



Combined transcriptomic and lipidomic analysis of D-4F ameliorating bleomycin-induced pulmonary fibrosis

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Background: Idiopathic pulmonary fibrosis (IPF) is a progressive lung disease that leads to respiratory failure, and for which there is no effective treatment. Apolipoprotein A-1 (ApoA-1) has been reported to ameliorate the bleomycin (BLM)-induced IPF model.

Methods: To examine the function of D-4F, an ApoA-1 mimetic polypeptide, in IPF, we used an *in-vivo* BLM-induced model. We assigned mice into the following 3 groups: the Blank Group (BLK Group), the Bleomycin Treatment Group (Model Group), and the D-4F Interference Group (Inter Group). The BLM-induced fibrosis was examined by hematoxylin and eosin, Masson's trichrome (M-T) staining and immunohistochemical staining. An untargeted lipidomic and transcriptomic analysis were used to examine the function of D-4F.

Results: There were 35 differentially altered lipids (DALs) in the BLK, Model and Inter Groups. A Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that glycerophospholipid metabolism was the most highly enriched of the 35 DALs. There were 99 differentially expressed genes (DEGs) in the BLK, Model and Inter Groups. The enriched KEGG pathway analysis showed that the mitogen-activated protein kinase (MAPK) pathway was 1 of the top 10 pathways. The results of the untargeted lipidomic and transcriptomic analysis showed that phospholipase A2 group 4c (Pla2g4c) was a crucial gene in both the MAPK pathway and glycerophospholipid metabolism. Pla2g4c was increased in the Model Group but decreased in the Inter Group.

Conclusions: It may be that D-4F prevented the BLM-induced pulmonary fibrosis model by inhibiting the expression of pla2g4c. Our findings suggest that D-4F may be a potential treatment of IPF.

Keywords: Idiopathic pulmonary fibrosis (IPF); D-4F; untargeted lipidomic analysis; transcriptomic analysis; pla2g4c

Submitted Jun 29, 2021. Accepted for publication Aug 26, 2021.

doi: 10.21037/atm-21-3777

View this article at: <https://dx.doi.org/10.21037/atm-21-3777>

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Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive lung disease that leads to respiratory failure. It is characterized by the excessive deposition of the extracellular matrix (ECM) in the pulmonary interstitium (1). fibroblast proliferation and deposition of the ECM are the major pathological changes of IPF (2). Epithelial-to-mesenchymal transition (EMT) describes the process by which epithelial cells transition into mesenchymal cells (3,4). Many factors can induce EMT, including the transforming growth factor β 1 (TGF- β 1), which is considered the major inducer of EMT (4). Research has shown that the level of apolipoprotein A-1 (ApoA-1) is decreased in IPF patients, and *apoa1* can ameliorate BLM-induced lung injury (5).

ApoA-1 is the major component of high-density lipoproteins (HDLs) and plays a key role in regulating normal lipid homeostasis by participating in reverse cholesterol transport (the process by which excess cholesterol in the periphery is transported to the liver and excreted from the body) (6). ApoA-1 is expressed in alveolar epithelial cells (AECs) and has anti-inflammatory effects in various lung diseases (7). The overexpression of ApoA-1 in experimental silicosis has been shown to decrease silica-induced lung inflammation and fibrotic nodule formation (8). Thus, ApoA-1 is a potential target for IPF.

D-4F (Ac-DWFKAFYDKVAEKFKKEAF-NH₂) is an 18-amino acid mimetic peptide of ApoA-1 (9). It contains a class A amphipathic helix with a polar and a non-polar face that binds lipids (10,11). It does not have a homologous sequence with ApoA-1 (10,11). D-4F was initially reported to be a drug treatment of the disease in the cardiovascular system; however, recently, it has been reported to be a drug treatment of the disease in the respiratory system that notably occurs in macrophages (12) and human AECs (12). It was hypothesized that D-4F ameliorates the IPF model *in vivo*. We conducted an untargeted lipidomic and transcriptomic analysis to investigate the effects of D-4F on the IPF model *in vivo*. We sought to identify a crucial target and explain the mechanism of D-4F in the IPF model.

We present the following article in accordance with the ARRIVE reporting checklist (available at <https://dx.doi.org/10.21037/atm-21-3777>) (13,14).

Methods

Animals

C57BL/6 male mice were purchased from SPF Biotechnology

Co., Ltd. (Beijing, China). This study began when all the mice were 8-weeks-old. Under specific pathogen free (SPF) conditions, the mice were fed an *ad-libitum* diet of laboratory pellet chow and tap water, and were kept on a 12-hour light/dark cycle at the Animal Centre of Shandong University. Experiments were performed under a project license (No.: 21109) granted by Laboratory Animal Ethical and Welfare Committee of Shandong University Cheeloo College of Medicine, in compliance with Shandong University Cheeloo College of Medicine guidelines for the care and use of animals. A protocol was prepared before the study without registration.

Induction of lung fibrosis

Bleomycin hydrochloride was purchased from Hisun Pfizer Pharmaceuticals Co., Ltd. (Zhejiang, China). D-4F (Ac-DWFKAFYDKVAEKFKKEAF-NH₂) was synthesized by Cloud-Clone Corp (purity >98%, Wuhan, China). Bleomycin and D-4F were dissolved in phosphate-buffered saline (PBS). Because of the minimum sample size of untargeted lipidomic analysis and the mortality of Bleomycin-induced IPF model, 36 C57BL/6 mice enter into this experiment. There were 3 groups in our study and there were 12 mice in each group. All C57BL/6 mice were randomly assigned to 3 groups: the Blank Group (BLK Group), Bleomycin Treatment Group (Model Group), and the D-4F Interference Group (Inter Group).

