corroborative evidence other than proteomics data. These statements are trouble-some. This requires further substantiation with experimental proof as described above in major point 7. Lung fibrosis is reversed but the manuscript lacks mechanistic understanding of the process. Moreover, pre-alveolar type-1 transitional cell state (PATS) described in reference 17 has been previous established using single cell transcriptomics. It might be important to establish a role for PATS with other methodologies along with the existing proteomics data.
6. One major weakness with the approach is that the study does not provide quantitative data for FAP expressing cells in lung fibrosis (or human IPF) to support the premise of the study. Authors state that there is a reduction in FAP expressing cells in S3C based on IHC, but this is not quantifiable and not convincing. This is a very important proof of conept control to include (ideally flow cytometry, which is doable as FAP is a surface marker). What are the FAP expressing cells, how many are there in healthy lungs versus fibrotic lungs? These are supposed to be on fibroblasts and recent paper highlight that there are several subtypes of fibroblasts in the fibrotic lung (e.g. PMID: 38987592). In detail: FAP is also expressed by healthy fibroblasts based on IPF cell atlas. It is not clear which fibroblasts are targeted by FAPCAR-T cells: healthy or myofibroblasts? Not clear from IHC. Depicting other fibroblastic markers and/or cell types using RNAscope on mouse lungs would be more convincing. What other cell types are ablated with FAPCAR-T cells in mouse lung? Perhaps single cell transcriptomics analysis would answer that. Without establishing FAP expression by RNA or protein in healthy vs bleo-injured lungs, it would be hard to interpret. Authors need to label or include arrows to indicate FAP staining in Fig 2D and Fig S3C.
7. Cholesterol analogues (b-sitoserol) were previously shown to improve endosomal escape and transfection efficiency in LNP-mRNA encapsulation (Nat Commun. 2020 Feb 20;11(1):983.). Authors need to refer to literature and cite the previous work appropriately to establish the rationale.
8. LNP-mRNA system for CAR-T cells has been established previously. It makes sense to clearly state how similar or dissimilar this manuscript is to reference 12 in terms of FAPCAR-T generation. Methods on generation of FAPCAR 2.1 and 2.2 needs to be elaborated and complete. What is the origin of scFv sequence used in FAPCAR-T cells? Is this similar to the one used in reference 12? Is FAPCAR 2.2 better than 2.1 because of mutated TM? What is the rationale here?
Minor:
1. The authors need to pay attention to the typographical errors. This manuscript will need a native English speaker for fixing incomplete sentences, spelling mistakes and grammar. For example, please see page 4, line 4 for an incomplete sentence. Examples where rewording or expansion of acronyms is required: ‘Effectively killing assay’, ‘Astral data-independent acquisition (DIA) Proteomic’. Figure S3: word ‘anterior’ is misspelled.
2. Details are missing in the methods section. For example, authors say ‘Union connector and peek tubes’. How were the LNPs synthesized? By a microfluidic device or in bulk?
3. Fig S5: Please indicate what B1-B4 and D1-D4 stand for.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #5

(Remarks to the Author)
This is an interesting manuscript demonstrating that idiopathic pulmonary fibrosis (IDF) can be potentially reversed in a mouse model of fibrosis using transient FAPCAR-T cells generated in vivo and delivered via LNP-mRNA system.

There are a number of aspects that require revision before it can be potentially accepted for publication.
For example, the grammar in the manuscript is poor at times with many grammatical errors throughout the manuscript. Even in the materials and methods the language interchanges between the present and the past tenses. Examples of some errors are below (too many to go through in detail so have just selected the Abstract to give some examples!)

Abstract
line 2/3 - 'Idiopathic pulmonary fibrosis (IPF) is a severe lung disease in the world, but has limited clinical therapies.' Change to 'Idiopathic pulmonary fibrosis (IPF) is a severe lung disease worldwide, but has limited clinical treatments.'
line 3 - 'Fibrosis maintains dynamic balance with overactivated fibroblasts.' - rewrite this sentence - poorly written.
line 4 - 'the lung may have self-repairing ability if fibrosis...' Change to 'the lung may have self-repairing abilities if fibrosis ...'
line 5 - 'The aim of the study is to evaluate therapeutic activity ..' Change to 'The aim of the study is to evaluate the therapeutic activity ...'
line 6 - '..address the relevant mechanism to epithelial cell polarization' Change to 'understand the relevant mechanisms of epithelial cell polarization'
line 9 - 'limited cell's self-healing ability.' Change to 'limited the self-healing ability of the cells.'
lines 11/12 -'The decreased ECM stiffness and the improvement of extracellular environment facilitated re-establishment of cell polarity and restored the self-repairing ability of epithelial cell.' Change to 'The decreased ECM stiffness and the improvement of the extracellular environment facilitated the re-establishment of cell polarity and restored the self-repairing ability of epithelial cells.'

Page 3, line 2/3 - 'This stimulated numerous anti fibrosis studies given limited therapies.' Rewrite this sentence to make it clearer to the reader.

Pg 4, line 13 - define 'BW'

Please clarify FAPCAR-T cells earlier in the manuscript.

Section - Proteomes for 0, 14 and 28 days after administration of bleomycin
This section needs some rewriting and clarification as the authors need to specifically clarify what samples have been compared, how many mice (i.e. the power of the study, n=?). What controls were used, etc.? It's very difficult to interpret these results without including this detail.

The data in figure 1B and figure 4 is not clear.

For the K-means clustering did all 4126 DEPs fall into the 6 clusters that have been shown? Again, detail is lacking. What comparative analysis gave 4126 proteins? Was this a combination of the 14 and 28 days of treatment? What about the 'time 0'?

In the materials and methods - what is meant by 'Maximum number of variable modifications was 1.' in the context of the previous sentence where 2 variable modifications were listed?

What specific software was used for differential proteomic analysis in order to yield the numbers of differentially expressed proteins.

How many matched peptides were used the determine that a protein was differentially expressed? Again, this sort of information is required before this section of the results can be properly reviewed.

Also please use a better title - example - 'Comparative proteomic analysis after treatment with bleomycin for 14 and 28 days'

or alternative!

In the sentence in this section 'Hence, we depicted the polarization molecular map of epithelial cells based on the KEGG analysis (Fig. 4G) albeit epithelial-mesenchymal transformation (EMT) and macrophage polarization might mislead bioinformatic enrichment results for epithelial cell polarization.' - what is meant by 'mislead the bioinformatic enrichment results'. This is unclear.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thanks to the author so much for revision of this manuscript. The authors addressed most of my concerns, I only have one more comment after the revision.

1. Figure 2I, could the author provide flow cytometry results of the LNP delivery to the splenic T cells? Or at least the immunostaining of the whole spleen section? Only a small area of T cell co-localization of LNP could not be strong enough as the in vivo T cell transfection is very low (~3%, Figure R1). And please also comment if ~3% CAR-T cells transfection in vivo is good enough for therapy (compared with previous studies).

Reviewer #2

(Remarks to the Author)
Final review:
The authors added hydroxyproline assay, wet-weight data, and elasticity modulus test that corroborate the results from bleomycin injury experiments
The authors added pirfenidone to the bleomycin experiments. Although it did not affect bleomycin injury, it shows that FAPCAR-T/CD5-LNPs are superior to FDA-approved fibrosis drugs.
The authors appropriately added citations and properly cited previous works.
The authors improved the methods sections by adding details on the design of FAPCAR-T.
The authors added flow cytometry to determine whether FAP-expressing cells were exclusively fibroblasts (pdgfra+) and their depletion upon treatment with FAPCAR-T/CD5 LNPs.
The authors should clarify Figure 3G and Page6; line 23: What are pulmonary cells? Please clarify.
Figure 5H should be together with the panels in Figure 4.
Page 9: Lines 17-18: Please rewrite this sentence. There is no logical progression between the two statements.
The new scRNA seq experiment showed the Col1a1 and Col3a1 expressing fibroblast populations were specifically depleted upon treatment with FAPCAR-T/CD5 LNPs.
The authors also reanalyzed the existing scRNA dataset GSE132771 and showed that FAP expression is IPF-specific.
The authors expanded on epithelial polarization experiments corroborating proteomics data.
The authors also showed PATS by way of scRNA seq that corroborated proteomics data.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #5

(Remarks to the Author)
All my comments have been addressed.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed my concerns and I now recommend publication.

Response to Reviewers' comments

Reviewer #1

In this manuscript, Yan and co-workers reported mRNA-LNP mediated FAP-CART transfection in the spleen to treat FAP-based disease in a preclinical IPF mice model. The authors used liver-directed benchmark MC3 LNP to incorporate β-sitosterol and further conjugate with CD5 antibody for targeted therapy. All of these design parameters have already published before. After IV injection with this design, the authors demonstrated delivery of FLuc mRNA to the spleen, and FAP-CAR mRNA delivery to the splenic T cell. In the lung fibrosis model, FAPCART eliminated overactivated fibroblast, decrease ECM stiffness, and restore epithelial cell polarization for therapeutic treatment. Even this study provides some interesting application of FAPCART for lung fibrosis therapy, however, the low novelty of current delivery platform, lack of enough data demonstration, and logic/writing stuff, making it not suitable for publication in its current form.

Answer: Thanks for your valuable suggestions. We revised the manuscript as suggested. Proteomic results were validated by substantial experiments. We also demonstrated the single-cell RNA sequencing of mouse lungs before and after CD5/βLNP-FAPCAR 2.2 treatment. Furthermore, we conducted a comprehensive validation in aged mouse (16-month-old) model as suggested by another reviewer. The "Healthy+CD5/βLNP-FAPCAR 2.2" control group and Pirfenidone (PFD) treatment group were added in the young mouse model. The revised manuscript has undergone the English Language Editing provided from Springer Nature. Please see the below responses. Thank you.

1. What this experiment for in Figure 2G? It is not clear why the authors detect cytokine release for co-culturing FAPCART and 3T3 cell.

Answer: Thanks for your valuable suggestion. The aim of the experiment was to detect the cytokine release level with different CAR sequence designs. In comparison with the traditional FAPCAR design (FAPCAR 2.1), CD28 transmembrane domain (TMD) was replaced by the specifically-designed sequence based on an ab initio Rosetta atomistic modeling (Weinstein et al. *PLOS Computational Biology*. 2019, 15:e1007318) and a de novo design programmable membrane proteins (proMPs) strategy (revised Reference 16).

In addition to the intracellular activation sequences (4-1BB&CD3ζ domains), previous studies have shown that transmembrane fragments derived from CD28 has the potential to recruit additional costimulatory signaling, which causes over-activation of CAR-T cells (Brudno et al. *Nature*

*Medicine.*2020, 26:270–280; Majzner et al. *Cancer Discovery.* 2020, 10:702–723). Although the enhanced potency and higher toxicity of CD28 TMD CARs might be beneficial in cancer treatment, it can cause nonessential cell activation and cytokine release in nononcology applications. Thus, in the present study, we detected the targeted lysis (Figure 2F) and cytokine release (Figure 2G) to screen the CAR designs. CD5/βLNP-FAPCAR 2.2 led to an ~30% reduction in cytokine release compared with CD5/βLNP-FAPCAR 2.1 without altering lysis capacity, indicating the superiority of the optimized TMD for pulmonary fibrosis treatment. This part has been added to the revised manuscript: Page 5, Line 4-9, 16-20.

2. How did the author get this copy number of mRNA in T cells?

Answer: The method for copy number experiment (revised Figure S2B) was described in **PCR strategy** part, **Methods** section (Page 44, Line 10-21). Briefly, we extracted total RNA from transfected T cells and performed RT-qPCR to obtain sample CT values. The standard CT values was performed with qPCR of gradient cDNA obtained from transcription of certain amount IVT mRNA. The concentration of standard mRNAs were transformed into copy numbers according to the formula: copy number (copies/μL)=$6.02\times10^{23}\times$((concentration ng/μL)$\times10^{-9}$/ base number×340) as described in a previous study (Kheirolomoom et al. *Biomaterials*. 2022, 281:121339. revised Reference 47). The standard curve was fitted with the CT value of the qPCR and the log value of the standard RNA copy number.

3. Figure 2I only showed the co-localization of LNP and T cell in the spleen. It is difficult to know if LNP can transfect T cell in the spleen. And it is also wired that why the authors did not show the co-localization of LNP and other splenic cells, like macrophages, DC, and B cells.

Answer: Thanks for your valuable suggestion. We added immunofluorescence experiments to show T cell transfection of LNPs in the spleen and lung. Splenic T cells, B cells, macrophages and DCs were stained with CD3, CD19, F4/80 and CD11c, respectively. And CD5/βLNP was labeled with PKH26 before tail vein injection. Analysis of splenic sections showed CD5/βLNP mainly colocalized with T cells and rarely colocalized with B cells or DCs. In addition, partial colocalization with F4/80 indicated the phagocytosis of exogenous LNPs by macrophages (revised Fig. 2I, S2D).

