

Study on the changes of ADRP+ cell population in the process of pulmonary fibrosis

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Research

Keywords: Idiopathic pulmonary fibrosis, ADRP, Macrophage, Lipofibroblast

Posted Date: May 8th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-26766/v1>

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Abstract

Background

ADRP is a marker of lung lipofibroblasts. Lipofibroblasts play an important role in assist type 2 alveolar epithelial cells function in peripheral lung tissue. Pulmonary fibrosis is characterized by continuous irreversible destruction of peripheral lung tissue. The expression of ADRP and the role of ADRP⁺ cells in pulmonary fibrosis are of interest to us.

Methods

Quantitative PCR as well as immunohistochemical multiplex staining were used to analyze the expression of ADRP during lung development, bleomycin-induced pulmonary fibrosis, and identify the type and function of ADRP⁺ cells during pulmonary fibrosis lesions.

Results

ADRP⁺ cells were found to decrease gradually from birth to adulthood. During pulmonary fibrosis, the expression of ADRP increased gradually, while another marker of lipofibroblast, the expression of PDGFR α decreased. There was a co-localization relationship between macrophage marker CD68 and ADRP. Both M1-type and M2-type macrophages can express ADRP in the early stage of pulmonary fibrosis. While in the subsequent pulmonary fibrosis process, M1-type ADRP⁺ macrophages gradually decrease, while the ADRP⁺ cells were mainly M2-type macrophages. ADRP⁺ M2-type macrophages can release S100A4 and distribute around the lesion area of pulmonary fibrosis. Pulmonary fibrosis gradually developed as M2-polarized macrophages replaced M1 to become the main population of pulmonary ADRP⁺ macrophages

Conclusion

In the process of pulmonary fibrosis, a large number of ADRP⁺ cells are macrophages, including M1 and M2 types successively. The aggregation site of ADRP⁺ M2-type macrophages indicates that fibrosis damage is about to or has already occurred. This study may provide a new research direction for the treatment of pulmonary fibrosis.

Background

Adipose differentiation related protein (ADRP) belongs to Perilipin protein family, is also called Perilipin 2 or Plin2. ADRP distributed on the surface of neutral lipid droplets in cytoplasm. Cytoplasmic lipid droplets degradation can be prevented when ADRP encapsulated. This results in accumulation of

intracytoplasmic lipid droplets. ADRP plays an important role in the balance between lipid droplets storage and lipid outflow[1]. On the other hand, as a marker of cell differentiation, ADRP expression is strongly induced to form ADRP⁺ cell when cells are faced with increased lipid load[2]. For example, mononuclear macrophages engulf a large amount of lipids to form foam cells in the atherosclerotic plaque, and ADRP is the marker protein of foam cells [3]. Knocking out ADRP gene affects the formation of foam cells and inhibits atherosclerosis[4]. Changes in ADRP expression patterns may indicate changes in the course of fibrosis. Decreased ADRP expression is associated with lipid loss and fibrosis in hepatic stellate cells [5]. Inducing the expression of ADRP can inhibit the expression of genes related to liver fibrosis in cultured cells, while inhibition of ADRP expression can reverse this process.[6]

ADRP is also expressed in lung tissue. There are ADRP⁺ cells in the lung interstitial tissue of normal newborn mice. These cells called lipofibroblasts for their cytoplasm contain neutral lipid droplets. Distributed near type II alveolar epithelial cells, lipofibroblasts play important role in lipid uptake and transport to type II alveolar epithelial cells, affecting the synthesis and secretion of surfactant[7, 8]. Lipofibroblasts contain active substances such as retinoic acid, which are closely related to alveolar growth, differentiation, homeostasis and repair. Decreased lipofibroblasts in newborn mice may lead to bronchopulmonary dysplasia (BPD)[9]. In the interstitial tissue of the terminal lung, the composition of lipofibroblasts is complex. Lipofibroblasts express many types of marker molecules, such as PDGFR α , CD90, PPAR γ , and ADRP[10, 11]. Among these molecular markers, ADRP is often used as a target for immunohistochemical examination of mature lipofibroblasts due to its coexistence with neutral lipid droplets. Most often, lipofibroblasts are ADRP⁺ cells in normal lung tissues.

Pulmonary fibrosis is a progressive and irreversible destruction of alveolar structures in peripheral lung tissues. In this process, the role of lipofibroblasts is of concern. It has been reported that lipofibroblasts transdifferentiated into myofibroblasts during pulmonary fibrosis induced by one-time perfusion of bleomycin through the airways, while lipofibroblasts reappear when fibrosis is alleviated[12]. Based on scRNA-seq technique, recent study has shown that pulmonary lipofibroblasts can also express immune function-related genes and some M2 macrophage-related genes[13]. Unlike one-time Bleomycin administration in the airway, which induces self-healing pulmonary fibrosis, multiple intraperitoneal administration causes repeated lung tissue damage and repair, which triggers irreversible fibrosis which is more similar to clinical pulmonary fibrosis to some extent[14]. In this case, we are interested in the expression pattern of ADRP, the fate of ADRP⁺ cells, and the relationship between ADRP⁺ cells and fibrosis. We found that macrophages can form intrapulmonary "ADRP⁺ cells", similar to foam cells in atherosclerotic plaques, and affect lesions in adjacent lung tissues.

Methods

Animal grouping, pulmonary fibrosis model preparation and lung section

8–12 weeks old healthy male C57 BL/6 mice, weighing 18–25 g, were purchased from Liaoning Changsheng Biotechnology Co., Ltd., China, (license no: SCXK [liao] 2010-0001. The experiment was approved by the Animal Welfare and Ethics Committee of China Medical University. The mice were randomly divided into 5 groups with 6 mice in each group, including 1 control group and 4 experimental groups which were induce pulmonary fibrosis. Experimental mice were intraperitoneally injected with Bleomycin according to the following protocol that is 1 USP/mouse on days 0 and 2, and 0.5 USP/mouse on days 4 and 6, and 0.25 USP/mouse on days 9, 12, 15, 18, 21, 24, and 27. The experimental groups were harvested on the 7th, 14th, 21st and 28th day respectively to observe the course of disease. The control group was injected with the same volume of normal saline and collected on the 28th day.

To evaluate the effect of different number of injection times on induced pulmonary fibrosis in mice, we truncated modeling process. The mice were randomly divided into 6 groups with 6 mice in each group, including 1 control group and 5 experimental groups. According to the modeling protocol, the mice in the five experimental groups completed 1,2,3,4 and all 11 times intraperitoneal Bleomycin injection respectively. The normal control group was injected with the same volume of normal saline. All six groups mice lung were collected on the 28th day.