The way of induction of lung fibrosis is as the article described (15). To induce pulmonary fibrosis, the mice were anesthetized with pentobarbital sodium salt and given a single intratracheal instillation of 60 μ L (2 mg/kg) or PBS as a control. After bleomycin or PBS instillation, D-4F (3 mg/kg) was intraperitoneally injected once a day for 4 weeks in the Inter Group. All the mice were anesthetized 4 weeks later. Blood and lung tissue samples were taken. The plasma samples were frozen immediately and stored at -80 °C. The lungs were perfused with PBS to remove blood, and portions of the left lungs were fixed in 4% paraformaldehyde.

Histopathology

The portions of the left lungs underwent hematoxylin and eosin (H&E), Masson's trichrome (M-T), and immunohistochemical staining. The alpha smooth muscle actin (α -SMA) antibody (Abcam Cat# ab124964, RRID:AB_11129103) was purchased from Abcam (Cambridge, UK). The Ashcroft scale was used to

evaluate lung fibrosis (16). The density of the M-T and immunohistochemistry staining of α -SMA were scored using an image J analysis program.

TGF- β 1 measurement

TGF- β 1 was measured using enzyme-linked immunoassay (ELISA) kits purchased from Beyotime Biotechnology (Shanghai, China).

Untargeted lipidomic analysis

There were 9 samples per group. The samples were analyzed as described previously (17-19). Detailed step-by-step descriptions are provided in the [Appendix 1](#). In brief, after lipid extraction, an untargeted lipidomic analysis, ultra-high performance liquid chromatography (UHPLC)-tandem mass spectrometry (MS)/MS analysis, data preprocessing and filtering, and a multivariate statistical analysis were performed on the samples. The results of the untargeted lipidomic analysis are provided in the available online: <https://cdn.amegroups.cn/static/public/atm-21-3777-01.xlsx>.

Transcriptomic analysis

There were 9 samples per group, which were mixed into 3 samples for the transcriptomic analysis. The samples were analyzed as described previously (20-24). Detailed steps are outlined in the [Appendix 2](#). In short, after ribonucleic acid (RNA) extraction and qualification, the samples were subject to a transcriptomic analysis, library preparation for transcriptome sequencing, clustering and sequencing. The results of the transcriptomic analysis were verified by a quantitative polymerase chain reaction (qPCR) using sequence-specific primers. The results of the transcriptomic analysis and qPCR are provided in the available online: <https://cdn.amegroups.cn/static/public/Appendix 4-5.zip>.

Statistics

Values are expressed as means \pm standard error of the mean (SEM). Significant differences were assessed by *t*-tests. GraphPad Prism software (ver. 8.0; RRID:SCR_002798 GraphPad, La Jolla, CA, USA) was used to perform all the statistical analyses. A value of $P < 0.05$ was considered significant.

Results

The histopathological effects of D-4F on BLM-induced pulmonary fibrosis

After instilling BLM into the mouse lungs, H&E and M-T staining of the Model Group showed significant pulmonary fibrosis. The lung structure showed severe distortion and collagen fibers were accumulated in the lung (see [Figure 1](#)). The Ashcroft score and image analysis of M-T staining in the Model group were significantly more severe than those of BLK group. However, the results of H&E and M-T staining revealed that the Inter Group showed significantly attenuated BLM-induced pulmonary fibrosis. The structure of the alveolar wall and diffuse fibrosis were reduced in the Inter Group. The Ashcroft score and the imaging analysis results of the M-T staining of the Inter Group were significantly lower than those of the Model Group (see [Figure 2](#)). The α -SMA is a kind of mesenchymal marker. α -SMA expression in the interstitial fibrosis was higher in Model Group than the Inter Group (see [Figure 3](#)), but was significantly more decreased in the Inter Group than the Model Group (see [Figure 2](#)). To gain insights into the pathological changes, the level of TGF- β 1 in the serum was analyzed and summarized (see [Figure 2](#)). The results showed that the serum level of TGF- β 1 in the Model Group was significantly higher than that in the BLK Group. The TGF- β 1 level of the Inter Group was significantly lower compared to that of the Model Group.

The untargeted lipidomic analysis in the D-4F group

The lipidomic of every group was analyzed by a principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) plots (see [Figure 4](#)). An obvious separation was observed in the OPLS-DA score scatter plots between the BLK and Model Groups (R^2Y : 0.977, Q^2 : 0.792, Q^2 intercept: -0.376), and the Model and Inter Groups (R^2Y : 0.957, Q^2 : 0.405, Q^2 intercept: -0.291).

Using a variable importance in projection (VIP) score > 1 and a $P < 0.05$, we identified 1,100 differentially altered lipids (DALs) in the BLK and Model Groups and 92 DALs in the Model and Inter Groups. There were 35 common DALs in the 1,100 DALs and 92 DALs (see [Figure 5](#)). The 92 DALs of the Model and Inter Groups comprised 65 glycerophospholipids, 6 sphingolipids, 16 glycerides, and 5 other lipids. The glycerophospholipids contained the following 13 classes: phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG),