In FAPCAR 2.2 IVT plasmid, a tdTomato sequence was added after the P2A peptide. We detected the fluorescence expression that showed the colocalization of tdTomato and CD3 in the spleen and

fibrotic lung. This indicated the CD5/βLNP transfected to T cells successfully and FAPCAR T cells could migrate to fibrotic area (revised Fig. 3E).

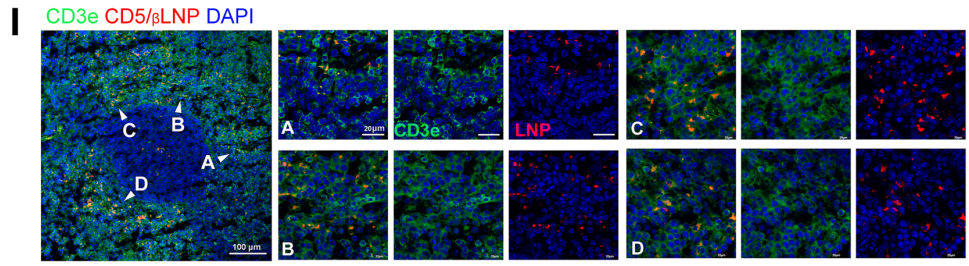


Figure 2I

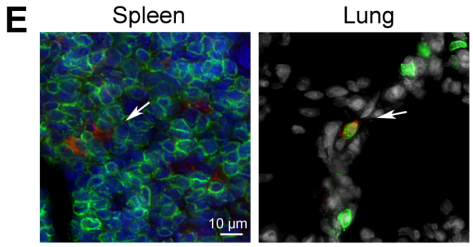


Figure 3E

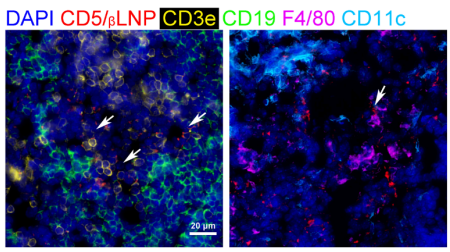


Figure S2D

4. What is the meaning of RBC in Figure 3c? I am not sure, but why the authors only give limited information to understand their paper.

Answer: We apologize for the confusion caused by the ambiguous description in the manuscript. The RBC in Figure 3C referred to the red blood cells remaining in the lung tissue. Although the sample tissues were fully perfused before the experiments, residual red blood cells still produced spontaneous fluorescence in both red and green channels (merged in yellow) during immunofluorescence imaging. To solve this problem, in the revised manuscript, red blood cell fluorescence quenching reagent was used to perform the CD3 immunofluorescence staining in fibrotic lung. The revised images have been added as Figure S3B.

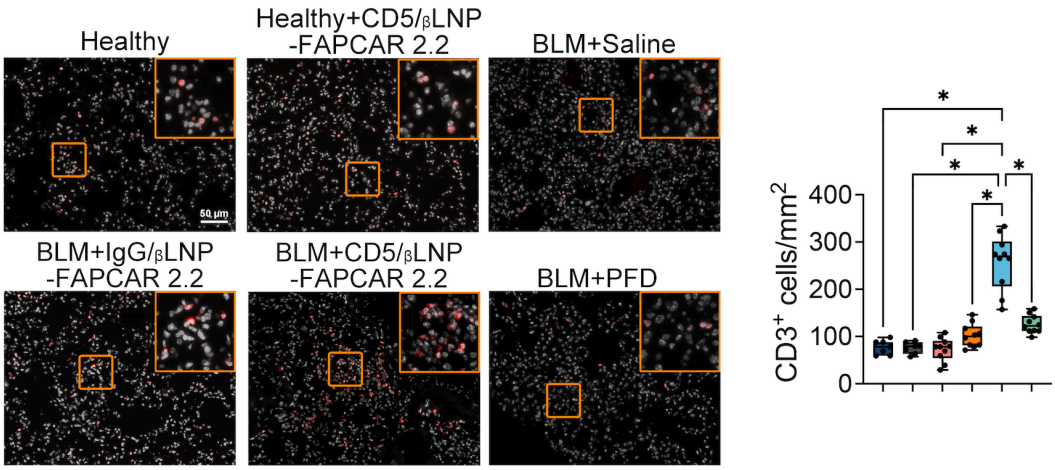


Figure S3B

5. Did the FAPCAR therapy change the macrophage polarization? how does M1 /M2 look like before and after the treatment?

Answer: Thanks for your valuable suggestion. We used flow cytometry, immunofluorescence staining and scRNA-seq to detect the macrophage phenotypes before and after treatment. The pulmonary cell suspension was labeled with F4/80, CD86 and CD163 to mark the macrophages and M1/M2 phenotype. The flow cytometry analysis showed the increased number of macrophages marked with F4/80 in inflammatory fibrotic lungs, which decreased after CD5/βLNP-FAPCAR 2.2 treatment (revised Fig. 6C). The analysis of the subphenotype revealed that the treatment decreased the number of M2 macrophages that were identified as the profibrotic phenotype (revised Fig. 6D, E and S6D, E). A subgroup, characterized by double-positive expression ($CD86^+/CD163^+$), increased in both CD5/βLNP and PFD treatment groups (revised Fig. S6F). Given recent findings that M1/M2 phenotypes are not mutually exclusive in macrophages, the cells in the therapeutic groups may undergo a state shift (Yuan F et al. *Front Immunol*. 2022, 13:798583; Shi Y et al. *Genome Biol*. 2022, 23:87). Similar results were also observed in the BLM model of aged mice (revised Fig. S6G, H). This part has been added to the revised manuscript: Page 10, Line 9-20.

Moreover, the scRNA-seq analysis was performed to show the cell lineage before and after CD5/βLNP-FAPCAR 2.2 treatment. The macrophages were divided into 10 clusters, of which 4 clusters (clusters 2, 4, 5, 7) steadily decreased and 3 clusters (clusters 0, 3, 8) increased after treatment. Clusters 0, 1, 2, 4, 5 and 6 were enriched for alveolar macrophage (AM) markers (Ear1, Ear2, Chil3, Plet1). The others were monocyte-derived macrophages (mono-M) that expressed Ccr5, CD33, Sell and Itgam (revised Fig. 8A-C, S8C). Clusters 2, 4 and 5 were identified as profibrotic lipid-associated macrophages (LAMs) because of high expression of profibrotic genes (Fn1, Spp1, CD44) and lipid

metabolism genes (Lpl, Trem2, Fabp4, Fabp5). Clusters 7 and 9 expressed inflammatory genes (Il1b, S1009a, S1008a) and typical bone marrow marker gene (CD33), indicating their monocyte lineage (revised Fig. 8C, S8D). Cluster 7 participated in profibrotic processes too. The decreased macrophages (clusters 2, 4, 5, 7) suggested that FAPCAR-T cells disrupted the viscous inflammatory-fibrosis cycle via the clearance of overactivated fibroblasts, which was consistent with the flow cytometry and proteomics results.

The population of the increased expression with treatment (clusters 0, 3, 8) may be involved in tissue reconstruction process. Clusters 3 and 8 had common features of high Apoe expression and CD11b positive, which were significantly elevated after CD5/βLNP-FAPCAR 2.2 treatment (revised Fig. S8C, D). Cluster 8 was also enriched with the highest MHC genes expression, indicating mature and activated features in the macrophage lineage. The two clusters expressed lipid related genes, but exhibited the low Lpl expression, which distinguished them from other LAMs that were mainly associated with fibrosis or inflammation. Therefore, we explored potential effects of the $Apoe^{+}$ mono-M population on tissue regeneration. Since Apoe is a type of secreted protein, we first evaluated cellular communications. CellphoneDB analysis revealed strong interaction between alveolar and $Apoe^{+}$ macrophages (revised Fig. 8D). Trem2 has been reported to be a receptor that triggers differentiation and development of myeloid cells. Protein interaction analysis showed that Apoe and Trem2 ligand pairs appeared only in the cluster 1/3 group (revised Fig. 8E). The findings suggested that $Apoe^{+}$ macrophages could help stimulate differentiation and development of myeloid cells. To further confirm the findings, pseudotime analysis revealed that clusters expressing high monocyte markers served as root cells, and macrophages differentiated through the intermediate cluster 3 to produce the activated mature innate immunologic cells or the mature alveolar macrophages (revised Fig. 8F, S8E). Accordingly, RNA velocity analysis showed the development path from the $Apoe^{+}$ mono-M to AM clusters (revised Fig. 8G, S8F). Overall, fibrosis-related alveolar macrophages were significantly reduced by the CD5/βLNP-FAPCAR 2.2 treatment, and $Apoe^{+}$ mono-M population participated in quantitative supplementation of alveolar macrophages to maintain their phenotype for the reconstruction of immunity.

This part has been added in revised manuscript: Page 13,14, Figure 8, S8.

Figure 6

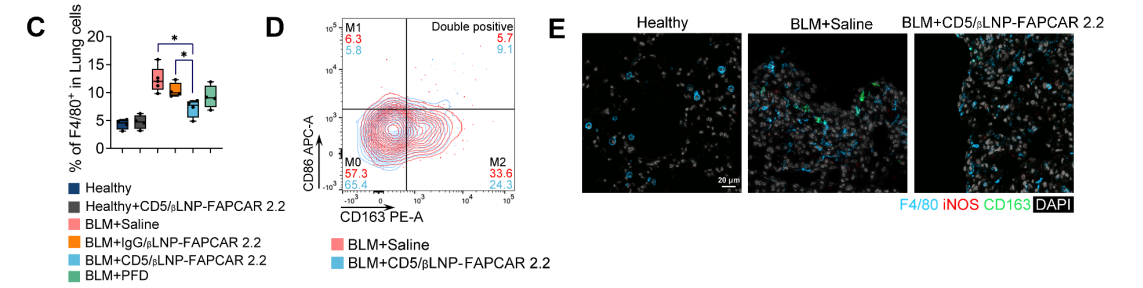


Figure S6

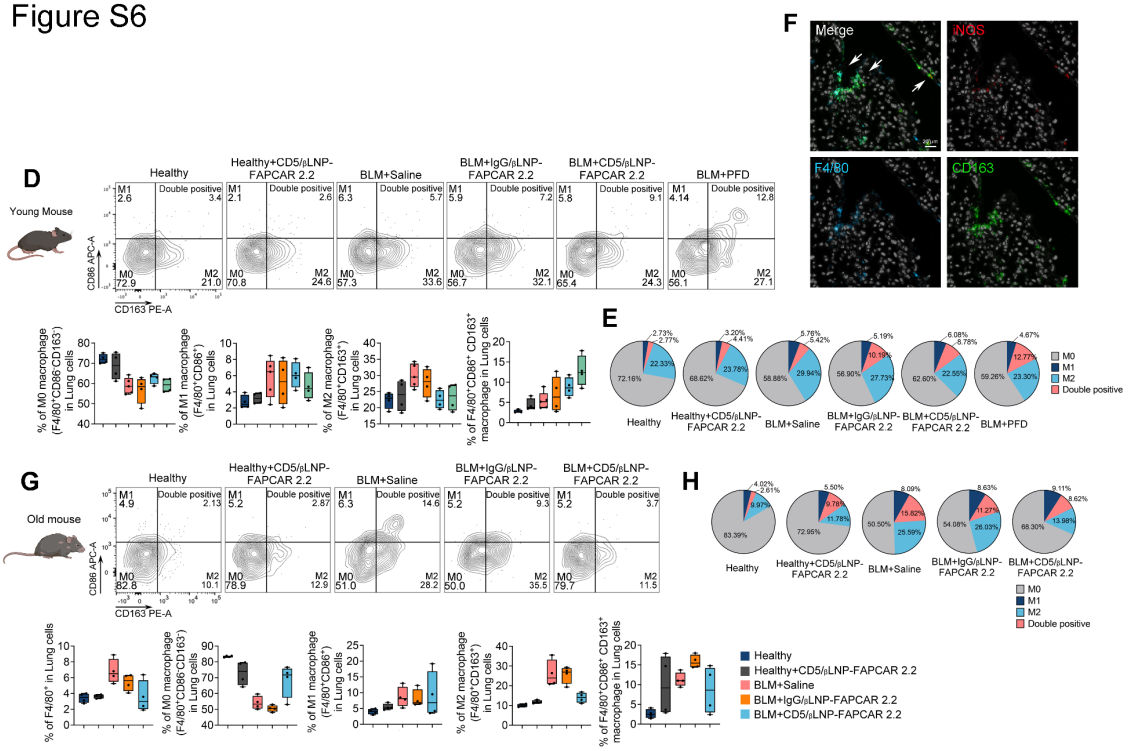


Figure 6C-E and Figure S6D-H.

