PORTLAND PRESS

Research Article

Cell-surface translocation of annexin A2 contributes to bleomycin-induced pulmonary fibrosis by mediating inflammatory response in mice

Yunlong Lei[1,2,*], Kui Wang[3,*], Xuefeng Li[4,*], Yi Li[1], Xuping Feng[3], Jing Zhou[3], Zhe Zhang[3], Canhua Huang[3] and
Tao Zhang[2]

[1]Department of Biochemistry and Molecular Biology, Molecular Medicine and Cancer Research Center, Chongqing Medical University, Chongqing, 400016, P.R. China; [2]The School of Biological Sciences and Technology, Chengdu Medical College, Chengdu, 610083, P. R. China; [3]State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, and West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University, and Collaborative Innovation Center for Biotherapy, Chengdu, 610041, P.R. China; [4]The Second Affiliated Hospital of Guangzhou Medical University, The State Key Laboratory of Respiratory Disease, Guangdong Provincial Key Laboratory of Allergy & Clinical Immunology; The Sixth Affiliated Hospital of Guangzhou Medical University, Qingyuan People's Hospital; Sino-French Hoffmann Institute, School of Basic Medical Sciences, Guangzhou Medical University, Guangzhou 511436, P.R. China

Correspondence: Tao Zhang (ztbillcmc@163.com) or Canhua Huang (hcanhua@hotmail.com)

Bleomycin, a widely used anti-cancer drug, may give rise to pulmonary fibrosis, a serious side effect which is associated with significant morbidity and mortality. Despite the intensive efforts, the precise pathogenic mechanisms of pulmonary fibrosis still remain to be clarified. Our previous study showed that bleomycin bound directly to annexin A2 (ANXA2, or p36), leading to development of pulmonary fibrosis by impeding transcription factor EB (TFEB)-induced autophagic flux. Here, we demonstrated that ANXA2 also played a critical role in bleomycin-induced inflammation, which represents another major cause of bleomycin-induced pulmonary fibrosis. We found that bleomycin could induce the cell surface translocation of ANXA2 in lung epithelial cells through exosomal secretion, associated with enhanced interaction between ANXA2 and p11. Knockdown of ANXA2 or blocking membrane ANXA2 mitigated bleomycin-induced activation of nuclear factor (NF)-κB pathway and production of pro-inflammatory cytokine IL-6 in lung epithelial cells. ANXA2-deficient (ANXA2$^{-/-}$) mice treated with bleomycin exhibit reduced pulmonary fibrosis along with decreased cytokine production compared with bleomycin-challenged wild-type mice. Further, the surface ANXA2 inhibitor TM601 could ameliorate fibrotic and inflammatory response in bleomycin-treated mice. Taken together, our results indicated that, in addition to disturbing autophagic flux, ANXA2 can contribute to bleomycin-induced pulmonary fibrosis by mediating inflammatory response.

Introduction

Bleomycin, a member of the glycopeptides group of antibiotics, is used for the treatment of testicular carcinoma, lymphoma, and squamous cell carcinoma [1]. However, continuous and high-dose medication of bleomycin causes severe pulmonary fibrosis, the most common and clinically significant adverse effect [1]. On the other hand, intratracheal administration of bleomycin has been widely used as an experimental model of lung fibrosis which resembles human pulmonary fibrosis biochemically and histologically [2–5]. Much of our understanding of the mechanisms underlying human idiopathic pulmonary fibrosis (IPF) has focused on the studies of bleomycin-induced pulmonary fibrosis in mouse models [5]. However, there is a lack of detailed, precise molecular mechanisms of bleomycin-induced pulmonary fibrosis, particularly its molecular targets. Identifying the direct target of bleomycin will shed light on better understanding of the pathologic mechanisms of pulmonary fibrosis.

Pulmonary fibrosis, the end stage of a chronic progressive disorder and a lethal lung disease, is characterized by the loss of normal functional alveolar structure [4,6–9]. The most common and aggressive form of pulmonary fibrosis is IPF, which is associated with high mortality and an uniformly poor prognosis, with an average survival of 3 years from the onset of dyspnoea [3,4]. IPF typically afflicts individuals of the age between 50 and 70 years old, with an estimated prevalence of 42.7 per 100000 and incidence of 16.3 per 100000 in the U.S.A. [6,10]. Although in recent years diagnostic approach and active research have been improved, IPF patients are still generally refractory to current available pharmacological therapies, and the only effective life-prolonging intervention is lung transplantation [10,11].