Mice were sacrificed by cervical dislocation and the dissected. Normal saline is injected from the right ventricle for pulmonary perfusion to clear the blood from the lungs until they become white. Lung was filled with 4% paraformaldehyde of 20 cm of water pressure, then it was ligated and fixed in a container containing the 4% paraformaldehyde for 16 hours at 4°C. Alcohol gradient dehydration, xylene transparent, and paraffin-embedded. Cut 5 µm thick tissue sections

HE staining, Masson staining

HE staining: Lung tissue sections were deparaffinized and rehydrated, stained with hematoxylin for 1 min, washed with distilled water for 20–30 times, differentiated with 0.5% ethanol for 20 s, washed with distilled water for 20 s; blue with 1% aqueous ammonia for 10 s, washed with distilled water for 20–30 times; washed with 95% ethanol two times, and stained with eosin for 1 min. Then the tissue sections were dehydrated conventionally, transparent, and sealed with neutral gum, then observed under the microscope.

Masson staining: The lung tissue sections were deparaffinized and rehydrated, washed with water; stained with orcein staining solution for 20 min, washed with water; stained with iron hematoxylin staining solution for 10 min; differentiated with hydrochloric acid ethanol for 20 s, washed with water; returned to blue with 1 × PBS for 20 s, washed with water; stained with Ponceau S - acid fuchsin staining solution for 10 min, washed with 0.2% glacial acetic acid for 1 min; color separation with 0.5% phosphomolybdic acid for 40–45 s, washed with 0.2% glacial acetic acid for 1 min; stained with 0.5% aniline blue staining solution for 1 min, washed with 0.2% glacial acetic acid for 1 min; blotting up excess staining solutions with filter paper; the tissue sections were routinely dehydrated, transparent, sealed with neutral gum, and dried, then observed under the microscope.

Immunochemistry Stain

The lung tissue sections were deparaffinized in xylenes. Hydrate sections gradually through graded alcohols, washed with 1 × PBS, treated with methanol/H₂O₂ solution at room temperature for 10 min, washed with 1 × PBS, boiled for 10 min with citrate buffer (10 mM Sodium Citrate, pH 6.0) for antigen retrieval, allow slides to cool in the buffer for 30 minutes in room temperature. To suppress non-specific binding of IgG, these sections were blocked with non-immune serum of the same species in which the secondary antibody is raised for 10 minutes at room temperature. The primary antibody CD206(R&D,AF2535,USA), CD86(Abcam,ab53004,China), CD68 (Servicebio,18080,China), ADRP(Abcam,ab52356,USA), α-SMA(Santa Cruz, sc-32251,USA), N-cadherin(Genetex,GTX127345,USA), S100A4(Cell Signaling Technology,13018S,USA), Caspase9(Cell Signaling Technology,9509S,USA), PCNA(Santa Cruz,sc-7907,USA) was diluted 1:100 or 1:500 with 1% BSA and incubated overnight at 4°C. Then incubate for 20 minutes with biotin-conjugated secondary antibody in at 37°C. The primary antibody was detected using Biotin-Streptavidin HRP detection system (ZSGB-BIO, China). Finally, the color was developed using an AEC Chromogenic Kit (BOSTER, China), and then the sections were washed with distilled water, counterstained with hematoxylin. Mount coverslip with aqueous mounting medium, then observed under the microscope.

Random observation of lung tissue visual field of three different lung fibrosis mice under 10 × 20 times microscope. If the cell is not stained, score 0, if the cell is stained, score 1. Taking the average value of the integral arithmetic of 3 fields as the final integral. The ADRP⁺ and CD68⁺ cells and co localization cells were counted, and the proportion of ADRP⁺ CD68⁺/ADRP⁺ cells in different periods was obtained. The same method was used to obtain the ratio of ADRP⁺CD86⁺/ADRP⁺ cells and ADRP⁺CD206⁺/ADRP⁺ cells in different periods.

Immunohistochemical antibody stripping multiple staining

Photographs were taken under microscope after the first immunohistochemical staining. Wash the AEC stain with gradient alcohol (25%-95%). Antibodies were stripped in the buffer containing 65 mM Tris-HCl pH6.8(Sigma, USA) 1% SDS Solarbio, USA 0.113 M 2- mercaptoethanol Sigma, USA 0.1 M NaCl 2 M Urea Sigma, USA, in 56°C water bath with agitation in the fume hood twice for 30 minutes each. After antibody stripping, the sections were washed in distilled water for 0.5 hours and replaced every 5 minutes in room temperature. Then the sections can be used for immunohistochemistry staining again according to the above protocol. Without adding first antibody, a negative control was setup to avoid any false staining due to incompletely antibody stripping.

Frozen tissue sections, Oil red O staining and Immunochemistry Stain

The model mice were sacrificed and the lungs were collected, perfused, and fixed with 4% paraformaldehyde for 16 hours following the above protocol. Then the lung samples were immersed in sucrose solutions with concentration gradients of 10%, 20% and 30%, respectively, for 24 hours in each gradient. Treated lungs were embedded with OTC and stored in -80 °C refrigerator, and Cut into 10 microns thick tissue sections under - 25°C.

The lung tissues frozen sections were stained with diluted Oil red O staining solution (Solarbio, USA) for about 10 minutes, 60% isopropanol color separation to the background colorless, counterstained with hematoxylin for 1 minute, washed with 1 × PBS, sealed with 70% glycerol and then took photograph. Next, the sections washed with 60% isopropanol and then ethanol hydrochloride washing to remove any color. The sections were used to stain ADRP following above immunohistochemistry protocol.

Realtime PCR

RNA was extracted from the left lobe of mouse lung. RNA samples were reverse transcribed into cDNA and then amplified and quantitated with Realtime PCR. The program was run on the ABI7500 Realtime PCR System instrument using TaKaRa SYBR PremixExTaq reagent (Cat# RR820A).

ADRP primer: GCGGGTGTGGTTAAGTCG

Ttctggggagtggtcagc

PDGFR α primer: AGGCTCTCATGTCTGAGCTG

TGTCCAGGTCTTTCTTCGGC

α -SMA primer: CCAACTGGGACGACATGGAA

GAGGCATAGAGGGACAGCAC

Collagen primer: ACATGTTTCAGCTTTGTGGACC

TAGGCCATTGTGTATGCAGC

CD68 primer: GGGGCTCTTGGGAACTACAC

GTACCGTCACAACCTCCCTG

CD86 primer: TCATTCCCGGATGGTGTGTG

TGAGCAGCATCACAAGGAGG

CD206 primer: TATAGGTGGAGAGCTGGCGA

CGGGAGAACCATCACTCCAG

Data analysis

All experiments were repeated 3 times. The measurement data were expressed as mean \pm standard deviation (mean \pm SD). The composition analysis was performed using GraphPad Prism5 data analysis

software. Comparison between multiple groups was performed by single factor analysis of variance. When $P < 0.05$, statistical significance was considered.