6. There is no evidence about the infiltration of FAPCART cell into the fibrotic tissue? how FAPCART entrapped into the fibrotic lung? what is the ratio of FAPCART can deliver from the spleen to the lung? how does the fibrotic lung look like after FAPCAR treatment? This is the most important part for FAPCART to recover lung damage, these detailed experiments should be added from how FAPCART infiltrated lung, and the efficacy to remove FAP.

Answer: Thanks for your valuable suggestion. We established a tdTomato report gene within FAPCAR 2.2 mRNA. In spleen and fibrotic lung, we detected the CD3 and tdTomato coexpression T cells, providing evidence for LNP transfection and FAPCAR T cell infiltration into lung tissue (revised Figure 3E). T cells in the blood circulation or spleen were transfected with LNP to express FAPCAR. FAPCAR T cells resulting from the spleen were mobilized into the peripheral circulating blood. When

blood flows through the fibrotic lungs, FAPCAR T cells, targeting FAP positive fibroblasts via antigen-antibody binding, are entrapped into the fibrotic lung.

We added the images of lung tissue, the ratio of FAPCAR T cells to $CD3^+$ pulmonary cells and the ratio of FAP positive fibroblasts to pulmonary cells before and after treatment. Lung tissue were fully perfused before harvest. Exterior images of the entire lung revealed that fibrotic lungs showed red lesion due to interstitial red blood cell leakage caused by tissue damage, whereas the lungs in the healthy group and in the CD5/βLNP treatment group were white (revised Fig. 3J). The ratios of FAPCAR T cells to T cells in lung and spleen of BLM mice were detected by the flow cytometry and only CD5/βLNP treatment group showed the infiltration of FAPCAR T cells in lung. There were ~3% splenic T cells and ~2% pulmonary T cells expressing FAPCAR (revised Fig. 3F, S3C, S3D). Although the spleen is an important organ for storing T cells, T cells in circulating blood, lymph nodes or lung residue T cells could also be the target cell of CD5/βLNP-FAPCAR 2.2 and all of them might be beneficial in clearing fibrosis. Hence, we did not identify the ratio of FAPCAR T cells migrated from spleen to lung. FAP positive fibroblasts, marked with Pdgfra and FAP, were analyzed with flow cytometry in pulmonary cells. The FAP^+ fibroblast population decreased significantly in the CD5/βLNP group (revised Fig. 3G, S3E). The analysis was also performed in BLM-model of aged mice (revised Fig. S4B).

In summary, these supplementary experiments provide more favorable evidence for the process of transient FAPCAR T cell clearance of pulmonary fibrosis. We added these results and figures in revised section, 'CD5/βLNP-FAPCAR 2.2 therapy reversed pulmonary fibrosis', Page 6.

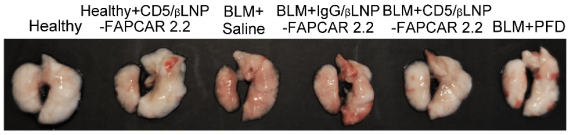


Fig. 3J

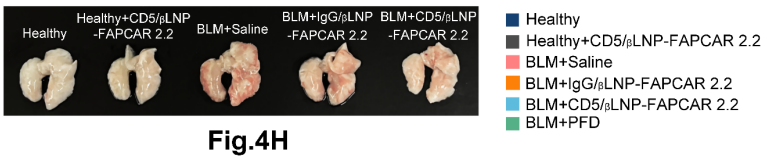


Fig.4H

Figure 3

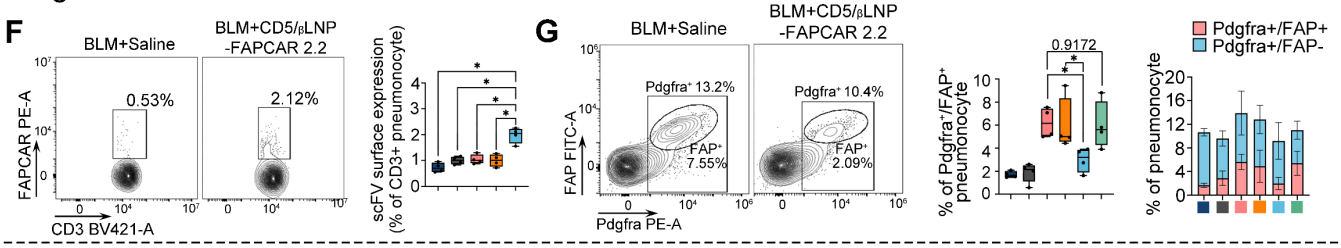


Figure S3

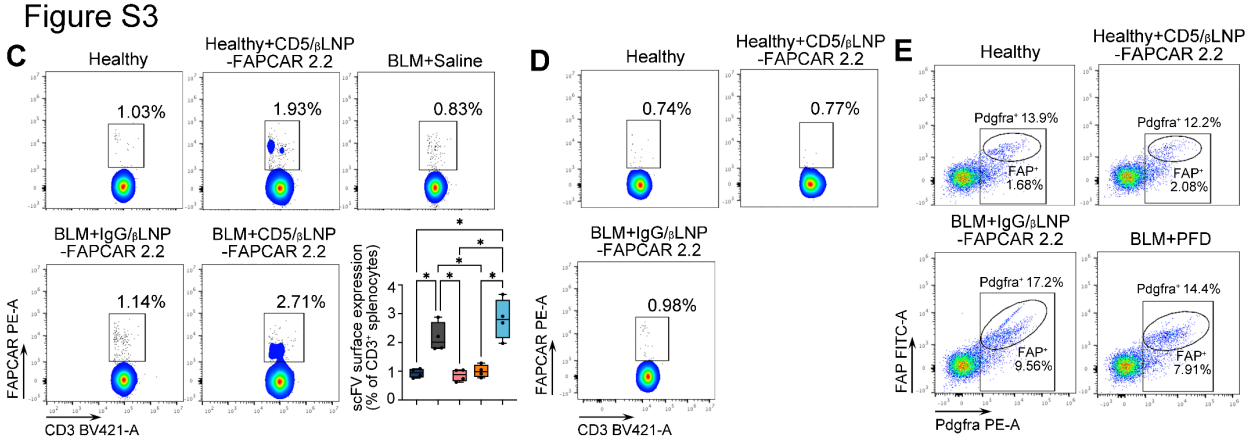


Figure 3F, G and Figure S3C-E

7. The total pathway (Figure 4G) should be demonstrated clearly by further experiments, rather than a schematic image.

Answer: We carried out immunofluorescence staining, scRNA-seq, western blotting and mechanical test to confirm the total pathway as suggested by the reviewer. Briefly, the proteomic results at different time points after BLM treatment suggested the accumulation of KRT8-positive cells (PATS). Previous studies have indicated that this is due to the stagnation of AT2 cell differentiation caused by the increase of ECM stiffness induced by fibrosis (Kobayashi Y et al. *Nat Cell Biol*. 2020, 22: 934-946. revised Reference 22). The elastic modulus of fibrotic lung tissue determined from the Mechanical Testing System significantly increased, while the tissue re-softened due to the fibrosis clearance after CD5/βLNP treatment (revised Fig. 5G, H). Immunofluorescence images revealed that the accumulation of PATS was particularly obvious in fibrotic area in BLM+Saline group while CD5/βLNP-FAPCAR 2.2 treatment reduced the number of PATS ($KRT8^+/SFTPC^+$ double-positive) (revised Fig. 5I, S5G).

For epithelial cell polarization, we initially detected Cdc42 expression level using the western blotting. The expression of Cdc42 in lung tissue increased after CD5/βLNP-FAPCAR 2.2 treatment (revised Fig. 6A). Moreover, immunofluorescence results showed that Cdc42 and PKCz were colocalized on the cell membrane and the colocalization tended to be on one side rather than the uniform distribution throughout the cell membrane after treatment (revised Fig. 6F, G). The cytoskeleton-associated tight junction was enhanced (revised Fig. 6H). This confirmed the mechanism that 'the signaling axis formed by Cdc42 and aPKC (Pkc-zeta) is the key to polarization regulation. ECM signals are sensed and transmitted by Crb3, which recruits Cdc42-aPKC-PAR6 to form complex at the apical domain. This complex represents a key step in excluding proteins from the apical domain, which promotes the polarity distribution of proteins between apical, lateral and basement membranes via regulating cytoskeleton (Buckley. C.E et al. *Nat Rev Mol Cell Biol.* 2022, 23:559-577. Apodaca. G. et al. *Nat Cell Biol.* 2012, 14:1235-43. Hayase. J. et al. *J Cell Biol.* 2013, 200: 635-50. revised Reference 27,28,29).' This part has been added in revised manuscript: Page 10, Line 21-25; Page 11, Line 1-6.

We hypothesized that the enrichment of cytoskeleton genes was the result of epithelial cell polarization. To verify this hypothesis, it is required to exclude macrophage polarization or epithelial-mesenchymal transformation (EMT) process because both processes might occur during the disease progression. The expression of EMT markers was analyzed by the western blotting (revised Fig. 6A). Mesenchymal cells, transformed from epithelial cells, expressed low level of E-cadherin (an adhesion factor) and high level of Vimentin. Expressions of these markers were close to the normal level after CD5/βLNP-FAPCAR 2.2 treatment. Hence, EMT was inhibited by the treatment and did not contribute to polarization signaling enrichment. This part has been added in revised manuscript: Page 10, Line 2-9.

For macrophage polarization analysis, as we described in **Response to Comment 5**, total pulmonary macrophages and M2 phenotype macrophages decreased after CD5/βLNP-FAPCAR 2.2 treatment. These results suggested that there was no significant polarization of macrophages.

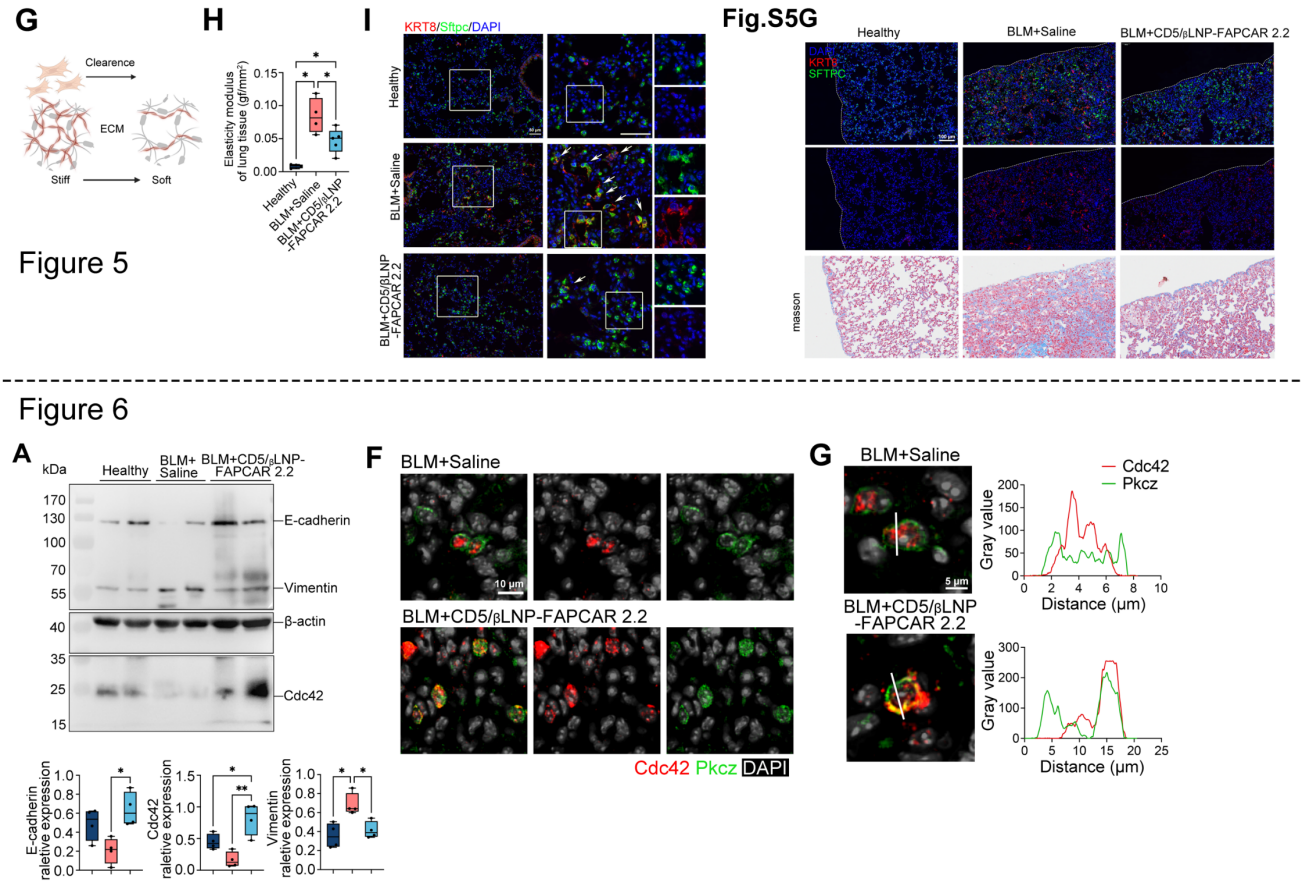


Figure 5G, 6A, F, G and Figure S5G

8. Can the author provide more comparison experiment about the efficacy of FAPCART in treatment lung fibrosis? Some commercial benchmark should be added to show the advantage of FAPCART therapy in this study.