The precise pathogenic mechanisms of pulmonary fibrosis remain largely unknown. But it has been widely accepted that chronic inflammation, cytokine production, autophagy imbalance and oxidative stress can injure the lung epithelial cells and modulate fibrogenic process, thus leading to the formation of end-stage fibrotic scar [3,4,6]. The role of inflammation in IPF is controversial. Although an influx of inflammatory cells is not observed in lung pathology and immunosuppression exhibits no obvious effect for IPF treatment, chronic inflammation is still considered as a critical event in pulmonary fibrosis [12]. For example, in some clinical studies, the increased inflammatory cells, such as neutrophilia and eosinophilia, have been found to be associated with worse prognosis and mortality of pulmonary fibrosis [4,6]. Balestro *et al.* [13] found that an innate and adaptive inflammation appears to be a prominent feature in the lung of IPF patients and could contribute to determining the rate of disease progression. Inflammatory cells contribute to the development of pulmonary fibrosis by their ability to secrete various cytokines, which could further promote the secretion of inflammatory cytokines, chemokines and growth factors from surrounding cells to a large quantity, resulting in inflammation and proliferation of mesenchymal cells [4,5,14]. Several transcription factors, including especially nuclear factor (NF)-κB, are involved in this process, and inhibition of NF-κB pathway could ameliorate pulmonary fibrosis in different fibrosis models [4,5,14].

The annexins are a family of widely distributed, calcium-dependent, anionic phospholipid-binding peripheral membrane proteins. Multiple members of the annexin family, including annexin A1, A2, A3, A5 and A11, have been reported to be associated with the fibrotic disease [15–19]. ANXA2 (p36, calpactin I heavy chain, lipocortin II) is unique among the annexin family, since it exists as a monomer or a heterotetramer form [20–22]. ANXA2 can be distributed in different cellular compartments, including nucleus, cytoplasm, organelle membranes, inner and outer plasma membrane [17,23]. Monomeric ANXA2 is mainly located in cytoplasm, while its heterotetramer form, composed of two ANXA2 monomers and two S100A10 calcium-binding protein subunits (p11), is distributed on the plasma membrane [17,22,24]. Since ANXA2 lacks a signal peptide for trafficking to the cellular plasma membrane through the classical endoplasmic reticulum (ER)-Golgi route, the surface translocation of ANXA2 was mainly dependent on exosomal secretion [24–26]. Different subcellular localization and various forms of ANXA2 appear to allow for its diverse and distinct biological functions, such as TLR4-mediated activation of macrophages by surface ANXA2 tetramer [27], the promoted DNA synthesis and cell proliferation by nuclear ANXA2 monomer [28], as well as induction of endocytosis, exocytosis, cytoskeletal rearrangement, and intracellular signal transduction by cytoplasmic ANXA2 monomer [23,24,29].

In our previous study, we determined that Glu139 of ANXA2 directly binds to the sugar moiety of bleomycin in lung epithelial cells, which could impede autophagic flux by inactivating transcription factor EB (TFEB) and lead to pulmonary fibrosis [30]. In the current study, we further found that ANXA2 was involved in bleomycin-induced pulmonary fibrosis by mediating inflammatory response, suggesting a novel function of ANXA2 in regulating pulmonary fibrosis.

Materials and methods
Ethics statements
The research has been carried out in accordance with the World Medical Association Declaration of Helsinki. Human tissue ethics approval was obtained from the Institutional Ethics Committee of Sichuan University. The animal studies involved in ANXA2$^{-/-}$ mice were performed in the University of North Dakota, and other animal studies were performed in Sichuan University. All animal studies were reviewed and approved by the Institutional Animal Care and Treatment Committee of Sichuan University, and the University of North Dakota Institutional Animal Care and Use Committee. All institutional and national guidelines for the care and use of laboratory animals were followed.

Cell treatment and transfections
A549 cells and 16HBE cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in RPMI1640 medium (Gibco, U.S.A.) containing 10% fetal calf serum (Hyclone, U.S.A.), penicillin

PORTLAND PRESS

(10^7 U/L), and streptomycin (10^7 U/L) at 37°C in a humidified chamber containing 5% CO_2. For ANXA2 silencing, the ANXA2 shRNA plasmid and control vector with no homology to any human gene were separately transfected into the indicated cells and the stable transfectants were selected in the presence of G418 (details about shRNA was described in our previous study [31]).