Results

1. ADRP⁺ cell in mice lung development and pulmonary fibrosis

ADRP is generally considered to be a marker molecule of lipofibroblasts in the lung. We examined the distribution of ADRP⁺ cells in the lungs of mice at different developmental stages by immunohistochemistry. ADRP⁺ cells gradually decreased with the formation of alveoli after birth and the ADRP⁺ cells were very rare in normal adult mice lung (Fig. 1a). Pulmonary fibrosis was induced in adult mice by intraperitoneal injection of bleomycin (FigS1). Lung samples were taken at the 7th (D7), 14th (D14), 21st (D21) and 28th days (D28) after modeling. The expression levels of some important cell differentiation markers were detected by Realtime-PCR. Compared with control group, α -SMA and Collagen gene expression levels increased significantly from the beginning of the D21. This was associated with increased myofibroblasts leading to pulmonary fibrosis. Lipofibroblasts related marker gene PDGFR α expression level decreased significantly from the 7th day, until D28. However, in contrast to PDGFR α , the expression of ADRP in D28 days was significantly increased compared with that in the control group (Fig. 1b).

We further examined the ADRP in lung tissue on the 7th and 28th day after modeling by immunohistochemical staining. Compared with the sample on the 7th day the number of ADRP⁺ cells on the 28th day' sample was increased (Fig. 1c). Lung tissue frozen sections were prepared from the 28th day's sample and stained with Oil red O. Oil red O positive stained cells were found. After Oil red O staining was removed from the same section, immunohistochemical staining showed that the positive cells with Oil red O staining were ADRP⁺ cells (Fig. 2). Found in the lung tissues of fibrosis model mice and containing neutral lipid particles, these ADRP⁺ cells have different distribution and shape from the general understanding lipid fibroblasts. Many of them were located in the alveolar cavity, with large diameter and single nucleus. These cells have morphological characteristics similar to that of macrophages in alveolar cavity.

2. During pulmonary fibrosis ADRP⁺ macrophage polarization pattern changes

We analyze the expression level of macrophage-associated molecular markers CD68, CD86, CD206 on the 7th, 14th, 21st and 28th days during the process of pulmonary tissue lesion in fibrosis model mice with Realtime-PCR techniques. It was found that the expression level of macrophage molecule marker CD68 gradually increased during the modeling process, and the significant difference began to appear on the 14th day after the modeling compared with that of the control group. The expression of M1-polarized macrophage molecular marker CD86 was also increased, and the significant difference began to appear

on the 28th day after modeling. Although the expression of M2-polarized macrophage molecular marker CD206 was increased in the same period, but the difference was not significant (Fig. 3a).

To identify whether ADRP⁺ cells are macrophages, we examined the co-localization relationship of ADRP with CD68⁺CD86 and CD206 on the 7th, 14th, 21st and 28th days of modeling mice lung section using multiple immunohistochemical staining. It was found that the ADRP⁺ cells increased gradually in the lung tissues of the model mice. These cells distributed in the lung tissues and alveolar cavity. In total ADRP⁺ cells, the proportion of ADRP⁺CD68⁺ cells were close to 80% on the 7th day and gradually increased to almost 100% on day 28 (Fig. 3b). There is no significant difference of ADRP⁺CD68⁺ cells proportion in these two time points. About half of the ADRP⁺ cells were ADRP⁺CD86⁺ cells on the 7th day in modeling mice lung section, and then to D28, the ADRP⁺CD86⁺ cells gradually approached to almost disappear (Fig. 3C). In the lung sections of mice collected on the 7th day after modeling, about half of the ADRP⁺ cells were ADRP⁺CD206⁺, while most of ADRP⁺ cells were ADRP⁺ CD206⁺ on day 14 and it lasted until the 28th day (Fig. 3d).

In summary, there is an ADRP⁺ macrophage polarization pattern changes during pulmonary fibrosis. In the early stage of bleomycin induced lung injury (D7), most of the ADRP⁺ cells were macrophages, that is M1-type or M2-type. As the damage continued (D14), the proportion of ADRP⁺ M2 macrophages exceeded that of ADRP⁺ M1 macrophages and nearly all ADRP⁺ cells were M2 macrophages after the peak of fibrosis (D21 and D28) in the model mice.

3. M2 polarized ADRP⁺ macrophage associated with pulmonary fibrosis

We detected ADRP, N-cadherin and α -SMA expression pattern in serial sections of the 21st day of modeling mice lung tissue by immunohistochemical staining. There were sporadic fibrosis lesions in the lungs of model mice. A large number of α -SMA and N-cadherin positive cells were found in the fibrosis lesion area. ADRP⁺ cells distributed in the area adjacent to fibrotic foci (Fig. 4a). While in the area without fibrosis lesion, no ADRP⁺ cells were found (Fig. 4b). This indicating that the appearance of M2 polarized ADRP⁺ macrophages is associated with fibrotic lesions.

In order to analysis of the relationship between ADRP⁺ macrophage appearance and the degree of lung injury, we reduced the number of intraperitoneal bleomycin administration, and only completed the first, second, third and fourth intraperitoneal injections respectively. After completion of the corresponding injection, modeled mice were fed to the 28th day and then lung samples were collected. Fibrosis foci were found in lung histopathology specimens of modeled mice which received three or more times intraperitoneal bleomycin injection (FigS2). There are aggregated α -SMA⁺ cells in fibrosis foci. ADRP⁺ cells distributed around the fibrosis foci (Fig. 5a). However, the lung structure was normal in the model

mice which were injected bleomycin only 1 or 2 times, and there were no fibrotic foci and ADRP⁺ cell aggregation (FigS2).

The expression level of CD68 gene, a macrophage marker, increased with the number of injections. The expression of lipofibroblast marker PDGFR α showed a significant decreasing trend after 2 administration, while the expression of ADRP increased significantly after 4 times administration (Fig. 5b). We examined the co-localization relationship of ADRP with CD206 in modeling mice with three intraperitoneal injections using multiple immunohistochemical staining. The results showed that ADRP⁺ M2 macrophages appeared around the fibrosis lesions (Fig. 5c). It is suggested that the formation of ADRP⁺ M2 macrophages is related to pulmonary fibrosis again.

4. M2-polarized ADRP⁺ macrophages were present at the site of peripheral lung tissue injury

The proliferation and differentiation status of ADRP⁺ cells were analyzed using multiple immunohistochemical staining. We found that PCNA was not expressed in ADRP⁺ cells in D21 model mice lung tissue. This indicate that ADRP⁺ cells did not proliferate. But adjacent to these ADRP⁺ cells, some of the interstitial cells expressed PCNA and showed proliferative status (Fig. 6a). We noticed that most of the ADRP⁺ cells expressed Caspase9. Moreover, in adjacent regions of ADRP⁺ cells, many alveolar epithelial cells were also expressed Caspase9, suggesting that this was the alveolar epithelial injury region (Fig. 6b). Furthermore, a large number of ADRP⁺S100A4⁺ double positive cells were observed in the lung tissue of D21 model mice (Fig. 6c). It suggests that M2-polarized ADRP⁺ macrophages may affect adjacent cells by releasing S100A4.