Answer: Thanks for your kindly suggestion. We added two additional experiment groups, "Healthy+CD5/βLNP-FAPCAR 2.2" and "BLM+Pirfenidone (PFD)". The previous description, "IPF", was replaced with "BLM" in revised manuscript (BLM, bleomycin) as suggested by another reviewer. A comprehensive study in aged mice (16-month-old mouse) was also added to the study. Micro CT, Masson staining, picrosirius red staining, flow cytometry and hydroxyproline assay were used to characterize the therapeutic effect of CD5/βLNP-FAPCAR 2.2 (following the guideline of "An Official American Thoracic Society Workshop Report: Use of Animal Models for the Preclinical Assessment of Potential Therapies for Pulmonary Fibrosis", *Am J Respir Cell Mol Biol.* 2017, 56(5):667-679, revised Reference 20). The results revealed that healthy mice injected with CD5/βLNP-FAPCAR 2.2 had no significant discomfort and side effects. In comparison with three weeks of intragastric treatment with PFD (a commercial drug used in clinic to treat IPF), CD5/βLNP-FAPCAR 2.2 treatment had

advantage on inhibition of pulmonary fibrosis, including fast clearance of pulmonary fibrosis and recovery of lung function. It usually takes a long time for PDF to take effect.

CD5/βLNP-FAPCAR 2.2 treatment also achieved considerable therapeutic efficacy in aged mice. The survival rate was significantly higher and lung coefficients were lower in the CD5/βLNP group than those in the BLM+Saline and IgG/βLNP groups (revised Fig. 4E-G). The micro-CT images showed that BLM-induced pulmonary fibrosis was more severe in aged mice compared to the young when they received the same dose, i.e., larger area of regional consolidation, higher HU density and less air volume in the lung (revised Fig. 4H-J, S4A). The flow cytometry analysis showed that the population of FAP-positive fibroblasts was reduced in aged mice, demonstrating the successful generation of transient FAPCAR-T cells (revised Fig. S4B). Moreover, Masson staining and HYP quantification revealed the significant decrease of fibrosis and collagen content in aged mice after CD5/βLNP-FAPCAR 2.2 treatment (revised Fig. 4K, L, S4C). This part was added in revised section 'Antifibrotic response in aged mice', Page 7.

9. The authors need to provide detailed information about the MC3 mediated T cell transfection. Based on our understanding, even using CD5 antibody for modification, ~80% transfection of T cell is still very high to be reached.

Answer: Thanks for your valuable suggestion and we agree that your concern is justified. Two ionizable lipids were selected in our preliminary study, and found that ALC-0315 (used in COVID-19 vaccine) had ~50% positive rate under the same transfection conditions (see the below Figure R1), while D-Lin-MC3 showed a higher positive rate in T cells in all experiments. As you mentioned, we obtained ~80% transfection rate from *in vitro* experiments. *In vitro*, 3×10^5 primary T cells were transfected with 5 μg LNP (mRNA quality) and the ratio of LNPs to T cells was ~2.67×10^6:1.

According to the Lipofectamine™ 2000 (Thermo Fisher Scientific) standard transfection protocol, using 4 μg DNA or 5 μg siRNA per 5×10^5 cells could transfect ~70% of cells. In addition, in Rurik et al.'s anti-cardiac fibrosis study, mRNA expression peaked at 24 h *in vitro*, where ~81% T cells expressed GFP and ~83% expressed FAPCAR (ionizable lipid L319 was used in research) (Rurik et al. *Science.* 2022, 375:91-96). Similarly, in Tombácz et al's and Parhiz et al.'s studies, targeted cells had ~80% ZsGreen positive ratio and over 85% endothelial cells were transfected with LNPs (Tombácz et al. *Molecular Therapy*. 2021, 29:3293-3304; Parhiz et al. *Journal of Control Release*. 2018, 291:106-115). Parayath et al. delivered 1928z CAR in T cells and reached a maximum transfection efficiency of ~92% (Parayath et al. *Nature Communications*. 2020, 11:6080). Due to the simplest cell culture and LNP stimulation environment, *in vitro* transfection could achieve high efficiency.

In vivo, our results showed that ~3% of the entire splenic T cells were CAR-positive T cells. And a previous study isolated T cells from splenic cells first and then showed ~20% FAPCAR-positive rate after injection of 10 μg LNP-mRNA *in vivo* (Rurik et al. *Science.* 2022, 375:91-96). In the transfection study with DCs, the ratio of EGFP-positive DCs in the entire splenocyte after EGFP-LPX injection was approximately 5% (Kranz et al. *Nature*. 2016, 534:396-401). Furthermore, the copy number in FAPCAR-T cells were significantly improved compared with previous studies (10^9 in our experiment, 10^7 in previous study (manuscript Reference 47), indicating that β-sitosterol promoted LNP endosome release and the CAR sequence intracellular expression.

Based on these, we conclude that ~80% *in vitro* and 3% *in vivo* transfection efficiency are reliable.

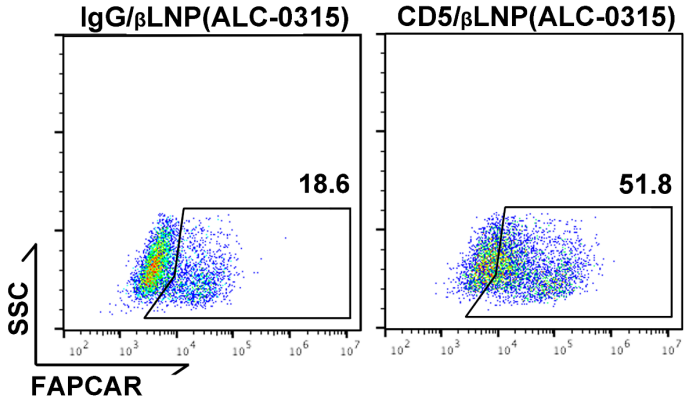


Figure R1. Flow cytometry assay of FAPCAR 2.1 expression in T cells after transducted with IgG or CD5-treated βLNP containing ALC-0315 as ionizable lipid.

Reviewer #2

Summary:

In this manuscript, Yan et al. describe a modified LNP-mRNA system for generation of FAPCAR-T cells to target FAP-expressing cells in a mouse bleomycin model. Authors showed that FAPCAR-T cells reversed fibrosis markers in a single experimental lung fibrosis model. They subjected the whole lungs from the intervention approach to proteomic profiling, which showed a decrease in fibrotic ECM proteins and an increase in epithelial cell polarization marker.

The topic of this study is very timely and of interest, it follows upon recent papers using the same approach for cardiac fibrosis (e.g. Rurik et al Science 2022) by applying FAPCAR-T cells as a potential new therapeutic approach. Here, the authors used the same approach but investigate the effect on an experimental lung fibrosis model. While this approach is novel in the lung, the overall approach is not very original and there are several shortcomings in the assessment of lung fibrosis as well as the lack of mechanistic insight above what is known, which dampen the enthusiasm for this study. In addition, data demonstrating the potential human relevance for IPF patients is missing. The manuscripts barely acknowledge relevant literature in the field (both for LNPs as well as lung fibrosis). Overall, the manuscript seems immature and a rushed without providing the rigorous investigation that this potentially important new therapeutic approach requires.

Answer: Thanks for your valuable suggestions. We revised the manuscript as suggested. In aged mouse (16-month-old) model, we conducted a comprehensive validation. The "Healthy+CD5/βLNP-FAPCAR 2.2" control group and Pirfenidone (PFD) treatment group were added in the young mouse model. Proteomic results were validated by substantial experiments. Furthermore, we carried out the single-cell RNA sequencing of mouse lungs before and after CD5/βLNP-FAPCAR 2.2 treatment. The analysis provides the first insight into the changes of pulmonary cell profile after treatment. Please see the below responses. Thank you.

Major points:

1. This manuscript from Yan et al. builds completely on the work by Rurik et al (2022) Science. 2022 Jan 7; 375(6576): 91-96. (reference 12), and most parts of figure 1 and 2 are incremental. Also limits overall originality in the approach. FAPCAR-T cells (reference 12) were previously used in cardiac injury model in mouse. Although FAPCAR-T cell targeting FAP expressing fibroblasts is not novel, its use in bleomycin model is novel and potentially pathbreaking. A thorough analysis of fibrosis-clearing pathways using molecular and computational methods is

beneficial to the field of lung fibrosis. Authors characterized the FAPCAR-T cells similar to what was done in reference 12 but the lung fibrosis part of the manuscript is lacking proper analysis. The authors refer to the bleomycin model as an "IPF model", which is not correct and well known and discussed in the lung community. Moreover, they analysis of lung fibrosis is limited and not well described. It misses lung function, hydroxy proline assessment and additional fibrosis marker readout, all well accepted gold standard for lung fibrosis assessment (PMID: 28459387).

Answer: Thanks for your valuable suggestion. After further review of the references, we revised the "IPF model" into "pulmonary fibrosis model" or "BLM model" in manuscript.

In the previous version, we provided several lung fibrosis assessment strategies, including: Micro-CT (indicating the mean lung density and total air volume), Masson's trichrome staining, Ashcroft score, Sirius red stain with polarized light assay. Compared with the gold standard for fibrosis assessment mentioned above, direct detection of collagen content was missing. We supplement the hydroxyproline (HYP) assessment using the kit from Sigma. Results showed CD5/βLNP-FAPCAR 2.2 treatment reduced the entire lung HYP content in both in young and aged mice (revised Fig. 4D, L). The assessment panel considers pulse oximetry as a potential lung function measurement, although they do not recommend pulmonary function as a routine part of the preclinical assessment of drugs. Due to limited technology and equipment, we were unable to complete pulse oximetry analysis in mice. However, micro CT results showed increased lung air volume, as well as increased expression of genes associated with gas exchange in proteomics, could indicate recovery of lung function. We will continue to refine this approach to assess pulmonary fibrosis in future studies.

And in revised manuscript, the corresponding experiments results have been described in revised section 'CD5/βLNP-FAPCAR 2.2 therapy reversed pulmonary fibrosis' and 'Antifibrotic response in aged mice', Page 6, 7.

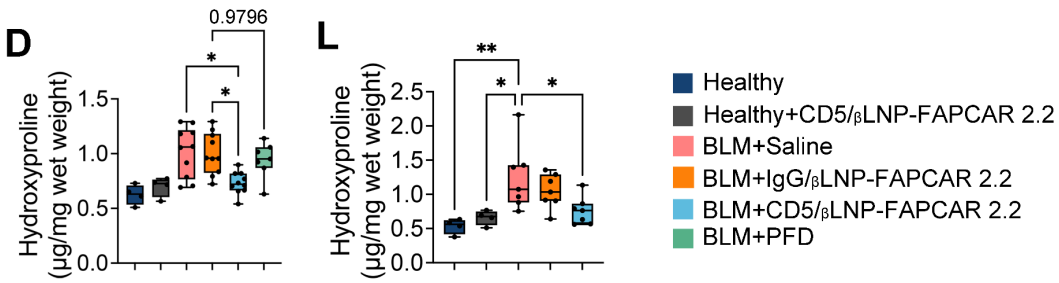


Figure 4D and L.

2. In addition, the single bleomycin model has some caveats as it is self-resolving and the authors would need to confirm they findings in a repetitive bleomycin model or using old mice.

Answer: Following your suggestion, we supplemented the BLM induced model with aged mice. Please see **Answer to Comment 4** in details. Thank you.

3. They are using a preventive approach, which is less informative and a therapeutic approach should be included. There is further a complete control arm missing with giving CAR-T cells in the healthy lung which needs to be included for rigorous and robust study design.

Answer: Following your suggestion, we supplemented the healthy+CD5/βLNP-FAPCAR 2.2 and Pirfenidone (PFD) treated BLM-mouse groups. Please see **Answer to Comment 4** in details. Thank you.