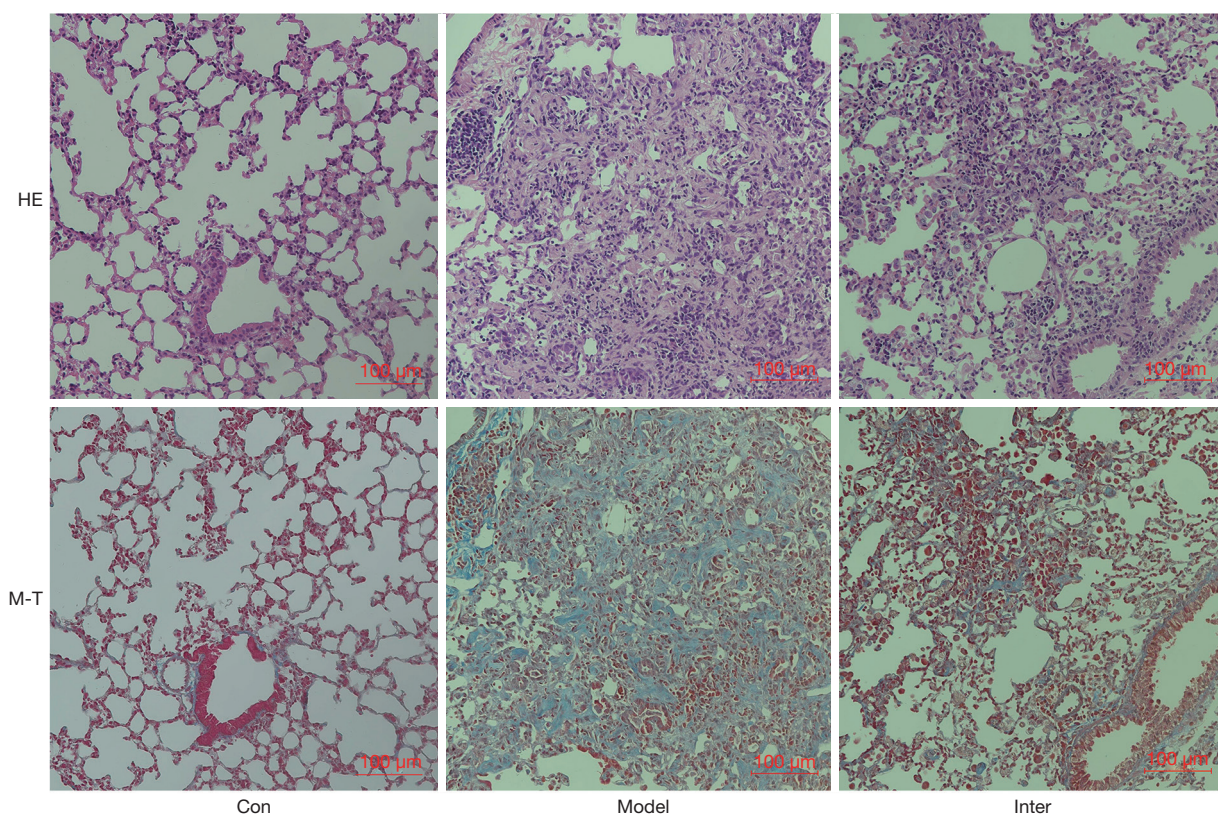


Figure 1 Effects of D-4f on BLM-induced pulmonary fibrosis. The histological results of H&E and M-T staining. (200× magnified). BLM, bleomycin; H&E, hematoxylin and eosin; M-T, Masson's trichrome.

phosphatidylethanol (PEt), phosphatidylethanolamine (PE), phosphatidylcholine (PC), lysophosphatidylserine (LPS), lysophosphatidylmethanol (LPM), lysophosphatidylinositol (LPI), lysophosphatidylethanolamine (LPE), lysophosphatidylcholine (LPC), dimethylphosphatidylethanolamine (dMePE), and lysodimethylphosphatidylethanolamine (LdMePE). The sphingolipids contained the following 3 classes: phytosphingosine (phSM), sphingomyelin (SM), and Gangliosides (GM3). The glycerides contained the following 2 classes: diglyceride (DG) and triglyceride (TG). The other lipids contained fatty acid (FA), Digalactosyldiacylglycerol (DGDG), and Sulfoquinovosyldiacylglycerol (SQDG) (see *Figure 6*).

To characterize any related lung lipids altered by D-4F treatments, we conducted a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis to identify the top 10 significant pathways in the 35 common DALs (see *Figure 5*). The potential target “glycerophospholipid metabolism” pathway was the most highly enriched (10 converted lipids; $P=7.662 \times 10^{-11}$).

The transcriptomic analysis in the D-4F group

To determine the lipidomic alterations between the Model Group and the Inter Group, transcriptomic changes were assessed using RNA-sequencing. Using a fold change ≥ 2 or < 2 and a $P < 0.05$, we identified 2,179 DEGs in the BLK and Model Groups, and 217 DEGs in the Model and Inter Groups (see *Figure 7*). The top 10 significant enriched KEGG analysis pathways were shown (see *Figure 8*). The Ras signaling pathway ($P=0.02734$) was in these pathways, and the genes involved in this pathway were *Fgf23*, *Fgfr3*, *Flt1*, phospholipase A₂ group 4c (*Pla2g4c*), *Plce1*, and *Rapgef5*.

There were 99 common DEGs in the BLK, Model Group and Inter Groups. To identify the key pathways associated with D-4F, a KEGG analysis was performed on the 99 common DEGs. The top 10 significant pathways in these DEGs are shown in *Figure 9*. We chose the mitogen-activated protein kinase (MAPK) signaling pathway as a key pathway. This pathway was obviously enriched (4 gene counts; $P=0.0152$).