Discussion

Our work confirms that ADRP cannot be used as a marker for lipofibroblasts in studies of pulmonary fibrosis. Pulmonary fibrosis leads to irreversible destruction of pulmonary alveolar structure. Lipofibroblasts, a kind of lung interstitial cells, are generally considered to play an important role in the formation and stabilization of alveolar structure. Therefore, appropriate molecular markers of lipofibroblasts should be selected for the study of pulmonary fibrosis. A variety of lung interstitial cells express PDGFR α . Some of these PDGFR α ⁺ cells are involved in the cell niches that maintains AEC2 in appropriate differentiation status[15, 16]. And CD34⁺ PDGFR α ⁺ lung interstitial cells contain high levels of neutral lipids[17]. Thus, lipofibroblasts are included in the pulmonary PDGFR α ⁺ cell population. We found that the expression level of PDGFR α was significantly lower than that of control group in model mouse lung. This indicated that PDGFR α ⁺ cells, including lipofibroblasts, decreased or disappeared due to transdifferentiation or apoptosis during pulmonary fibrosis process. It has been reported that PDGFR α ⁺ cell lineage involved in the formation of fibrotic lesion[18]. The decrease or even disappearance of PDGFR α ⁺ cells directly altered the cell niches required for the normal functioning of AEC2 and affected the homeostasis of the terminal lung tissue. A very contradictory result is that the classical lipofibroblast

marker molecule ADRP expression is significantly elevated in model mouse lung compared with that of control group. The reason for this phenomenon is that ADRP is a differentiation marker related to cell lipid load, and a group of high lipid load ADRP⁺ cells appeared in the lungs of model mice. Our experiments confirmed that a large amount of lipids was stored in cytoplasm of ADRP⁺ cells (Fig. 2), and ADRP⁺ cells express macrophages marker (Fig. 3). Many ADRP⁺ cells were distributed in the alveolar cavity, with typical morphology of macrophages. It can be inferred that intraperitoneal injection of bleomycin caused damage to the peripheral lung tissues. Macrophages in the lung tissues engulfed lipid-rich alveolar surfactant and apoptotic alveolar epithelial cells. The lipid load of macrophages increased. This results in significantly increased expression of ADRP, eventually forming ADRP⁺ cells in the lung. This process may be similar to the formation of ADRP⁺ foam cells during atherosclerosis[19]. Study based on single-cell sequencing has found that lipofibroblasts cells can express the immune-related gene CD206 of M2-type macrophages[20]. Although the modeling method for inducing fibrosis is different from ours, it cannot rule out that a considerable part of ADRP⁺ cells are macrophages rather than intended lipofibroblasts.

Our work confirms that the ADRP⁺ macrophages polarization mode is associated with the course of pulmonary fibrosis. Macrophages are an important part of the body's immune defense system. In the modeling process of pulmonary fibrosis induced by intraperitoneal injection of bleomycin, ADRP⁺ macrophages changed regularly with the course of the disease. Intraperitoneal injection of bleomycin first caused an inflammatory response in the lungs, followed by fibrosis and progressive aggravation[14]. We found that many ADRP⁺ cells simultaneously expressed CD86 in the 7-day model samples. These cells are M1-type macrophages. Activated M1 macrophages produce pro-inflammatory cytokines, which are associated with early inflammatory responses. After 14 days of modeling, most of the ADRP⁺ macrophages in the lungs began to express CD206, although there was still inflammatory response in the lungs of the model mice. During the process of pulmonary fibrosis formation, in ADRP⁺ cells population M1-type macrophages are constantly reduced and eventually almost all of ADRP⁺ cells are M2-type macrophages. M2 macrophages can promote fibrosis and resist inflammatory. On the 21 days after modeling, markers of fibrosis, α -SMA and N-cadherin, appeared in adjacent regions of ADRP⁺ M2-polarized macrophages. The model mice began to form fibrotic foci.

Pulmonary fibrosis lesions, formed after a one-time intratracheal perfusion of bleomycin administration in mice, were self-healing over time delay. While multiple intraperitoneal injections of Bleomycin caused repeated tissue damage and repair, it will trigger irreversible fibrosis. The course of pulmonary lesions is similar to that of fibrosis observed clinically[14]. To verify the association of ADRP⁺ M2 macrophage aggregation emergence with pulmonary fibrosis, we truncated the modeling process. It was found that 1–2 times intraperitoneal injections of Bleomycin did not cause significant and persistent pulmonary fibrosis injury, and no ADRP⁺ cells aggregation. At least 3 intraperitoneal injections of bleomycin triggered irreversible pulmonary fibrosis injury. Significant expression of α -SMA was observed in lung tissues

(Fig. 5). There is ADRP⁺ cells aggregation in modeled mice lung (Fig. 5). Again, ADRP⁺M2 macrophages were associated with pulmonary fibrosis.

We note that many ADRP⁺ macrophages express Caspase9 which suggests that these cells may undergo apoptosis in the future (Fig. 6). This also explains that although pulmonary fibrosis lesions can be detected in D28 lung tissue sections after three injections, the number of ADRP⁺ macrophages in the lung was small. More injections of bleomycin are needed to induce more extensive fibrosis damage in order to see a significant increase in ADRP by Realtime-PCR in the total RNA extracted from lung tissue (Fig. 5).

We found that fibrosis damage is imminent or has occurred at ADRP⁺ macrophage aggregation sites. Lung specimens of model mice showed that some ADRP⁺ cells and their adjacent epithelial cells expressed Caspase9 (Fig. 6). This is a signal of cell injury and apoptosis. Repeated alveolar epithelial cell damage can trigger the occurrence of pulmonary fibrosis. At the late stage of modeling, ADRP⁺ cells were M2 macrophages, among which a large proportion expressed S100A4 (Fig. 6). This is an important indicator of epithelial mesenchymal transformation. We observed that some adjacent cells of ADRP⁺ macrophages expressed PCNA. These cells were in an active state of proliferation. This suggests that ADRP⁺ macrophages may affect the differentiation and proliferation status of surrounding cells by releasing S100A4. Studies have suggested that S100A4 can be generated and secreted by M2-polarized alveolar macrophages and enhance the proliferation and activation of lung fibroblasts[21]. S100A4⁺ cells in IPF lung tissue are distributed in the borderline region between the focal site of myofibroblasts and the normal alveolar structure, which is defined as the pre-fibrosis activity[21]. These phenomena are mutually confirmed by our observations. It was further suggested that the appearance of ADRP⁺ M2-polarized macrophages was one of the necessary conditions for the occurrence of pulmonary fibrosis.

Further investigated should be done to elucidate how macrophage polarization affects the differentiation and proliferation of surrounding cells and its relationship with pulmonary fibrosis. The presence of ADRP⁺ macrophages or their secreted proteins in bronchoalveolar lavage fluid or sputum may be used to predict the possibility of pulmonary fibrosis induced by pulmonary toxic drugs. For patients with pulmonary fibrosis symptoms, targeted inhibition of M2-polarized ADRP⁺ macrophages may be a new idea to delay or prevent the deterioration of pulmonary fibrosis.