4. Overall, given these limitations, the authors arrived at several bold conclusions that are not backed sufficiently.

Answer: In response to comments 2-4, we carried out more experiments, including comprehensive experiments using aged mouse model, a healthy control group with CD5/βLNPs injection, and a BLM-model group treated with Pirfenidone (PFD) by oral gavage, as suggested by the reviewer. Injecting CD5/βLNP-FAPCAR 2.2 to healthy young/aged mice twice showed negligible effects on their feeding status, weight, blood tests, and organ examinations. In comparison with PFD treatment with for 3 weeks, CD5/βLNP-FAPCAR 2.2 treatment had advantage on inhibition of pulmonary fibrosis, including fast clearance of pulmonary fibrosis and recovery of lung function. It usually takes a long time for PDF to take effect.

CD5/βLNP-FAPCAR 2.2 treatment also achieved considerable therapeutic efficacy in aged mice. The survival rate was significantly higher and lung coefficients were lower in the CD5/βLNP group than those in the BLM+Saline and IgG/βLNP groups (revised Fig. 4E-G). The micro-CT images showed that BLM-induced pulmonary fibrosis was more severe in aged mice compared to the young when they received the same dose, i.e., larger area of regional consolidation, higher HU density and less air volume in the lung (revised Fig. 4H-J, S4A). The flow cytometry analysis showed that the population of FAP-positive fibroblasts was reduced in aged mice, demonstrating the successful generation of transient FAPCAR-T cells (revised Fig. S4B). Moreover, Masson staining and HYP quantification revealed the significant decrease of fibrosis and collagen content in aged mice after

CD5/βLNP-FAPCAR 2.2 treatment (revised Fig. 4K, L, S4C). This part was added in revised section 'Antifibrotic response in aged mice', Page 7.

The aim of the study is to develop a comprehensive anti-fibrotic treatment targeting FAP-positive fibroblasts. As the logistic starting point, we evaluated possibility of clearing FAP fibroblasts in lung and the effect on the fibrosis progression. The following studies will evaluate therapeutic activities at different time instances in various animal models. Furthermore, in patients with IPF, studies have shown that FAP is highly expressed (Yang P et al. *Am J Respir Crit Care Med*. 2023, 207(2):160-172; Röhrich M et al. *J Nucl Med.* 2022, 63(1):127-133). In future clinical trials, we will carry out the detailed evaluation of therapeutic activities of FAPCAR-T at different time instances. Thank you.

5. The proteomic profiling is an elegant addition to the study, however, there is very limited analysis of these data, especially to known new pathways and cell types that are relevant in lung fibrosis (such as ADI cells). Further corroboration and mechanistic insight are needed to advance the potential impact of this study compared to what is known already. In detail: A lot is inferred from proteomics data/KEGG pathway analysis with bold assertions. Statements such as "EMT markers were reduced" or "epithelial cell polarization" or "slowed down AT2 to AT1 differentiation" require corroborative evidence other than proteomics data. These statements are trouble-some. This requires further substantiation with experimental proof as described above in major point 7. Lung fibrosis is reversed but the manuscript lacks mechanistic understanding of the process. Moreover, pre-alveolar type-1 transitional cell state (PATS) described in reference 17 has been previous established using single cell transcriptomics. It might be important to establish a role for PATS with other methodologies along with the existing proteomics data.

Answer: Thanks for your valuable suggestion. We carried out more experiments to validate the relevant signaling pathways identified by proteomics as suggested by the reviewer.

First, we determined the elastic modulus of fibrotic lung tissue using the Mechanical Testing System, which significantly increased due to the fibrosis clearance after CD5/βLNP-FAPCAR 2.2 treatment (revised Fig. 5G, H). Immunofluorescence images showed the accumulation of PATS in fibrotic area in BLM mice, which was reduced by the CD5/βLNP-FAPCAR 2.2 treatment (revised Fig. 5I, S5G).

Second, expression of EMT markers was analyzed by the western blotting (revised Fig. 6A). Mesenchymal cells, transformed from epithelial cells, expressed low level of E-cadherin (an adhesion factor) and high level of Vimentin. Expressions of these markers were close to the normal level after

CD5/βLNP-FAPCAR 2.2 treatment. Hence, EMT was inhibited by the treatment and did not contribute to polarization signaling enrichment.

For macrophages, previously studies have shown pulmonary fibrosis could increase macrophages number and promoted cell differentiation into pro-fibrotic or inflammation state. (Sanin DE et al. *Sci Immunol*. 2022, 7(70):eabl7482; Peyser R et al. *Am J Respir Cell Mol Biol.* 2019, 61(1):74-85; Lv J et al. *Front Immunol*. 2023, 14:1230266). The flow cytometry analysis showed the increased number of macrophages marked with F4/80 in inflammatory fibrotic lungs, which decreased after CD5/βLNP-FAPCAR 2.2 treatment (revised Fig. 6C). The analysis of the subphenotype revealed that the treatment decreased the number of M2 macrophages that were identified as the profibrotic phenotype (revised Fig. 6D, E and S6D, E). A subgroup, characterized by double-positive expression ($CD86^{+}/CD163^{+}$), increased in both CD5/βLNP and PFD treatment groups (revised Fig. S6F). Given recent findings that M1/M2 phenotypes are not mutually exclusive in macrophages, the cells in the therapeutic groups may undergo a state shift (Yuan F et al. *Front Immunol*. 2022, 13:798583; Shi Y et al. *Genome Biol*. 2022, 23:87). Similar results were also observed in the BLM model of aged mice (revised Fig. S6G, H). Overall, the decrease of total macrophage population and M2 subphenotype excluded the macrophages polarization from the pathway enrichment after CD5/βLNP-FAPCAR 2.2 treatment.

For epithelial cell polarization, we initially detected Cdc42 expression level using the western blotting. The expression of Cdc42 in lung tissue increased after CD5/βLNP-FAPCAR 2.2 treatment (revised Fig. 6A). Moreover, immunofluorescence results showed that Cdc42 and PKCz were colocalized on the cell membrane and the colocalization tended to be on one side rather than the uniform distribution throughout the cell membrane after treatment (revised Fig. 6F, G). The cytoskeleton-associated tight junction was enhanced (revised Fig. 6H). This confirmed the mechanism that 'the signaling axis formed by Cdc42 and aPKC (Pkc-zeta) is the key to polarization regulation. ECM signals are sensed and transmitted by Crb3, which recruits Cdc42-aPKC-PAR6 to form complex at the apical domain. This complex represents a key step in excluding proteins from the apical domain, which promotes the polarity distribution of proteins between apical, lateral and basement membranes via regulating cytoskeleton (Buckley. C.E et al. *Nat Rev Mol Cell Biol.* 2022, 23:559-577. Apodaca. G. et al. *Nat Cell Biol.* 2012, 14:1235-43. Hayase. J. et al. *J Cell Biol.* 2013, 200: 635-50. revised Reference 27,28,29).' This part has been added in revised manuscript: Page 10, Line 21-25; Page 11, Line 1-6.

To explore the lung regeneration process following successful fibrosis clearance in details, we mapped cellular profile by the scRNA-seq analysis of lungs between BLM+Saline and CD5/βLNP groups via the 10×Genomics platform (revised Fig. 7A). The changes in cell profile after treatment

showed that the enhanced plasticity of alveolar cells and the supplementation of AT1 cells were the main contributors to lung regeneration. The number of PATS (KRT8^{+}, sftpc^{+}) and the intermediate cluster Hopx^{high} AT1 (Krt8^{+}, Hopx^{+}, Ager^{+}) were significantly reduced by the treatment. Moreover, the proliferating alveolar progenitor cells (PAPCs) were identified. We re-clustered PAPCs with alveolar cells (revised Fig. 7M) and performed the cell lineage trajectory inference using the RNA velocity and Slingshot. The developmental trajectory of PAPCs was found toward to alveolar cells (revised Fig. 7N, S8B). Moreover, AT1 cells were the differentiation endpoint of AT2, Hopx^{high} AT1 and PATS clusters. This part has been added in revised manuscript: Page 12, Line 7-25.

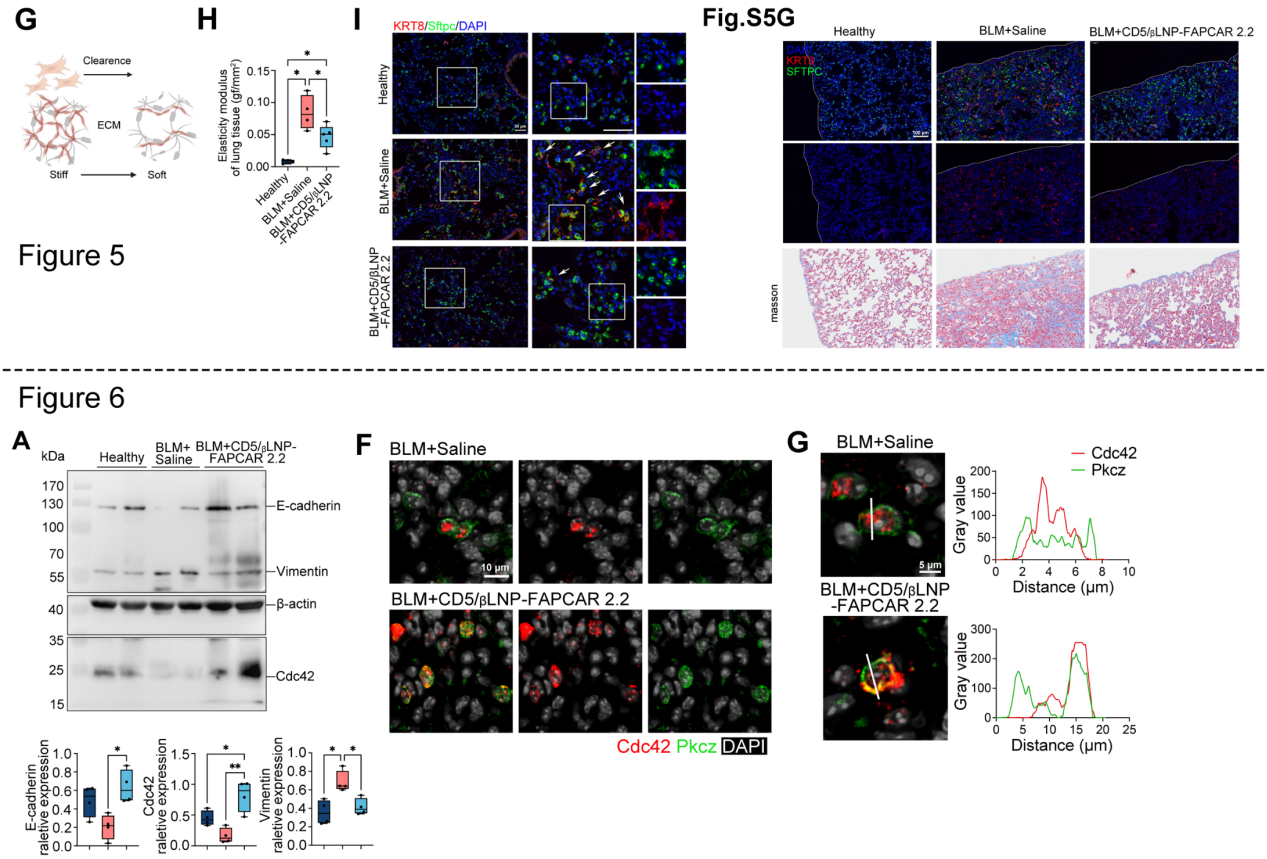


Figure 5G-I, Figure 6A, F, G and Figure S5G

6. One major weakness with the approach is that the study does not provide quantitative data for FAP expressing cells in lung fibrosis (or human IPF) to support the premise of the study. Authors state that there is a reduction in FAP expressing cells in S3C based on IHC, but this is not quantifiable and not convincing. This is a very important proof of concept control to include (ideally flow cytometry, which is doable as FAP is a surface marker). What are the FAP expressing cells, how many are there in healthy lungs versus fibrotic lungs? These are supposed

to be on fibroblasts and recent paper highlight that there are several subtypes of fibroblasts in the fibrotic lung (e.g. PMID: 38987592). In detail: FAP is also expressed by healthy fibroblasts based on IPF cell atlas. It is not clear which fibroblasts are targeted by FAPCAR-T cells: healthy or myofibroblasts? Not clear from IHC. Depicting other fibroblastic markers and/or cell types using RNAscope on mouse lungs would be more convincing. What other cell types are ablated with FAPCAR-T cells in mouse lung? Perhaps single cell transcriptomics analysis would answer that. Without establishing FAP expression by RNA or protein in healthy vs bleo-injured lungs, it would be hard to interpret. Authors need to label or include arrows to indicate FAP staining in Fig 2D and Fig S3C.

Answer: Thanks for your valuable suggestion. We added flow cytometry assay for FAP fibroblasts and scRNA-seq to evaluate pulmonary cell states before and after FAPCAR-T treatment, as suggested by your suggestions.