For colony formation assay, the cells were seeded at a density of 1000 cells/well in 6-well plates, the medium was changed every 3 days. After 14 days, the colonies were fixed with methanol and stained with Crystal Violet (Sigma, St. Louis, MO, U.S.A.) for 30 min.

Integrin-linked kinase (ILK) small interfering RNA (siRNA) was chemically synthesized by GenePharma company (Shanghai, China) with target sequence as UCAGAGCUUUGUCACUUGC TT. The target sequence of control siRNA is UUCUCCGAACGUGUCACGU TT. The siRNA was transfected into the indicated cells using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions.

Animals exposure to bleomycin and Histopathologic assessment

C57BL/6 wild-type (WT) mice were purchased from Beijing HFK Bioscience Co., Ltd (Beijing, China) and ANXA2$^{-/-}$ mice is provided by Dr. Katherine A. Hajjar at Weill Medical College of Cornell University. All the mice used in the present study were 6–8 weeks old (18–20 g body weight) and maintained in the animal facilities under specific pathogen-free conditions. As described in previous study [32], mice were anesthetized intraperitoneally with 80 μl of a ketamine and xylazine solution (3.2 and 0.16 mg/kg, respectively) before intratracheal delivery, and then administered 5 mg/kg bleomycin or saline intratracheally.

The lungs of 0, 14, and 21 days were removed after bleomycin or saline administration. Following fixation, the lungs were embedded in paraffin. The sections were then stained with hematoxylin and eosin (H&E), and Masson's trichrome stain. The grade of pulmonary fibrosis in the lung sections was scored on a scale of 0–8 using Ashcroft scoring method [33]. The severity of fibrotic changes in each histological section of the lung was assessed as the mean score of severity from observed microscopic fields. After the examination of 50 randomly chosen regions in each sample at a magnification of Σ 400×, the mean score of all the fields was taken as the fibrosis score. Grading was done in a blinded manner by three independent observers. Hydroxyproline (Sigma–Aldrich, MAK008) contents of whole lungs were performed according to the manufacturer's instructions.

In the treatment group, mice were challenged with bleomycin, and then treated with saline or TM601 (Morphotek) (10 mg/kg, every third day) for 21 days.

Human samples

A total of 34 IPF tissues and 28 paired adjacent normal lung tissues were obtained under informed consent from patients who underwent surgical resection at either West China Hospital or Sichuan Provincial People's Hospital (Chengdu, China). Patient demographics and clinical characteristics are shown in Supplementary Table S1. Excised Lungs were fixed with 4% paraformaldehyde and embedded with paraffin for immunohistochemical examination.

Cell surface elution

Cellular surface ANXA2 was eluted by the extracellular calcium-chelating agent EGTA as previous reported [24,34]. Briefly, 1.5×10^6 A549 or 16HBE cells were grown in 10-cm culture plates for 24 h and then treated with different concentrations of bleomycin for 24 or 48 h. After bleomycin treatment, cells were washed three times with ice-cold Hepes-buffered saline (HBS: 11 mM Hepes,137 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM glucose). Then cells were eluted with HBS buffer containing 20 mM EGTA and protease inhibitors at 4°C for 30 min. The eluates were collected, centrifuged 400 ***g*** for 5 min, and then detected by Western blot. In some experiments, cells were pretreated with methyl-β-cyclodextrin (MβCD) for 1 h before bleomycin treatment.

Western blot and co-immunoprecipitation

Proteins were extracted in RIPA buffer and quantified using the DC protein assay kit (Bio-Rad, U.S.A.). Samples proteins were separated by 12% SDS/PAGE and then transferred to PVDF membranes (Amersham Biosciences, U.S.A.). The membranes were blocked overnight with PBS (phosphate buffered saline) containing 0.1% Tween 20 in 5% skimmed milk at 4°C, and subsequently probed using the primary antibodies: rabbit-anti-ANXA2 (diluted 1:800, Santa Cruz, U.S.A.), rabbit-anti-p11 (diluted 1:800, Santa Cruz, U.S.A.), mouse-anti-tubulin (diluted 1:1000, Santa Cruz, U.S.A.), rabbit-anti-p65 (diluted 1:1000, Cell Signaling, U.S.A.), rabbit-anti-Phospho-p65 (S536) (diluted 1:500, Cell Signaling, U.S.A.), rabbit-anti-ILK (diluted 1:800, Cell Signaling, U.S.A.), rabbit-anti-IκBα (diluted 1:1000, Cell Signaling, U.S.A.), mouse-anti-Phospho-IκBα (Ser32/36) (5A5) (diluted 1:1000, Cell Signaling, U.S.A.),