Conclusion

In the process of pulmonary fibrosis, most of ADRP⁺ cells are macrophages. During the process of pulmonary fibrosis foci formation, in ADRP⁺ cells population M1-type macrophages are replaced by M2-type macrophages. The sites of ADRP⁺ M2 macrophage aggregation indicate that fibrosis damage is imminent or has already occurred here. This study may provide a new research direction for the treatment of pulmonary fibrosis.

Abbreviations

ADRP: Adipose differentiation related protein; BPD: bronchopulmonary dysplasia; α -SMA: alpha-Smooth Muscle Actin; PDGFR: platelet-derived growth factor receptor; AEC: Alveolar Epithelial cells; PCR: Polymerase Chain Reaction

Declarations

Acknowledgements

The laboratory of respiratory medicine of the first affiliated hospital of China medical university gave the necessary support.

Funding

This work was supported by the National Natural Science Foundation of China Project #31271231.

Authors' contributions

Cheng Chen design experiment and finally completed the paper writing. Wei Tan completed the main experimental work to write a draft, Yaru Wang gave the necessary assistance. Yuhua Chen gave part of the guidance. All authors have read and approved the manuscript.

Ethics approval and consent to participate

All the animal experiments were performed according to Guidelines for Animal Care in China Medical University (CMU) and were approved by the CMU Animal Care and Use Committee (IACUC Issue No. 14031M). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Competing interests

The authors declare that they have no conflict of interest.

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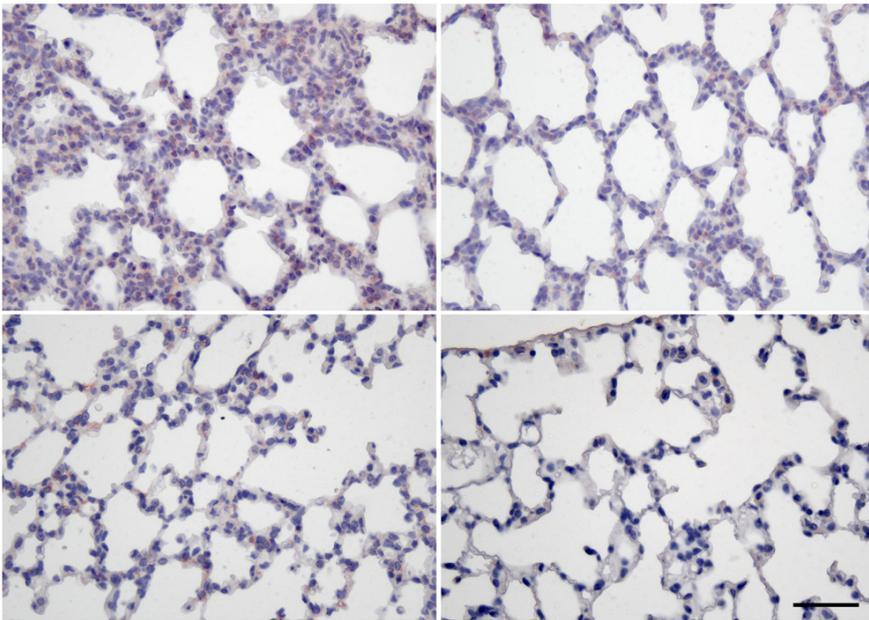
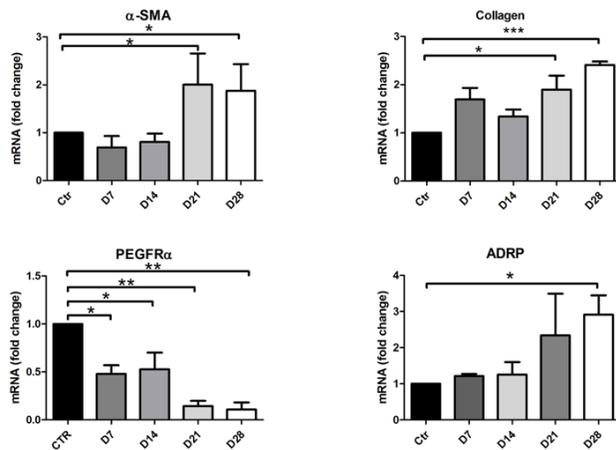
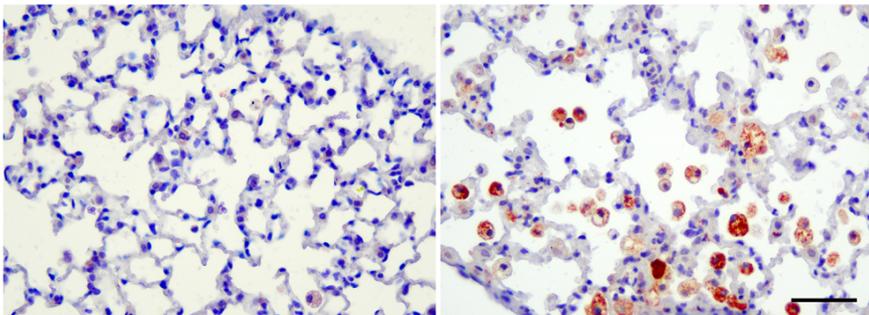
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Supplemental Figures

FigS1. HE staining and Masson staining in different stages of modeling. Histopathological specimens of pulmonary fibrosis at different stages, 7th, 14th, 21st, and 28th day, were stained. Compared with the Control group, the alveolar structure of lung histopathological specimens gradually increased, the alveolar wall thickened, fibrotic lesions formed, and a large amount of collagen deposition appeared in the severely damaged area in BLM group on the especially on the 14th day. Scale bar = 50 μ m.

FigS2. HE staining and Masson staining of the different number of intraperitoneal bleomycin administration. HE staining and Masson staining of lung histopathological specimens collected on the 28th day after the first intraperitoneal bleomycin injection was performed to observe the structural changes and collagen deposition with different number of injection times. Compared with the Control group, the alveolar damage of the lung tissue pathological specimens of the bleomycin administration group was gradually aggravated, and pulmonary fibrosis lesions and collagen deposition appeared after injection 3 times. Scale bar = 50 μ m.

Figures

A**B****C****Figure 1**

Changes in ADRP during lung development and pulmonary fibrosis in mice. a Immunohistochemical staining for ADRP in the lung sections prepared on the 1st, 3rd, 14th day postnatal and adult (8-12 weeks) mice. b Realtime-PCR result to show α -SMA, Collagen, ADRP, and PDGFR α expression change in mice lung tissues collected on the 7th (D7), 14th (D14), 21st (D21) and 28th (D28) days after pulmonary

fibrosis modeling. c Immunohistochemical staining for ADRP in D7 and D28 pulmonary fibrosis modeling mice lung. * is $P < 0.05$; ** is $P < 0.01$; *** is $P < 0.001$. Scale bar = $50\mu\text{m}$.