We used flow cytometry to show the FAP positive cell changes before and after CD5/βLNP-FAPCAR 2.2 treatment in both young and aged mice. Fibroblasts were marked with Pdgfra. The CD5/βLNP FAPCAR 2.2 treatment significantly downregulated the $Pdgfra^{+}/FAP^{+}$ fibroblasts. The population of $Pdgfra^{+}/FAP^{-}$ had relatively less change. Moreover, FAP had no expression in Pdgfra-negative population. The results indicated the FAPCAR-T cells targeted clearance of FAP^{+} fibroblasts in fibrotic lung (revised Fig. 3G, S3E, S4B). FAP is a marker of activated fibroblast and data from Uniprot.org and related researches have shown that it is rarely expressed in normal fibroblasts (Fitzgerald et al. *Cancer Metastasis Review.* 2020, 39:783-803), but highly expressed in adipocytes, fetal dermal fibroblasts (not expressed in adults), granulation tissue of healing wounds, activated hepatic stellate cells (HSC) and myofibroblasts from cirrhotic liver (not expressed in normal liver), and glioblastomas and glioma cells (Scanlan et al. *Proc Natl Acad Sci USA*.1994,91: 5657–5661; Waldele et al. *Arthritis Res Ther.* 2015,17:12; Aghajanian et al. *Nature*. 2019, 573: 430–433). Here, transient FAPCAR-T was used to eliminate overactivated fibroblasts in fibrotic lung and minimized the impact on normal tissue repair.

We subsequently reviewed literature of FAP expressions in pulmonary fibrosis. We reanalyzed FAP expressions in several fibroblast clusters using recent single-cell RNA-sequencing data (GSE132771) of fibroblast in pulmonary fibrosis (Tsukui et al. *Nature Communications*. 2020,11:1920; Tsukui et al. *Nature.* 2024,631:627-634). Results showed that, compared with the four fibroblast clusters, FAP was expressed in fibrotic fibroblasts and stress-activated fibroblasts. FAP expression was hardly detected in normal lung fibroblasts (Figure R2). Meanwhile, single-cell data from IPF patients

(GSE135893) showed that FAP was expressed in myofibroblasts and HAS1^{+} fibroblasts clusters in IPF lungs (the latter is a cell group that promotes the synthesis of hyaluronic acid, one of the components of ECM) (Figure R3) and FAP positive fibroblasts (404) accounted for 91% of FAP positive cells (443) (Yang et al. *Am J Respir Crit Care Med*. 2023, 207:160-172). Therefore, in the FAPCAR-T treatment, activated fibroblasts are the target.

We performed the scRNA-seq analysis to show that fibroblasts were comprised of three distinct clusters, including inflammatory, fibrotic and mesothelial cells. Cluster 0 was identified as inflammatory fibroblasts with high expressions of Cxcl12 and Cxcl13. Fibrotic fibroblasts were characterized by Fbln2 and other pathological ECM genes. Notably, the two clusters overexpressing Col1a1 and Col3a1 were significantly reduced after CD5/βLNP-FAPCAR 2.2 treatment. Moreover, mesothelial fibroblasts were characterized by Msln and remained unchanged in all groups (revised Fig. 7E, F, S7C-E). In addition, FAP was expressed in both fibrotic and inflammatory clusters. Most of cells in the two clusters were shown in the BLM group, suggesting that FAPCAR-T cells could effectively eliminate the target cells without affecting other cell populations (revised Fig. 7G). This part has been added in revised manuscript: Page 11, Line 23-25; Page 12, Line 1-6.

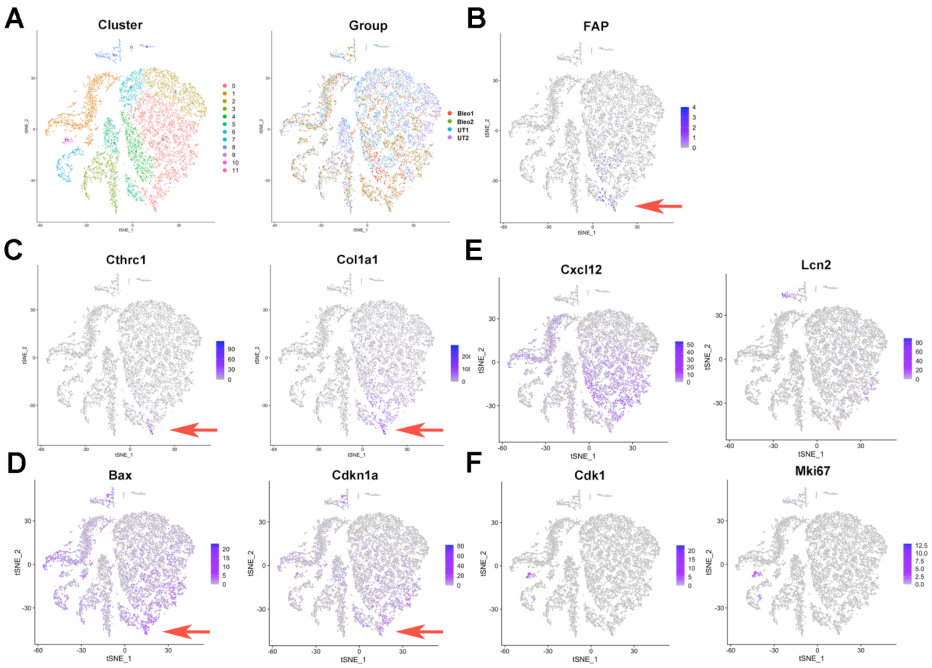


Figure R2. Reanalyzed tSNE data for comparing FAP expression and markers of alveolar fibroblast subtypes. (A) The clusters and sample groups distribution of alveolar fibroblasts. (B) FAP expression. (C-F) Markers expression of fibrotic fibroblasts (C), stress-activated fibroblasts (D), inflammatory fibroblasts (E) and proliferating fibroblasts (F).

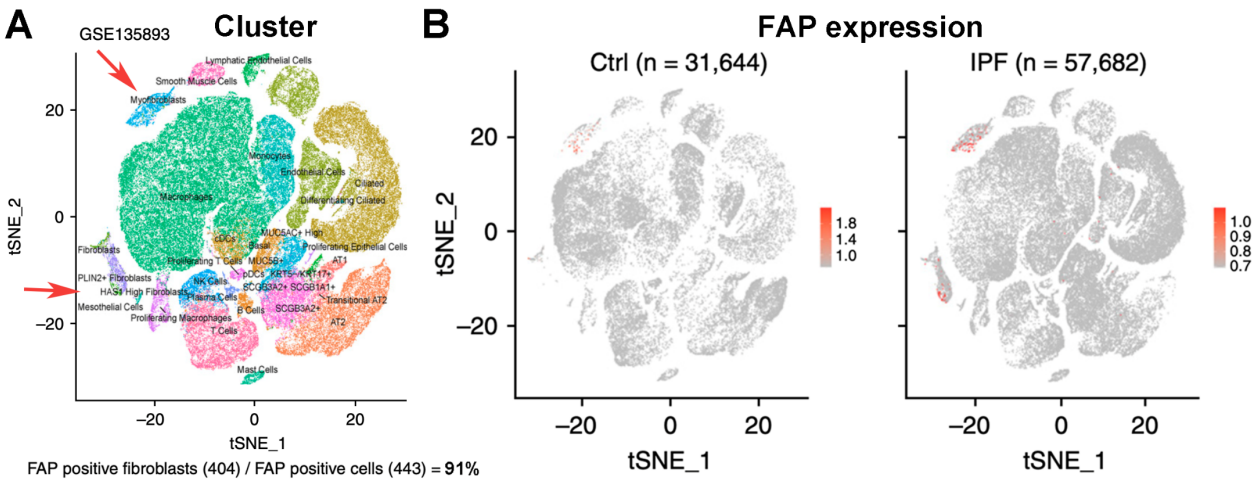


Figure R3. Single-cell analysis reveals the specific expression of FAP in myofibroblasts and HAS1^{+} fibroblasts. (Yang et al. 2023.)

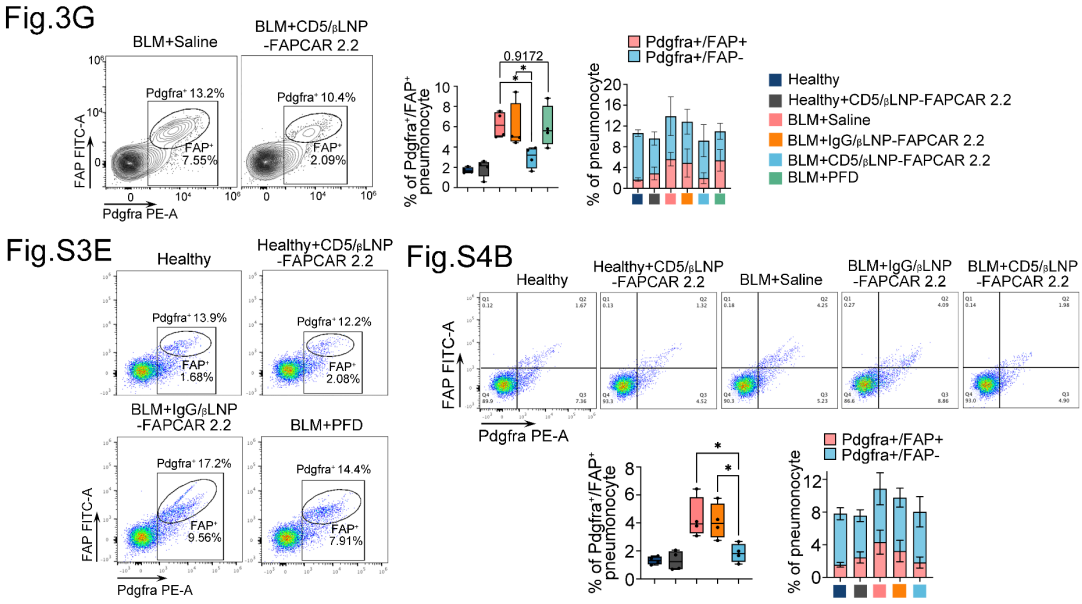


Figure 3G, S3E and S4B

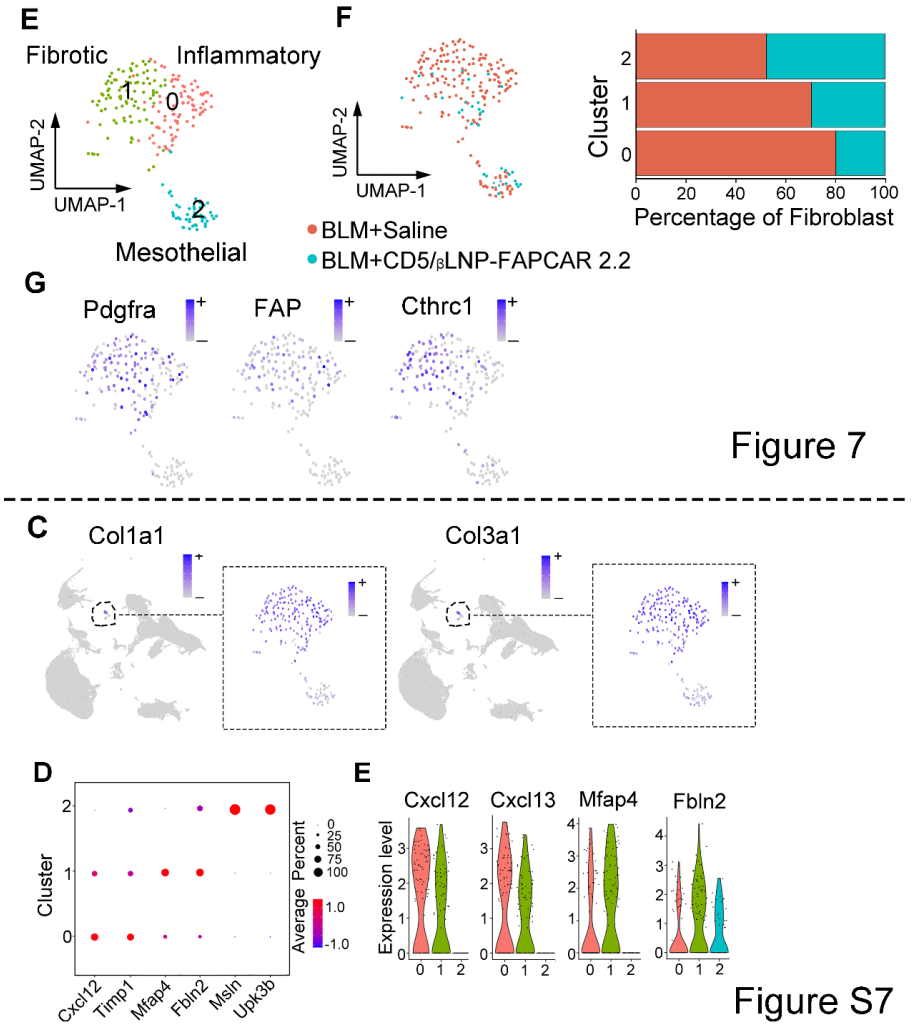


Figure 7E-G and Figure S7 C-E

7. Cholesterol analogues (b-sitoserol) were previously shown to improve endosomal escape and transfection efficiency in LNP-mRNA encapsulation (Nat Commun. 2020 Feb 20;11(1):983.). Authors need to refer to literature and cite the previous work appropriately to establish the rationale.