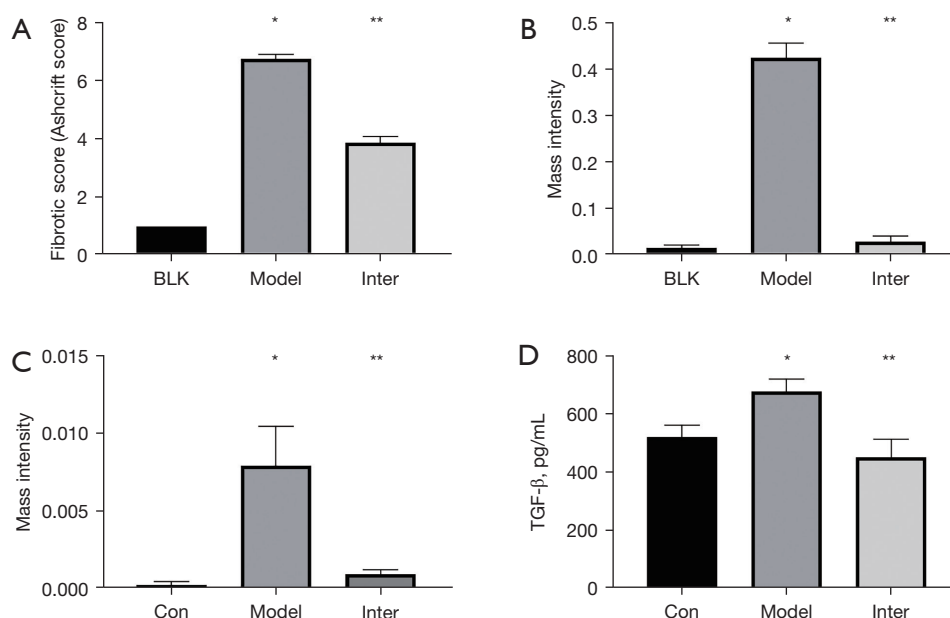


Figure 2 The fibrotic score and image analysis. (A) Fibrotic scores (Ashcroft scores) were calculated to analyze the 3 groups. The fibrotic score of the Model Group increased significantly compared to that of the BLK Group. Additionally, the fibrotic score of the Inter Group was significantly reduced compared to that of the Model Group. (B) The M-T score increased remarkably in the Model Group. The score of the Inter Group was decreased compared to that of the Model Group. (C) The results of the immunohistochemistry staining of α -SMA was similar to the M-T score. (D) The level of TGF- β 1 in the plasma. n=3 in the BLK Group; n=8 in H&E; n=5 in M-T staining and immunohistochemistry staining of α -SMA. *, P<0.05 compared to the BLK Group; **, P<0.05 compare to the Model Group. H&E, hematoxylin and eosin; M-T, Masson's trichrome; α -SMA, alpha smooth muscle actin; TGF- β 1, transforming growth factor β 1.

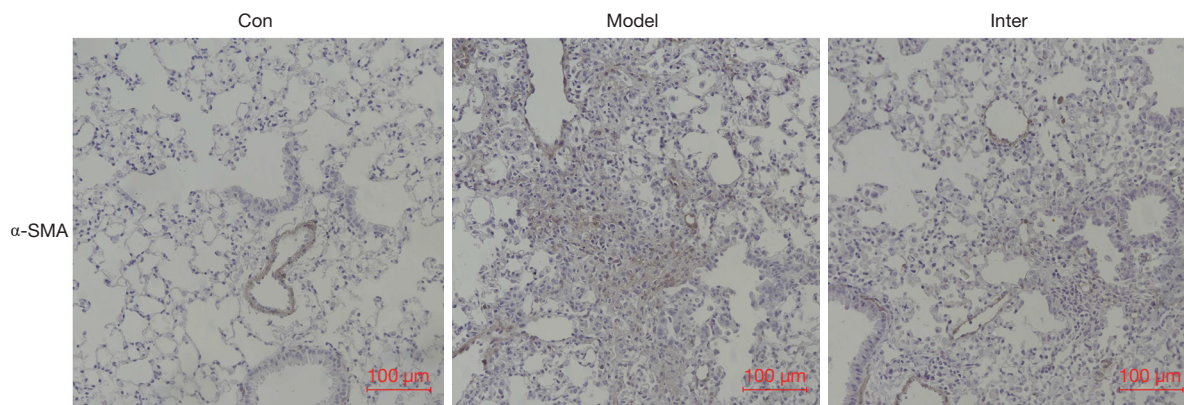


Figure 3 The histological results of immunohistochemistry staining of α -SMA. (200 \times magnified). α -SMA, alpha smooth muscle actin.

The lipid-transcript correlative analysis

Based on the results of the lipidomic and transcriptomic analysis of the Model and Inter Groups, the protein-protein interaction network (PPI network) was employed. This network was constructed from 217 DEGs and 92 DALs.

The PPI network comprised 10 pathways, and we selected glycerophospholipid metabolism based on the results of the lipidomic analysis (see *Figure 10*).