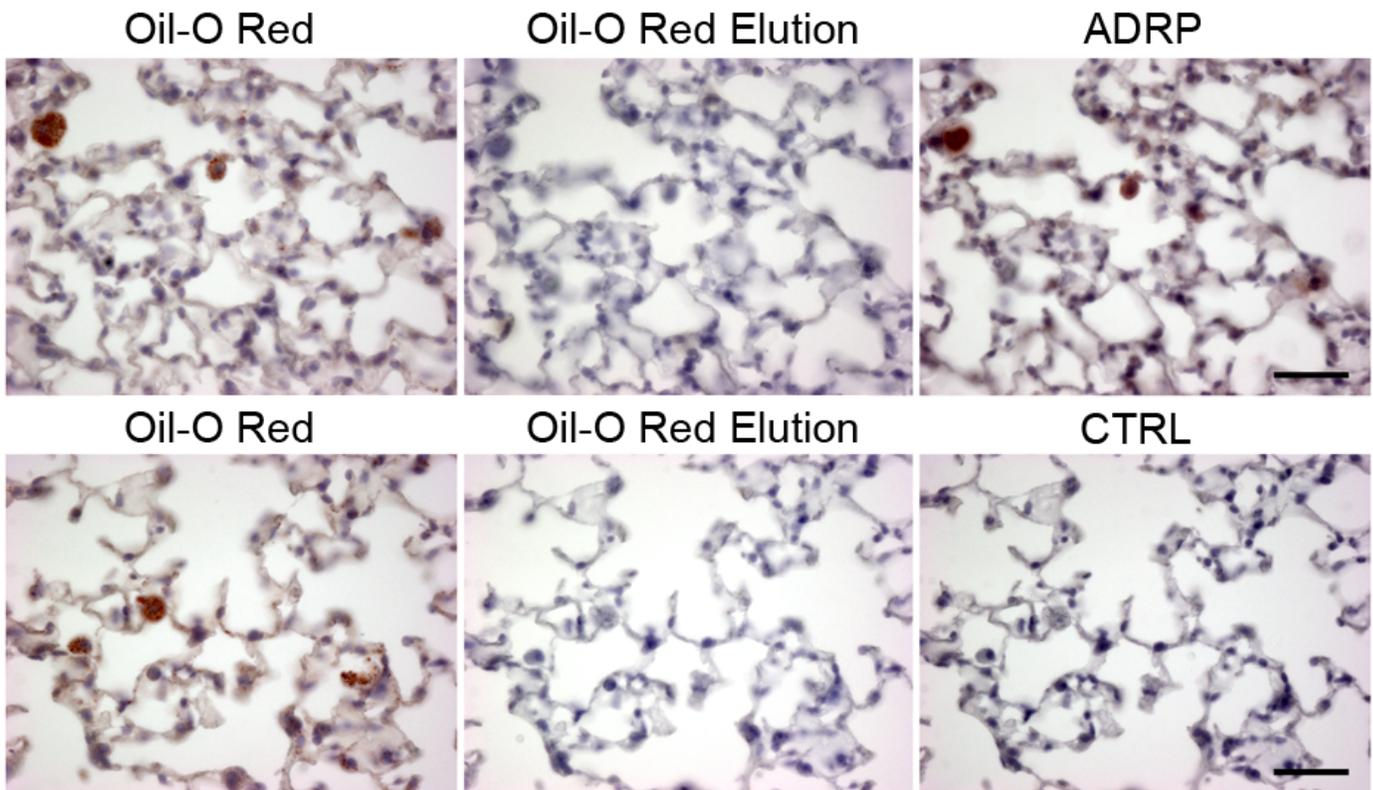


Figure 2

Oil red staining positive cells expressed ADRP during pulmonary fibrosis and distributed in alveolar cavity. Pulmonary fibrosis modeling mice lung frozen section (D28) stained with Oil red O, and then eluted (Oil red O elution). ADRP was detected in the same section by immunohistochemical staining (ADRP). CTRL, a parallel immunohistochemical control group, showed that the preceding oil red staining and elution did not affect the immunohistochemical staining. Scale bar = $50\mu\text{m}$.