mouse-anti-IL-6 (diluted 1:1000, Santa Cruz, U.S.A.). Blots were incubated with the respective primary antibodies for 2 h at room temperature and washed three times in PBST (phosphate buffered saline with Tween-20). Subsequently, the blots were incubated with secondary antibody conjugated to Horseradish Peroxidase for 2 h at room temperature. Target proteins were detected by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, U.S.A.). β-actin was used as an internal control. In some experiments, bleomycin-treated A549 or 16HBE cells were pre-neutralized with 10 µg/ml rabbit-anti-ANXA2 (Santa Cruz, U.S.A.) or control IgG.

Co-Immunoprecipitation assay was carried out as described in previous study [35]. Briefly, A549 or 16HBE cell lysates (400 µg of protein) were incubated with 40 µl (1:1 slurry) of protein A/G-Sepharose and 4 µg of polyclonal ANXA2 antibody. Following incubation, beads were washed quintic with lysis buffer. Proteins were eluted with Laemmli SDS sample dilution buffer and then detected by Western blot.

Immunofluorescence

For immunofluorescence, cells were fixed with 2% paraformaldehyde in PBS for 10 min. After being washed twice with ice-cold PBS, fixed cells were blocked with PBS containing 5% BSA at room temperature for 30 min, and incubated with anti-ANXA2 (diluted 1:200, Santa Cruz, U.S.A.) or anti-p11 (diluted 1:100, Santa Cruz, U.S.A.) antibody at 4°C overnight. The cells were washed twice with PBS and treated with fluorescein-conjugated secondary antibodies (diluted 1:100) in PBS for 1 h at room temperature, followed by Hoechst staining of the cell nucleus (blue). In the permeabilized control, cells were first fixed with 2% paraformaldehyde and then permeabilized with 0.4% Triton X-100 at room temperature for 30 min. Images were obtained using a Zeiss Imager.Z1 fluorescence microscope equipped with an AxioCam MRc5 digital CCD camera (Carl Zeiss Microimaging Inc.)

Cytokine profiling and assays

Cytokine concentrations in the first 0.6 ml bronchoalveolar lavage fluid (BALF) collected at the indicated times were measured by standard ELISA kits following the manufacturer's instructions (eBioscience Inc., San Diego, CA).

Exosome isolation

As described in previous study [24], the culture medium containing 10% FBS was centrifuged overnight at 110000 ***g*** to remove endogenous exosomes. After bleomycin stimulation, the culture medium was collected and centrifuged at 300 ***g*** for 10 min to remove suspended cells. The supernatant was then centrifuged at 2000 ***g*** for 10 min to remove the dead cells. After centrifugation at 10000 ***g*** for 30 min to remove cell debris, the exosomes from the supernatant were centrifuged at 110000 ***g*** for 70 min. The exosomal pellet was washed once in PBS and then resuspended in 200 ml PBS.

Statistics

All quantitative data were recorded as mean $\pm$ S.D., and comparisons between two groups were performed by Student's *T*-test. The differences among multiple groups were assessed by one-way ANOVA analysis (Tukey's multiple comparisons test), while statistical significance was defined as *P<0.05*.

Results
The expression of ANXA2 was not altered in bleomycin-triggered inflammation process