To conform to the PPI network of glycerophospholipid metabolism, the 35 common DALs in the D-4F group were divided into the following 9 classes: C00157, C04230,

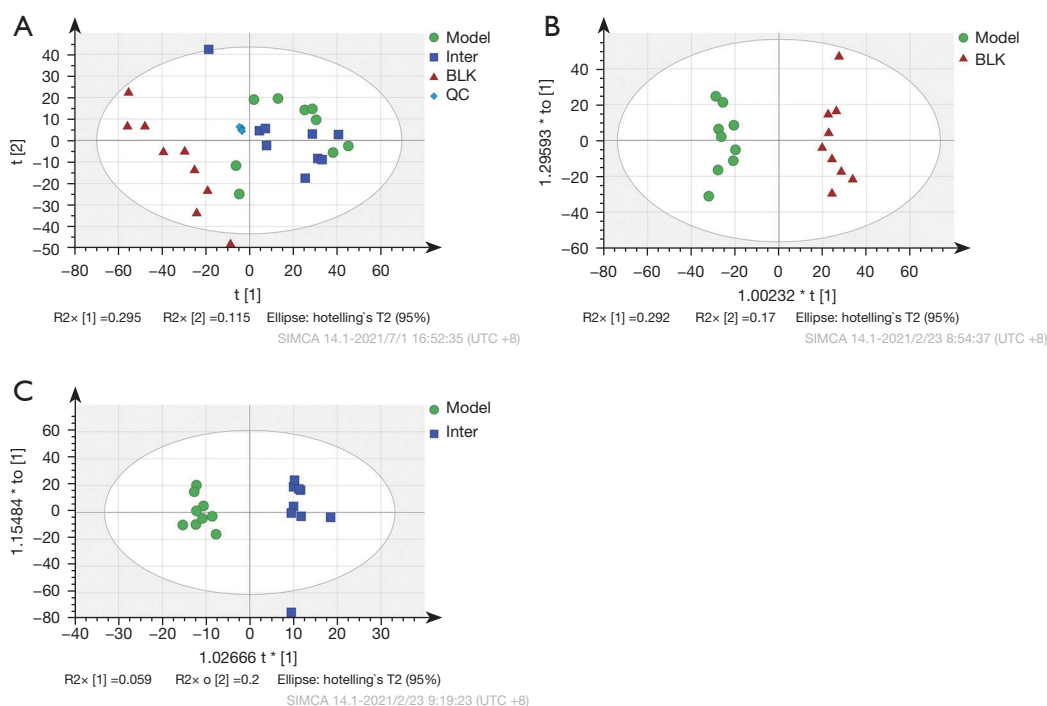


Figure 4 PCA and OPLS-DA. (A) PCA plot for the BLK, Model and Inter Groups and QC samples. OPLS-DA plot for the (B) BLK and Model Groups, (C) Model and Inter Groups. PCA, principal component analysis; OPLS-DA, orthogonal partial least-squares discriminant analysis; QC, quality control.

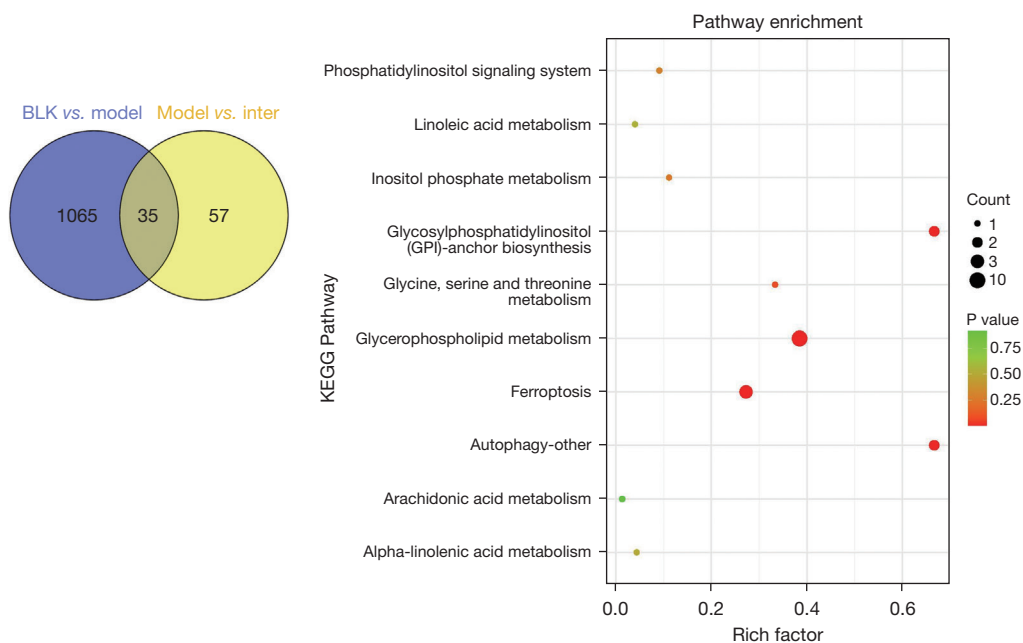


Figure 5 Lipidomic data analysis in the D-4F group. To acquire significant DALs in the D4-F group, the BLK versus Model dataset (purple) was compared to the Model versus Inter dataset (yellow). A KEGG analysis was performed on the 35 common DALs found in the D-4F group. DAL, differentially altered lipid; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Lipid type	Lipid class	addr.	Number of molecules
Glycerophospholipids	Phosphatidylserine	PS	9
	Phosphatidylinositol	PI	2
	Phosphatidylglycerol	PG	6
	Phosphatidylethanol	PEt	1
	Phosphatidylethanolamine	PE	13
	Phosphatidylcholine	PC	13
	Lysophosphatidylserine	LPS	1
	Lysophosphatidylmethanol	LPMe	1
	Lysophosphatidylinositol	LPI	1
	Lysophosphatidylethanolamine	LPE	1
	Lysophosphatidylcholine	LPC	12
	Lysodimethylphosphatidylethanolamine	LdMePE	4
	Dimethylphosphatidylethanolamine	dMePE	1
	Sphingomyelin	SM	2
Sphingolipids	Phytosphingosine	phSM	3
	Gangliosides	GM3	1
Glycerides	Diglyceride	DG	1
	Triglyceride	TG	15
Other lipids	Fatty acid	FA	1
	Digalactosyldiacylglycerol	DGDG	1
	Sulfoquinovosyldiacylglycerol	SQDG	3

Figure 6 Numbers of detected individual lipid molecules in the Model and the Inter Groups.