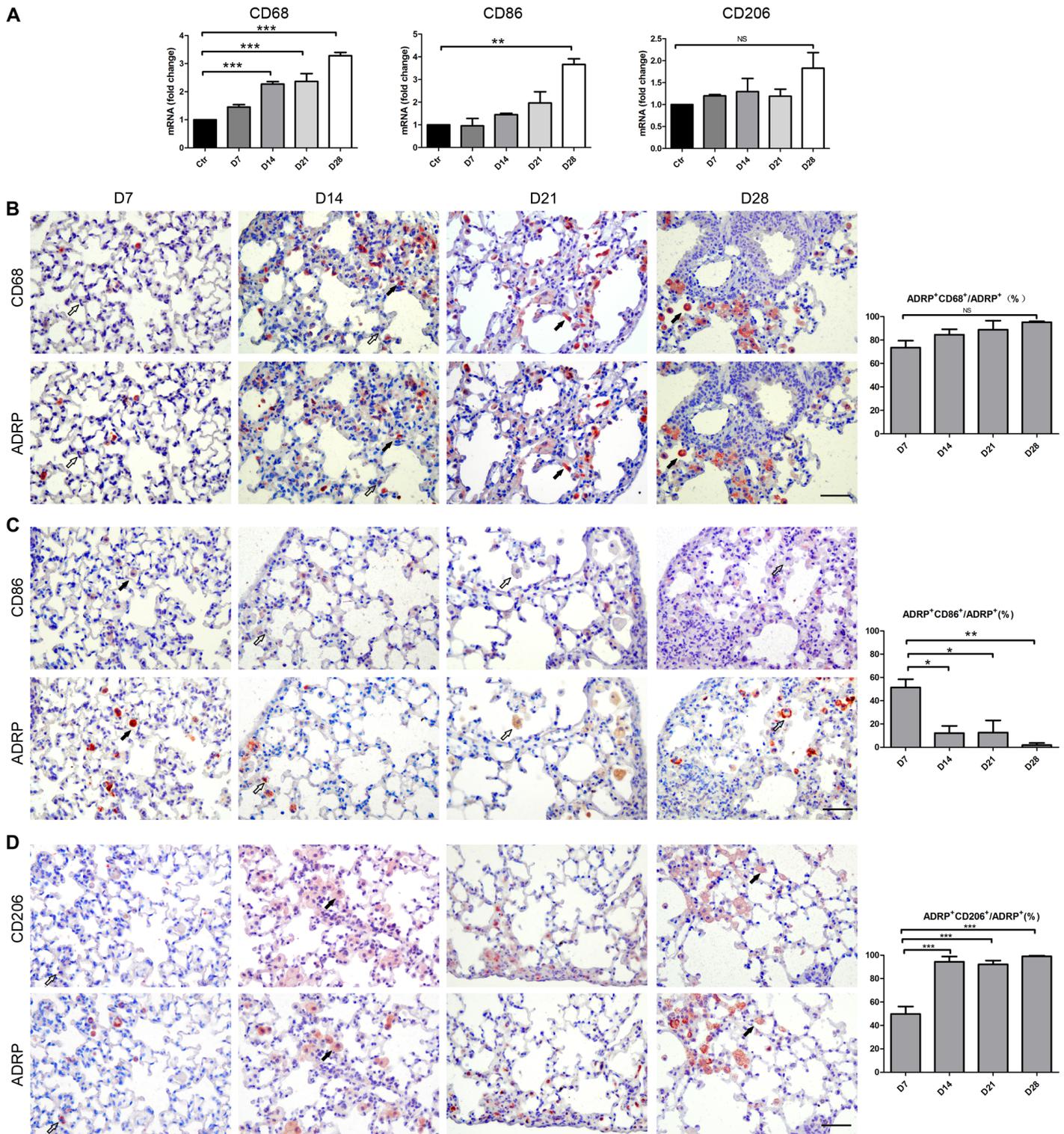


Figure 3

Relationship between ADRP+ cells and different types of macrophages in pulmonary fibrosis process. Lung specimens were collected at different stages of pulmonary fibrosis modeling, D7, D14, D21, and D28. a Macrophage-associated molecular markers CD68, D86 and CD206 expression levels were detected by Realtime-PCR in lung tissues of control and pulmonary fibrosis modeling mice. Immunohistochemical antibody stripping multiple staining was used to detect CD68, CD86 and CD206, colocalization

relationship with ADRP in lung. Representative immunohistochemical results were shown in b, c and d respectively and the ratio of ADRP+CD68+, ADRP+CD86+ and ADRP+CD206+ to ADRP+ cells was counted. All experiments repeated in three lung tissue samples. The hollow arrow shows that two molecular markers cannot co-locate in the same cell, while the solid arrow shows that two molecular markers can co-locate in the same cell. NS is $P>0.05$; * is $P<0.05$; ** is $P<0.01$; *** is $P<0.001$. Scale bar = 50 μ m.

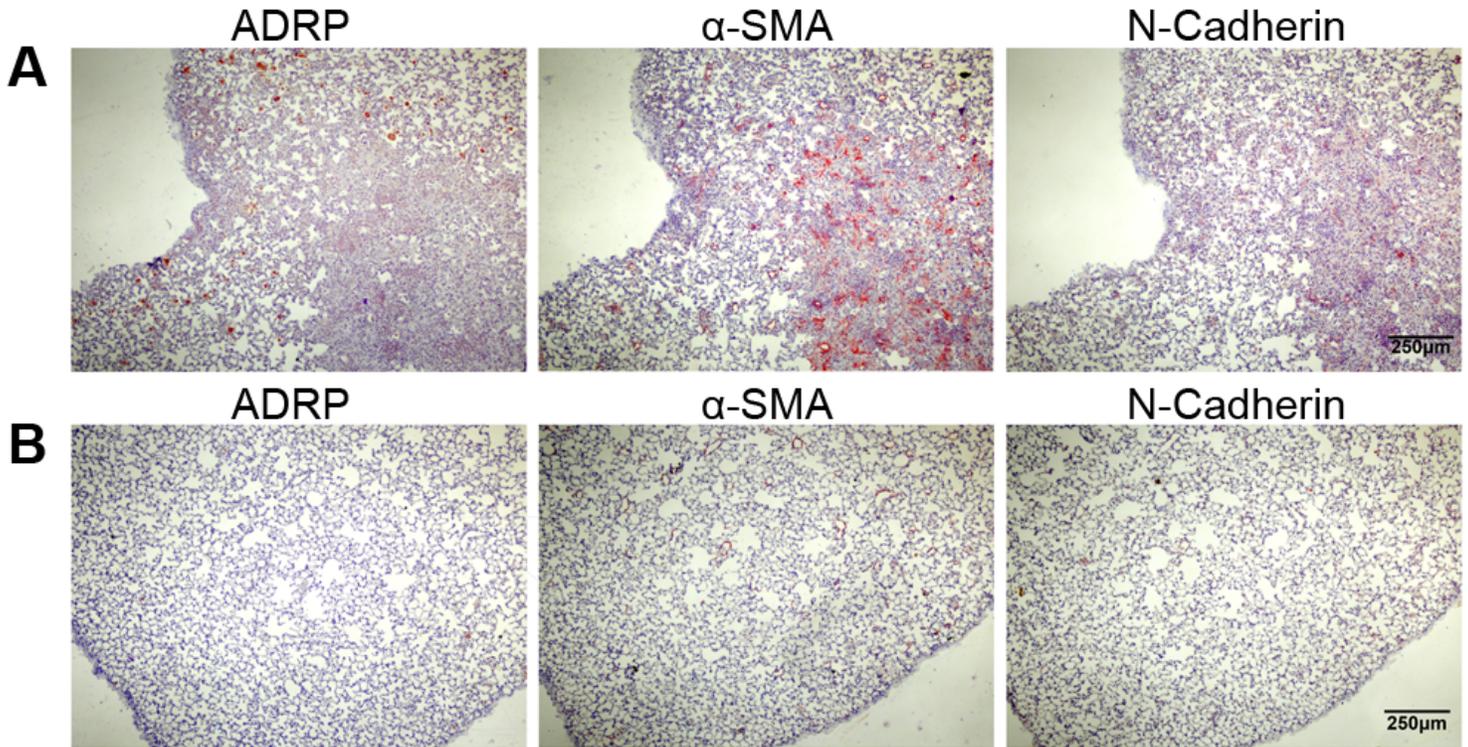


Figure 4

Distribution of ADRP and α -SMA and N-cadherin in lung of model mice with pulmonary fibrosis. ADRP, α -SMA and N-cadherin expression pattern were detected by immunohistochemical staining in serial sections of pulmonary fibrosis tissue prepared on the 21st day after the start of modeling. a Distribution of these target molecules in the fibrotic lesion area. b Distribution of these target molecules in undamaged areas. Scale bar = 250 μ m.