Answer: Thanks for your valuable suggestion. The study has been cited in revised manuscript as Reference 15.

8. LNP-mRNA system for CAR-T cells has been established previously. It makes sense to clearly state how similar or dissimilar this manuscript is to reference 12 in terms of FAPCAR-T generation. Methods on generation of FAPCAR 2.1 and 2.2 needs to be elaborated and complete.

What is the origin of scFv sequence used in FAPCAR-T cells? Is this similar to the one used in reference 12? Is FAPCAR 2.2 better than 2.1 because of mutated TM? What is the rationale here?

Answer: Thanks for your valuable suggestion. Revised method description is as follows: FAPCAR 2.1 design is based on the second-generation CAR sequence principle. CAR domains are comprised of CD8α leader, anti-muFAP scFV, CD8α hinge, CD28 transmembrane (TMD) and 4-1BB & CD3ζ intracellular signal. Amino acid sequences of protein fragments are extracted and backtracked to DNA sequences according to the functional annotation on Uniprot.org. The light chain (VL) and heavy chain (VH) amino acid sequences of the anti-muFAP scFV segment (clone73.3) are derived from previous study (Wang et al. *Cancer Immunol Res*, 2014, 2,154-66, i.e., revised Methods Reference 1). The VH is linked distal to the VL (kappa) by a 3-time repeated G4S linker and then genetic codes are optimized for mouse species. Four cysteine (Cys) are introduced to form disulfide bonds for scFv stability. Two Cys are placed at both ends of the linker (C terminus of VL and N terminus of VH) and two in VL-VH domain (L100-H44) (Boucher et al. *Mabs*. 2023, 15:2195517, i.e., revised Method Reference 2). The domains of FAPCAR 2.2 are the same as FAPCAR 2.1 except that CD28 TMD is replaced by a TMD designed with programmable membrane proteins (proMPs) strategy (Elazar. A. et al. *Elife.* 2022, 11:e75660, i.e., revised Method Reference 3). The complete DNA sequence was synthesized by a company (Sangon Biotech, Shanghai, China). This part has been added in revised manuscript, Methods section: Page 37, Line 18-25; Page 38, Line 1-4.

Clone 73.3 is one of the FAP antibody types commonly used in experiments and also used in reference 12 which used VH-linker-VL linkage without modification. For the optimization of FAPCAR 2.2, we referred to Elizar et al.'s research (Elazar. A. et al. *Elife.* 2022, 11:e75660, i.e., revised Method Reference 3). In addition to the necessary intracellular activation domain in CAR design, the TMD fragment from endogenous T cell proteins could participate in molecular interactions, resulting in additional stimulatory signals and unpredictable cytokine release. In CAR design with CD28 TMD, 'co-stimulation is explicitly encoded through the signaling tail incorporated into the CAR protein but is also amplified through a specific sequence signature in the CD28 TMD that recruits endogenous CD28 into activated CAR complexes.' Furthermore, exogenous TMD sequence directly influence receptor oligomeric state, which regulates cytokine release. In the present study, CD5/βLNP-FAPCAR 2.2 led to an ~30% reduction in cytokine release compared with that of CD5/βLNP-FAPCAR 2.1 without altering lysis capacity (revised Fig. 2G), indicating the superiority of the optimized TMD and the increased suitability for pulmonary fibrosis treatment.

Minor:

1. The authors need to pay attention to the typographical errors. This manuscript will need a native English speaker for fixing incomplete sentences, spelling mistakes and grammar. For example, please see page 4, line 4 for an incomplete sentence. Examples where rewording or expansion of acronyms is required: "Effectively killing assay", "Astral data-independent acquisition (DIA) Proteomic". Figure S3: word "anterior" is misspelled.

Answer: Thanks for your kindly suggestion. The revised manuscript has undergone the English Language Editing provided from Springer Nature. The verification code is: **C73E-8C6B-E045-ADC7-C317**.

"Effectively killing assay" is revised to "*Ex vivo* cytotoxicity assay". "Astral data-independent acquisition (DIA) Proteomic" is revised to "Data-independent acquisition (DIA) Proteomic assay". Word "anterior" in Figure S3 has been corrected.

2. Details are missing in the methods section. For example, authors say "Union connector and peek tubes". How were the LNPs synthesized? By a microfluidic device or in bulk?

Answer: Thanks for your kindly suggestion. We revised methods section and added necessary details. The LNP synthesis protocol was: mRNAs were dissolved in 20 mM citric acid buffer (pH=4) and LNPs were in ethanol at a concentration of 10 mg/mL with 3:1 volume ratio of the two solutions. The syringes containing mRNA solution and lipid solution were connected to a tee union connector (0.05 in thru hole, #P-728; IDEX, Oak Harbor, WA) with HPLC peek tube (0.02 in ID for mRNA and 0.01 in ID for lipid solution, IDEX). Another peek tube (0.04 in ID) was used to collect products into a 50 mL tube. The laboratory encapsulation total flow rate was 16 mL/min (12mL/min for mRNA solution and 4 mL/min for lipid solution). Prepared suspension was immediately diluted 100 times with PBS containing 25 mM EDTA (pH=7.4) and then concentrated to mRNA of ~1 μg/μL. This part has been added in revised manuscript, Methods section: Page 39, Line 1-6.

3. Fig S5: Please indicate what B1-B4 and D1-D4 stand for.

Answer: Thanks for your reminding. The numbers "B1-B4, D1-D4" referred to different lungs of the BLM and CD5/βLNP-treated groups. We take into account that the two groups have been distinguished

by color blocks at the top of the heat map (green for BLM+Saline and orange for BLM+CD5/βLNP-FAPCAR 2.2) such that these numbers were removed from the revised figures. Thank you again.

Reviewer #5:

This is an interesting manuscript demonstrating that idiopathic pulmonary fibrosis (IDF) can be potentially reversed in a mouse model of fibrosis using transient FAPCAR-T cells generated in vivo and delivered via LNP-mRNA system.

There are a number of aspects that require revision before it can be potentially accepted for publication.

For example, the grammar in the manuscript is poor at times with many grammatical errors throughout the manuscript. Even in the materials and methods the language interchanges between the present and the past tenses. Examples of some errors are below (too many to go through in detail so have just selected the Abstract to give some examples!)

Answer: Thanks for your valuable suggestions. We revised the manuscript as suggested. The revised manuscript has undergone the English Language Editing provided from Springer Nature. The verification code is: C73E-8C6B-E045-ADC7-C317. Please see the below responses. Thank you.

Abstract

line 2/3 – "Idiopathic pulmonary fibrosis (IPF) is a severe lung disease in the world, but has limited clinical therapies." Change to "Idiopathic pulmonary fibrosis (IPF) is a severe lung disease worldwide, but has limited clinical treatments"

Answer: Thanks for your valuable suggestion. The sentence has been revised as "Idiopathic pulmonary fibrosis (IPF) is a severe lung disease occurring throughout the world; however, few clinical therapies are available for treating this disorder."

line 3 – "Fibrosis maintains dynamic balance with overactivated fibroblasts." - rewrite this sentence - poorly written.

Answer: Thanks for your valuable suggestion. The sentence has been revised as "Overactivated fibroblasts drive abnormal fibrosis accumulation to maintain dynamic balance between inflammation and extracellular matrix (ECM) stiffness."

line 4 – "the lung may have self-repairing ability if fibrosis..." Change to "the lung may have self-repairing abilities if fibrosis ..."

Answer: Thanks for your valuable suggestion. The sentence has been revised into "Given pulmonary cell can regenerate, the lung may possess self-repairing abilities if fibrosis is removed via clearance of overactivated fibroblasts."

line 5 – "The aim of the study is to evaluate therapeutic activity .." Change to "The aim of the study is to evaluate the therapeutic activity ..."
line 6 – "..address the relevant mechanism to epithelial cell polarization" Change to "understand the relevant mechanisms of epithelial cell polarization"

Answer: Thanks for your valuable suggestion. The sentence has been revised into "The aim of this study was to evaluate the therapeutic activity of transient antifibrotic CAR-T cells (generated via a novelly-designed LNP-mRNA system) and explore the regeneration mechanisms of lung in a mouse model of bleomycin-induced pulmonary fibrosis."

line 9 – "limited cells self-healing ability." Change to "limited the self-healing ability of the cells."
lines 11/12 –"The decreased ECM stiffness and the improvement of extracellular environment facilitated re-establishment of cell polarity and restored the self-repairing ability of epithelial cell." Change to "The decreased ECM stiffness and the improvement of the extracellular environment facilitated the re-establishment of cell polarity and restored the self-repairing ability of epithelial cells."

Answer: Thanks for your valuable suggestion. Since we add substantial experiments and rewrite the abstract, these sentences have been revised as "Here, we found that fibrosis-induced ECM stiffening impaired alveolar epithelial cell compensation. The proposed LNP-mRNA therapy eliminated overactivated fibroblasts to rescue pulmonary fibrosis. The restored ECM environment regulated the cellular profile. The elevated plasticity of AT2 and $Pclaf^{+}$ cells increased AT1 cell population via polarization. $Apoe^{+}$ macrophages and increased numbers of effector T cells were shown to reestablish pulmonary immunity." Thank you so much.

Page 3, line 2/3 – "This stimulated numerous anti fibrosis studies given limited therapies." Rewrite this sentence to make it clearer to the reader.

Answer: The sentence has been revised as "Given the limited therapeutic methods on pulmonary fibrosis, numerous studies have explored antifibrotic approaches and its pathogenesis." in Page 3, Line 3-4.

Pg 4, line 13 - define "BW"

Answer: "BW" was abbreviation for body weight and defined in revised manuscript.

Please clarify FAPCAR-T cells earlier in the manuscript.

Answer: "FAPCAR-T" has been clarified in Introduction section when it first appeared. Page 3, Line 13-14.

Section - Proteomes for 0, 14 and 28 days after administration of bleomycin
This section needs some rewriting and clarification as the authors need to specifically clarify what samples have been compared, how many mice (i.e. the power of the study, n=?). What controls were used, etc.? It's very difficult to interpret these results without including this detail.

Answer: Thanks for your valuable suggestion. For proteomics assay, each group included four mice. The traditional 'control group' is not needed when comparing multiple groups. Here, data were normalized for each 'row', based on which we computed the Z-score. Details are described in the **Answer** to the " **For the K-means clustering** …" comment.

This part has been revised into "We performed the principal component analysis (PCA) of three time instances (postoperative day 0, 14 and 28 after BLM administration) (4 mice per group), which revealed significant changes in proteomes with time in BLM mice (Fig. 5A). The K-means clustering algorithm divided 4126 DEPs (differentially expressed proteins) of the whole groups into 6 clusters, which was consistent with heatmap results. Based on the GOBP analysis, clusters were annotated as DNA repair, immune system process, ECM organization, intracellular signal transduction, protein transport, and lipid metabolic process (Fig. 5B)." in Page 8, Line 19-25.

The data in figure 1B and figure 4 is not clear.

Answer: Thanks for your kindly suggestions, we have revised the whole Figures.

For the K-means clustering did all 4126 DEPs fall into the 6 clusters that have been shown? Again, detail is lacking. What comparative analysis gave 4126 proteins? Was this a combination of the 14 and 28 days of treatment? What about the "time 0"?

Answer: Thanks for your valuable suggestion. All 4126 DEPs fall into the six clusters. The comparative analysis of three groups (D0, D14 and D28) together gives 4126 proteins, which is different from the two-group comparison. Briefly, the sample mass spectrometry intensities in dataset were normalized for each row with the formula: (x-mean)/sd. Normalized values in each row had the same mean and standard deviation. The normalized raw data were then assessed using the Z-score (the distance from the sample point to the population mean) to distinguish the distribution across the groups. Positive values indicated upregulation relative to the overall mean, while negative values indicated downregulation. Proteins with expression fold change>1.5 (up and down) and $p<0.05$ were identified as the different expression protein (DEP). These details are added in revised Methods: Page 46, Line 1-7.

In the materials and methods - what is meant by "Maximum number of variable modifications was 1." in the context of the previous sentence where 2 variable modifications were listed?

Answer: The sentence means 'At most one variable modification was set up for each peptide segment.' There could be many types of variable modifications, but only one modification site could be found on the obtained peptide segment. The sentence has been explained in revised Methods: Page 45, Line 17.