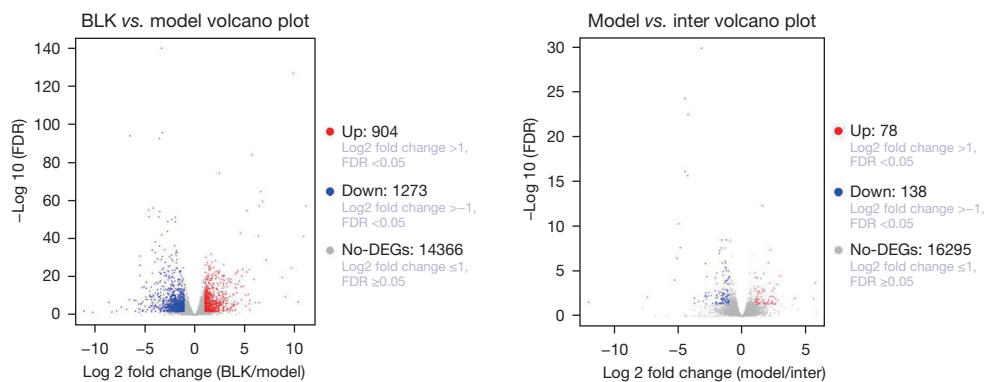


Figure 7 Volcano plot of BLK vs. Model and Model vs. Inter Groups.

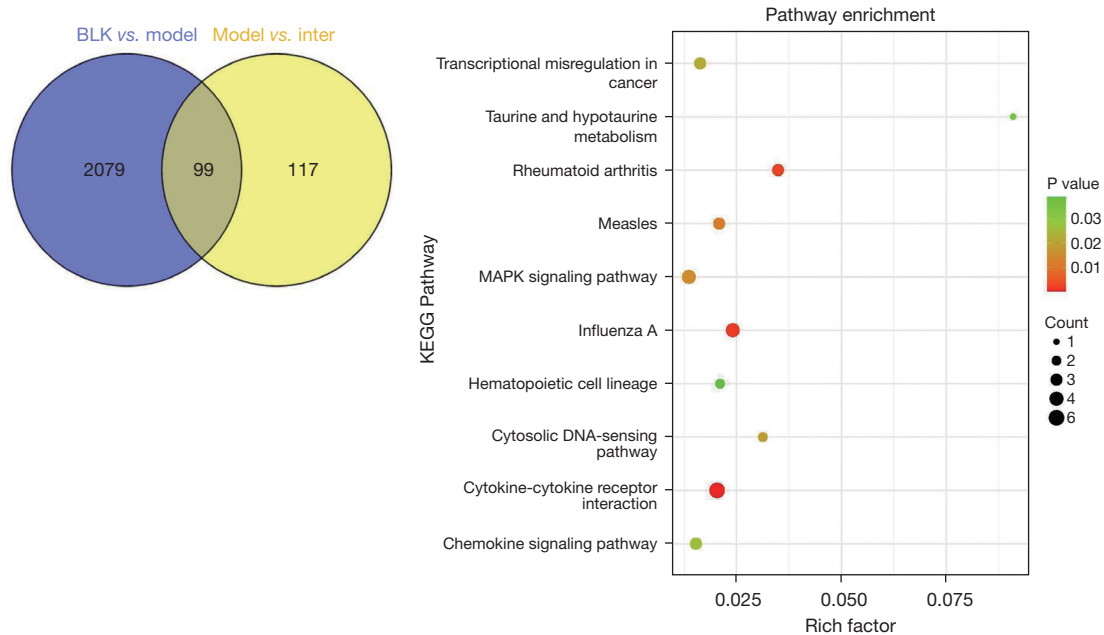
C00350, C01194, C02737, C04438, PG, LPS, and TG. Compared to that of the Model Group, the lipid classes of the Inter Group were significantly decreased in terms of TG (P=0.0189), C02737 (P=0.0013), and LPS (P=0.0081). and increased in terms of C04230 (P=0.0046), PG (P=0.0247), C01194 (P=0.0142), and C04438 (P=0.0423). C00157 and C00350 were no significant difference between the Model

Group and the Inter Group (see *Figure 11*).

Pla2g4c is a crucial gene in the MAPK pathway, and glycerophospholipid metabolism. Pla2g4c is a calcium+-independent PLA2 family (25,26) and was significantly decreased in the Inter Group. Pla2g4c is a member of the PLA2 family, and pla2g2a, which is another member of the PLA2 family, has been reported to be increased in BLM-

TermID	Description	Enrich factor	P value
ko04657	IL-17 signaling pathway	4.542	0.00463
ko05323	Rheumatoid arthritis	4.556	0.01112
ko01503	Cationic antimicrobial peptide (CAMP) resistance	71.764	0.01393
ko04015	Rap1 signaling pathway	2.760	0.02086
ko04640	Hematopoietic cell lineage	2.594	0.02734
ko04014	Ras signaling pathway	2.594	0.02734
ko00254	Aflatoxin biosynthesis	35.882	0.02768
ko04060	Cytokine-cytokine receptor interaction	2.326	0.02979
ko04070	Phosphatidylinositol signaling system	4.394	0.3926
ko04623	Cytosolic DNA-sensing pathway	4.394	0.3026

Figure 8 Top 10 significantly enriched KEGG analysis pathways among the DEGs in the Model and Inter Group. DEG, differentially expressed gene; KEGG, Kyoto Encyclopedia of Genes and Genomes.