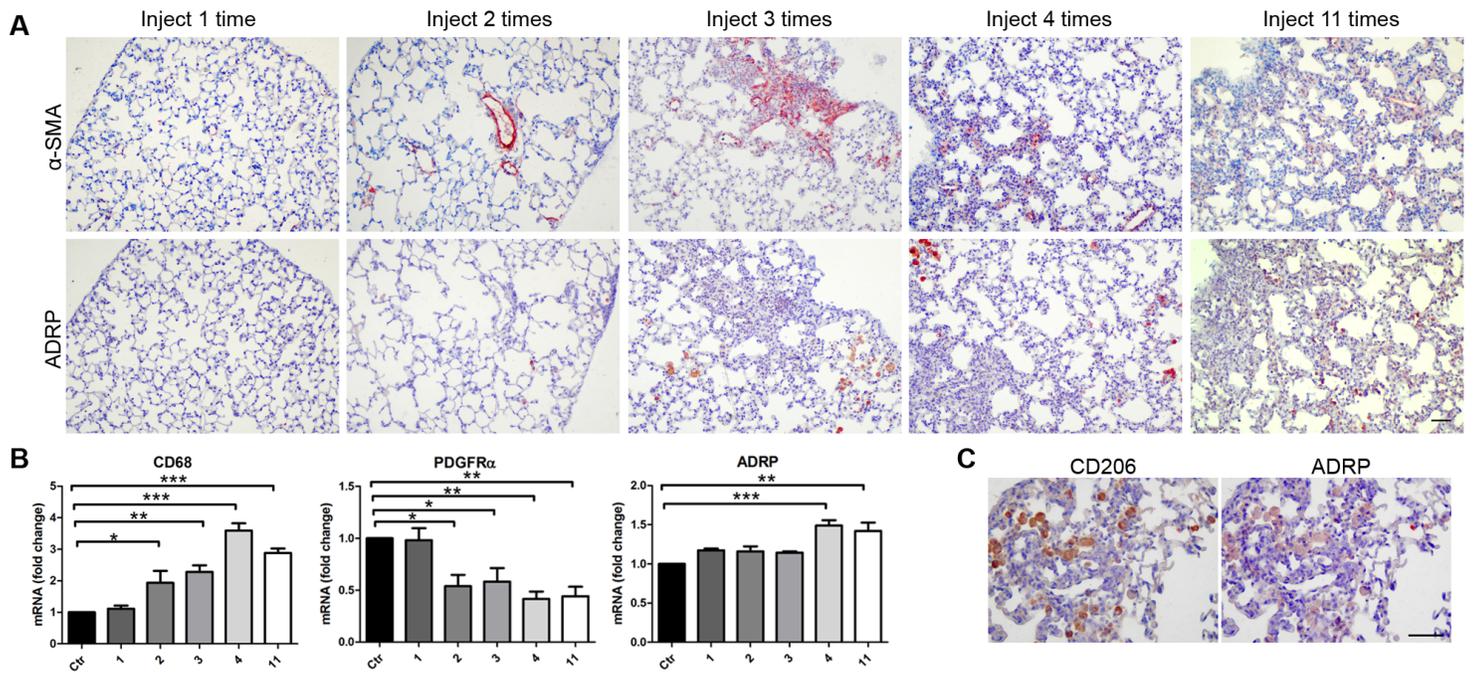


Figure 5

ADRP⁺ macrophage aggregation is associated with pulmonary fibrosis. The experimental mice were intraperitoneally injected with bleomycin for different number of times and fed to 28 days for sampling. a Immunohistochemical staining of α -SMA and ADRP from serial sections of lung tissue revealed that at least three times intraperitoneal injections of bleomycin were required to induce pulmonary fibrosis. b Changes in the mRNA expression level of CD68, PDGFR α and ADRP gene after different number of intraperitoneal bleomycin injection times. c Immunohistochemical antibody stripping multiple staining of CD206 and ADRP show that three intraperitoneal injections of bleomycin can induce the aggregation of ADRP⁺ M2 macrophages. * is $P < 0.05$; ** is $P < 0.01$; *** is $P < 0.001$. Scale bar = 50 μ m.

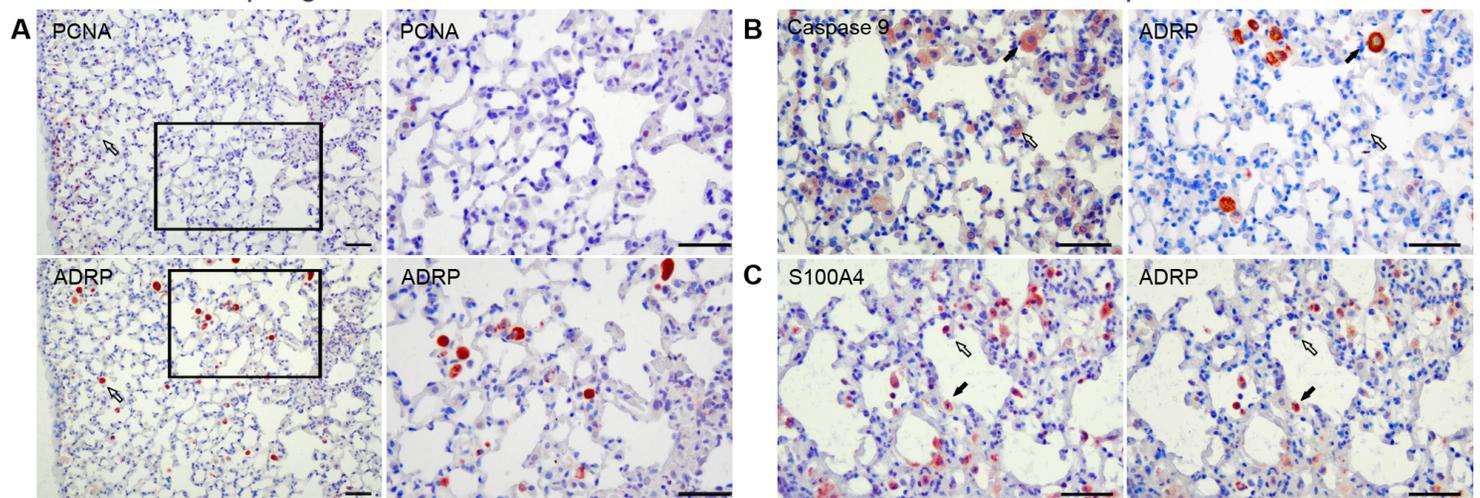


Figure 6

Effect of aggregation of ADRP+M2 macrophages on adjacent peripheral lung tissue cells. The co-localization relationship between ADRP and three target molecules: PCNA, S100A4 and Caspase9 in peripheral lung tissue of D21 fibrotic mice model was analyzed by immunohistochemical antibody stripping multiple staining technique. a The left immunohistochemical images of PCNA and ADRP showed that ADRP+ cells did not express PCNA. The image on the right is an enlargement of the box on the left to show that most PCNA+ cells are located in the lung interstitial and ADRP+ cells are mainly located in the alveolar cavity. b ADRP+ cells express Caspase9, while many alveolar epithelial cells that do not express ADRP also express Caspase9. c Lots of ADRP+ cells express S100A4 simultaneously. The hollow arrow shows that two molecular markers cannot co-locate in the same cell, while the solid arrow shows that two molecular markers can co-locate in the same cell. Scale bar = 50µm.

Supplementary Files

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