What specific software was used for differential proteomic analysis in order to yield the numbers of differentially expressed proteins.

Answer: Bioinformatic analysis were carried out with DIA-NN 1.8.1, Microsoft Excel and R statistical computing software. The *p* value and fold change (FC) were used in comparation between two groups. Student *t* test was used for calculating *p* value. The FC values were from: test group mean/control mean. Proteins with expression fold change>1.5 (up and down) and $p<0.05$ were identified as the different expression protein (DEP). Anova was used to calculate *p* values across multiple groups, and $p<0.05$ was defined as significant. This part has been added in revised Methods: Page 45, Line 23-25, Page 46, Line 5-9.

How many matched peptides were used the determine that a protein was differentially expressed? Again, this sort of information is required before this section of the results can be properly reviewed.

Answer: The obtained DIA original mass spectrometry file was imported into DIA-NN 1.8.1 for DIA analysis. The presence of one or more unique peptide segments indicated that the protein was detected. This part has been added in revised Methods: Page 45, Line 11-12.

Also please use a better title - example – "Comparative proteomic analysis after treatment with bleomycin for 14 and 28 days" or alternative!

Answer: Following your suggestion, we revised title to "Characteristics of proteins in fibrotic lung".

In the sentence in this section "Hence, we depicted the polarization molecular map of epithelial cells based on the KEGG analysis (Fig. 4G) albeit epithelial-mesenchymal transformation (EMT) and macrophage polarization might mislead bioinformatic enrichment results for epithelial cell polarization." - what is meant by "mislead the bioinformatic enrichment results". This is unclear.

Answer: Thanks for your kindly suggestion. We observed that the upregulated DEPs were mainly enriched in cytoskeleton and intercellular connectivity signaling pathways after CD5/βLNP-FAPCAR 2.2 treatment, all of which were known to be associated with the cell polarization. After treatment of pulmonary fibrosis, there may have three types of cell polarization: alveolar epithelial cell polarization, macrophage polarization, and epithelial-mesenchymal transformation (EMT). The differentiation of AT2 to AT1 cells is associated with dramatic changes in cell shape and structure, which are generally considered to be the epithelial polarization process. Such transformation is typically accompanied by many changes in expression of signaling and structural proteins. Hence, epithelial cells are assumed to be the main contributors to the pathway enrichment in lung recovery. To verify this hypothesis, it is required to exclude macrophage polarization or epithelial-mesenchymal transformation (EMT) process. We rewrote the description to make it more readable in revised manuscript: Page 9, Line17-25, Page 10, Line 1-5. Thank you again.

Manuscript：# NCOMMS-24-34989A-Z

Response to Reviewers' comments

Reviewer #1

Thanks to the author so much for revision of this manuscript. The authors addressed most of my concerns, I only have one more comment after the revision.

1. Figure 2I, could the author provide flow cytometry results of the LNP delivery to the splenic T cells? Or at least the immunostaining of the whole spleen section? Only a small area of T cell co-localization of LNP could not be strong enough as the in vivo T cell transfection is very low (~3%, Figure R1). And please also comment if ~3% CAR-T cells transfection in vivo is good enough for therapy (compared with previous studies).

Answer: We provide the immunostaining of the whole spleen section as suggested (Please see the below Figure R1). Thank you so much.

Kheirolomoom, A. et al. (Reference 47) revealed a 3% ~ 4% transfection efficiency of splenic T cells with mCherry-LNPs (~4×10^{13} particles/kg body weight carrying ~0.6 mg mCherry mRNA (2 nmol)) (Please see the below Figure R2), in agreement with our results. Furthermore, we computed the number of FAPCAR-T cells after transfection. Our previous experiments have shown 2~3×10^{8} spleen cells in mouse that include approximate 36% T cells. There are approximate 3×10^{6} FAPCAR-T cells in mouse spleen after one injection (not including transfected T cells in blood or lymphoid tissue). We performed a total of two injections that resulted in 6×10^{6} FAPCAR-T cells in mouse spleen. In Amor et al.'s study, one injection of 0.5~1×10^{6} m.uPAR-h.28z CAR-T cells reversed senescence (Amor C. et al. *Nature.* 2020, 583(7814):127-132). In Aghajanian et al.'s study, one injection of 1×10^{7} CAR-T cells rescued cardiac fibrosis in mouse (Aghajanian H. et al. *Nature.* 2019, 573(7774):430-433). Moreover, one injection of 1×10^{7} CAR T cells was applied to anti-tumor studies (e.g. Allen GM. et al. *Science.* 2022, 378(6625):eaba1624; Zhang AQ. et al. *Nat Biomed Eng.* 2023, 7(9):1113-1128). The number of CAR-T cells in the present study are similar with those in previous studies. Hence, approximate 3% CAR-T cells transfection in vivo is good enough for therapy. Thank you so much.

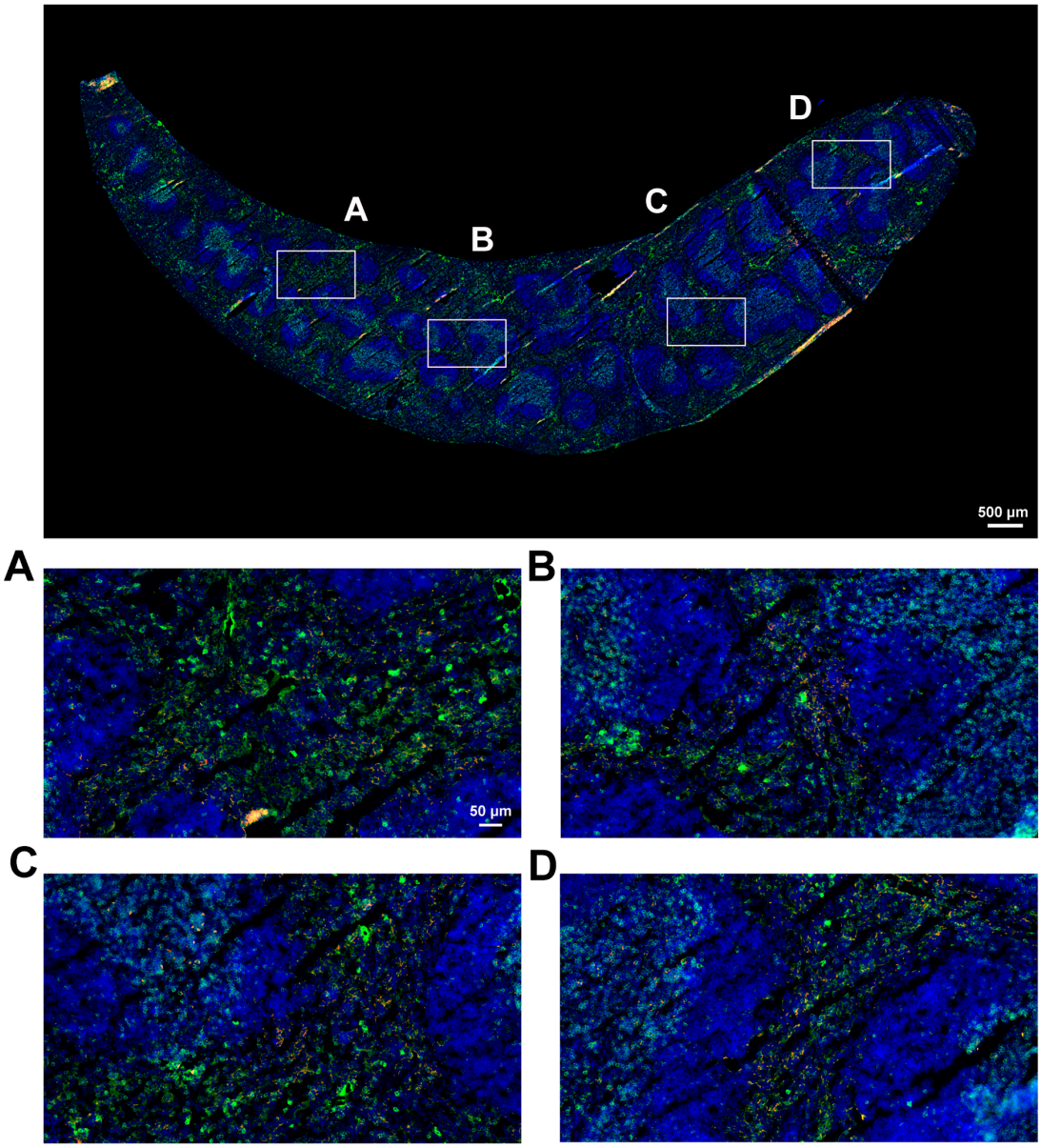
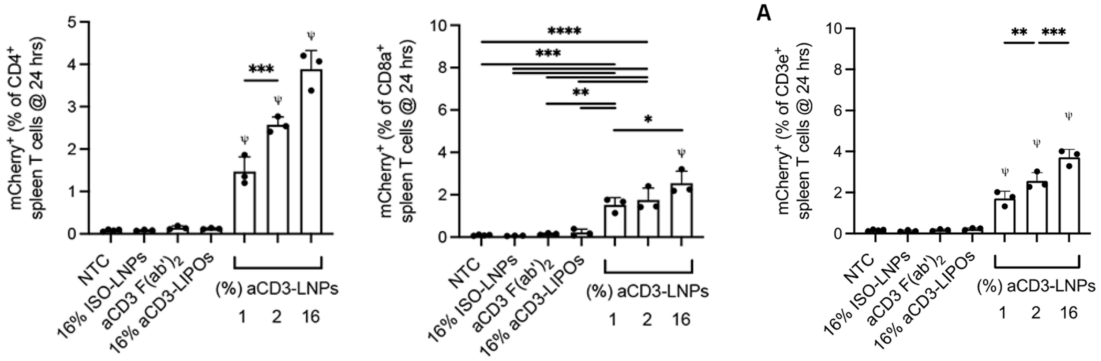


Figure R1. Co-localization of PKH26-labeled LNPs (red) and T cells (green) in the spleen.



Kheirolomoom. A. et al. *Biomaterials*, 2022.

Figure R2. Transfection efficiency in previous studies.

Reviewer #2

Final review:

The authors added hydroxyproline assay, wet-weight data, and elasticity modulus test that corroborate the results from bleomycin injury experiments.

The authors added pirfenidone to the bleomycin experiments. Although it did not affect bleomycin injury, it shows that FAPCAR-T/CD5-LNPs are superior to FDA-approved fibrosis drugs.

The authors appropriately added citations and properly cited previous works.

The authors improved the methods sections by adding details on the design of FAPCAR-T.

The authors added flow cytometry to determine whether FAP-expressing cells were exclusively fibroblasts (pdgfra+) and their depletion upon treatment with FAPCAR-T/CD5 LNPs.

The authors should clarify Figure 3G and Page6; line 23: What are pulmonary cells? Please clarify.

Figure 5H should be together with the panels in Figure 4.

Page 9: Lines 17-18: Please rewrite this sentence. There is no logical progression between the two statements.

The new scRNA seq experiment showed the Col1a1 and Col3a1 expressing fibroblast populations were specifically depleted upon treatment with FAPCAR-T/CD5 LNPs.

The authors also reanalyzed the existing scRNA dataset GSE132771 and showed that FAP expression is IPF-specific.

The authors expanded on epithelial polarization experiments corroborating proteomics data.

The authors also showed PATS by way of scRNA seq that corroborated proteomics data.

Answer: Thanks for your kindly suggestion.

In Figure 3G and Page6, pulmonary cells denote all lung cells (Tsukui et al. *Nature*. 2024, 631(8021):627-634). Pulmonary cells were revised as all lung cells in Figures 3G and S4B as well as the relevant figure legends. Moreover, we revised the sentence in Page6; Line 23 to "Moreover, flow cytometry revealed that the number of FAPCAR-positive T cells was increased in both spleens and lungs after CD5/βLNP-FAPCAR 2.2 treatment……". Sorry for the confusions.

Figure 5H was moved as the new Figure 4E as suggested. The corresponding text and figure legends were revised (Page 7; Line 16-18; Page 28; Line 5-6).

The sentence (Page 9; Line 17-18) was revised as "Proteomic analysis showed the clearance of fibrosis in the ECM after CD5/βLNP-FAPCAR 2.2 treatment. The Mechanical Testing assessment

showed a significant reduction in the elastic modulus of lung tissue after treatment (Fig. 4E). These findings demonstrated the reduction of extracellular tissue tension (Fig. 5G), which could contribute to the differentiation of AT2 to AT1. Furthermore, the immunofluorescence results showed a significant decrease in the number of PATS after treatment (Fig 5H, S5G), suggesting that the differentiation of AT2 to AT1 had returned to the normal." (Page 9; Line 19-24). Thank you so much.