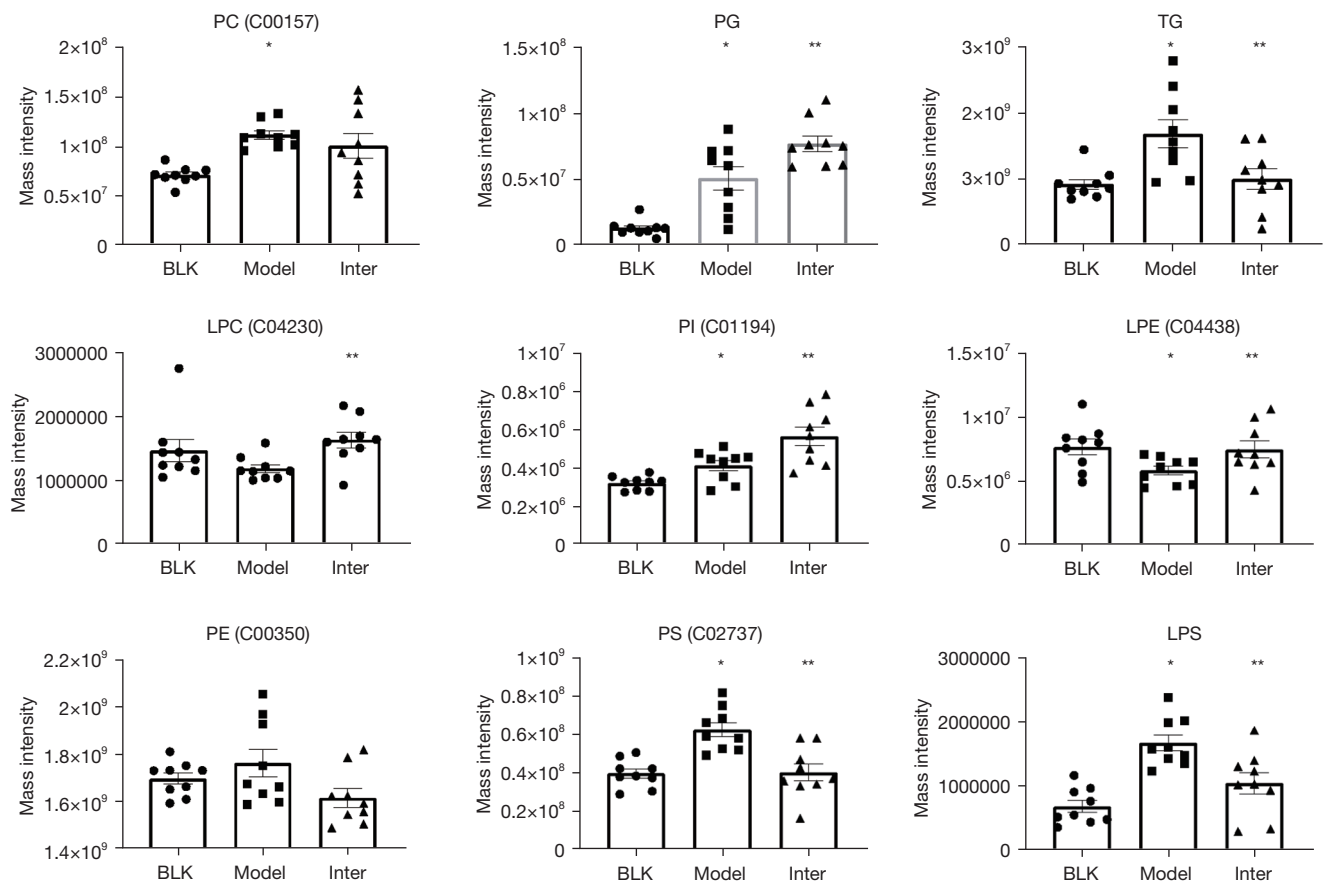


Figure 11 The common DALs in the D-4F group. Based on the PPI network, 35 common DALs were divided into 9 classes. *, $P < 0.05$, compared to the BLK Group; **, $P < 0.05$, compare to the Model Group. DAL, differentially altered lipid; PPI network, protein-protein interaction network.

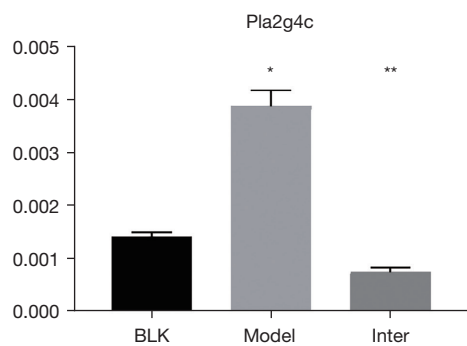


Figure 12 The expression of *pla2g4c* in the BLK, Model and Inter Groups using quantitative real-time PCR. *, $P < 0.05$, compared to the BLK Group; **, $P < 0.05$, compare to the Model Group.

alternative activation, and reduced TGF- β 1 transcription and translation (38). D-4F inhibits EMT and macrophage alternative activation *in vitro*, indicating that D-4F may attenuate IPF *in vivo*.

An untargeted lipidomic analysis was conducted to examine the effects of D-4F on BLM-induced mice with a lipid metabolites disorder. Overall, we found that the lipids in the lung were significantly changed in BLM-induced and D-4F-treated lungs. In this study, there were 35 common DALs in the D-4F group. A KEGG pathway analysis of those DALs revealed a significant alteration in glycerophospholipid metabolism. The PG and PI levels of the DALs were increased in the Model and Inter Groups. PG is a major component of lung, and has been reported to interact with macrophages Monocyte differentiation antigen

CD14 (CD14) and myeloid differentiation protein-2 (MD2) *in vitro* (39). Further, it was reported that PI binds to the CD14 plasma membrane and prevents monocyte activation by bacterial lipopolysaccharides (40). Saito *et al.* (15) conjectured that high levels of PG and PI counteract inflammation and thus ameliorate BLM-induced lung injury. Our study showed that PG and PI were more increased in the Inter Group than the Model Group, and D-4F can decrease inflammation in experimental asthma and influenza infection. Thus, D-4F increased PG and PI by reducing inflammation to improve BLM-induced lung injury.

Additionally, PS was significantly increased in the Model Group but decreased in the Inter Group. PS exists in all human cells, is an essential component on the inner leaflet of the cell membrane, and is externalized upon the induction of apoptosis, resulting in efferocytosis (41). The overexpression of PS enhances efferocytosis, leading to autoimmunity (42). Research has shown that apoptotic levels were elevated in the bronchoalveolar lavage macrophages of patients with IPF (43). Additionally, previous research has shown that the growing expression of TGF- β results from the uptake of apoptotic cells by macrophages, which is a growth factor in both anti-inflammatory and pro-fibrotic activities (44). Thus, in relation to the decreased levels of PS in the Inter Group, it may be that D-4F counteracted the apoptosis and suppressed macrophage activation.

The results of the transcriptomic and KEGG analyses showed that the MAPK pathway was involved in the ApoA-1-/- group and the D-4F group. MAPK is a cellular bioenergetic sensor metabolic regulator and plays an important role in growth differentiation and stress responses (45). The activated MAPK pathway is directly correlated with the severity of pulmonary fibrosis. In IPF patients, the MAPK pathway is activated in epithelial cells, smooth muscle cells, and fibroblasts (46). The TGF- β activated MAPK pathway is essential in the aberrant proliferation of pulmonary interstitial fibroblasts (47). Conversely, the specific inhibitor FR-167653, which inhibits MAPK signaling, ameliorated BLM-induced IPF in mice (48). D-4F decreased the level of TGF- β in the Model Group and inhibited the MAPK pathway to ameliorate BLM-induced lung injury. Additionally, the Ras signaling pathway was enriched in the Model and Inter Groups. The Ras signaling pathway plays an important role in regulating blood pressure and electrolyte balance. Recently, research has begun to unravel the role of Ras in inflammatory responses in the lungs (49). TGF- β is the most potent pro-

fibrotic cytokine and acts downstream of Ang II in vascular smooth muscles, myofibroblasts, and macrophages (50). EMT, which is a hallmark of IPF, was induced by TGF- β via the RAS and MAPK pathway (51). Additionally, Renin inhibition by aliskiren has been reported to attenuate lung fibrosis, which decreases TGF- β and prevents myofibroblasts activation and differentiation in the BLM-induced IPF model (52). Overall, D-4F ameliorated BLM-induced lung injury and decreased the levels of TGF- β via the Ras/MAPK pathway.

Pla2g4c [also referred to as cytosolic PLA2 γ (cPLA2 γ)] was identified as an ortholog cPLA2a belonging to the cPAL2 family and contains a lipase consensus sequence that lacks the C2 domain (53,54). cPLA2 γ is a major enzyme involved in phospholipid AA remodeling (55). Additionally, cPLA2 γ catalyzes CoA-dependent transacylation and exhibits lysophospholipase/transacylation activity, but it prefers lysophospholipase/transacylation to CoA-dependent transacylation reaction as acyl donors (53,54,56). Yamashita *et al.* suggested that purified cPLA2 γ catalyzes acyltransferase activity between 2 LPC molecules to form PC and glycerophosphocholine, and between 2 molecules of LPE to form PE and glycerophosphoethanolamine (53). In this study, the level of pla2g4c decreased, while the levels of LPC and LPE increased, which suggests that pla2g4c plays a crucial role in the treatment of D-4F on BLM-induced IPF.

In conclusion, this study demonstrated that D-4F ameliorating BLM-induced IPF is associated with glycerophospholipid metabolism and the Ras/MAPK pathway. Further, pla2g4c may be the core genes that alleviate BLM-induced IPF after D-4F treatment. Our results identified potential targets for treating IPF.

Acknowledgments

Funding: This work was supported by the Research Award Fund for Outstanding Young Scientists of Shandong Province (BS2015YY043), the Natural Science Foundation of the Shandong Province (ID-ZR2017MH122), and the Shandong Medicine and Health Science and Technology Development Plan Project (2014WS0139).

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <https://dx.doi.org/10.21037/atm-21-3777>

Data Sharing Statement: Available at <https://dx.doi.org/10.21037/atm-21-3777>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://dx.doi.org/10.21037/atm-21-3777>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (No.: 21109) granted by Laboratory Animal Ethical and Welfare Committee of Shandong University Cheeloo College of Medicine, in compliance with Shandong University Cheeloo College of Medicine guidelines for the care and use of animals.

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Cite this article as: Xia Y, Cheng M, Hu Y, Li M, Shen L, Ji X, Cui X, Liu X, Wang W, Gao H. Combined transcriptomic and lipidomic analysis of D-4F ameliorating bleomycin-induced pulmonary fibrosis. *Ann Transl Med* 2021;9(18):1424. doi: 10.21037/atm-21-3777