In previous study, we found that ANXA2-bleomycin binding inhibited TFEB-induced autophagy flux, which contributed to bleomycin-induced pulmonary fibrosis [30]. The pathologic mechanisms of pulmonary fibrosis is complex, in addition to autophagy, chronic inflammation and cytokines production were widely accepted to play a role in pulmonary fibrosis [5,36–39]. Several recent studies found that ANXA2 could induce the activation of NF-κB pathway which has been demonstrated as an important cue of inflammation and pulmonary fibrosis [5,36–39]. To determine whether the pattern of ANXA2 expression was associated with inflammation in bleomycin-induced pulmonary fibrosis, mice were intratracheally treated with bleomycin or saline control. To evaluate histopathologic changes and the extent of pulmonary fibrosis, tissue sections were subjected to H&E staining and Masson trichrome staining. As shown in Figure 1A, bleomycin challenging induced enlarged and thickened alveolar wall on day 14, while the pulmonary architecture was nearly destructed and replaced by collapsed box-like air spaces separated by wide patches of connective tissue on day 21. The accumulation of extracellular collagen is a hallmark of pulmonary fibrosis. Using Masson trichrome staining, we observed an increased collagen deposition in the thickened alveolar regions at days
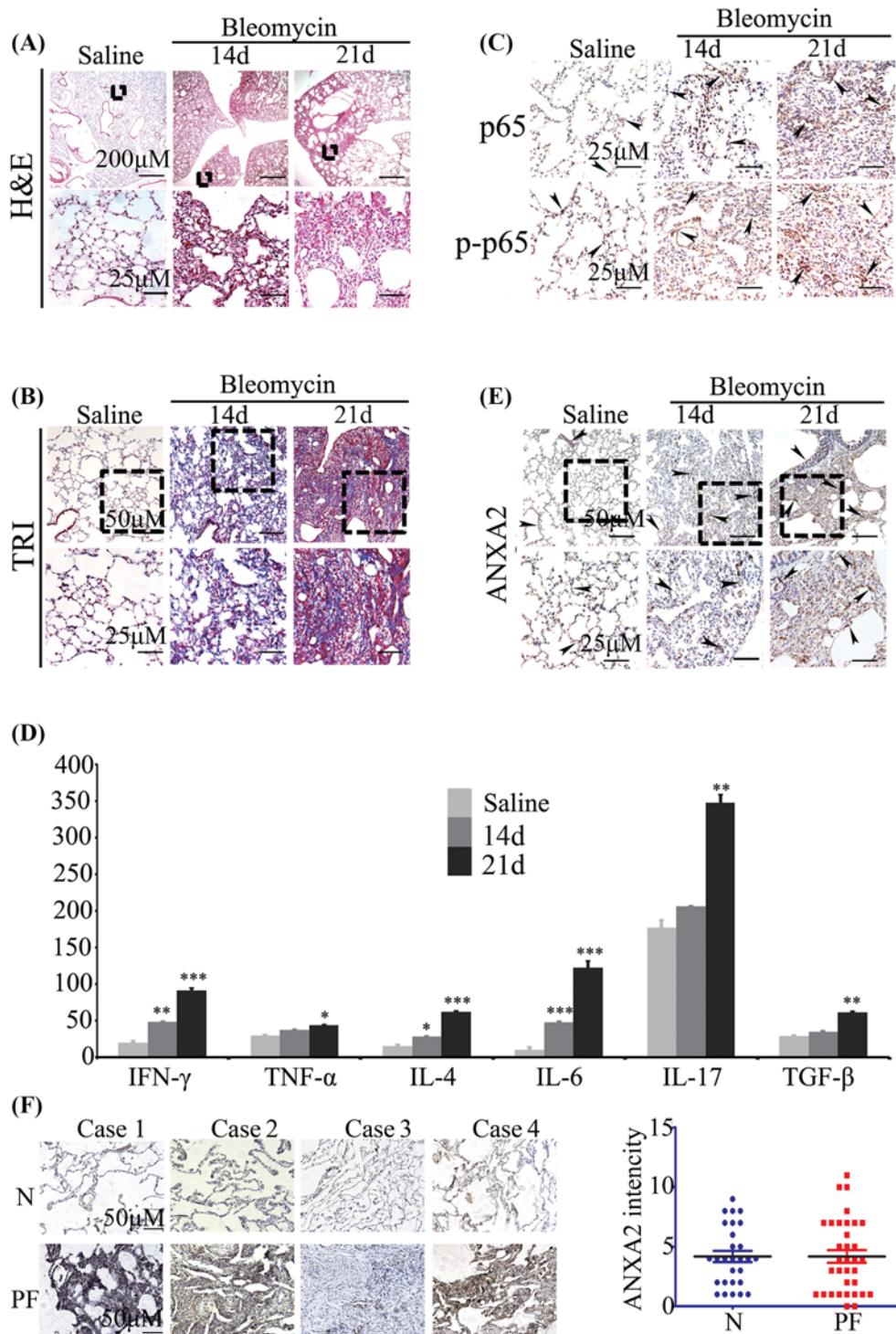
Figure 1. The expression of ANXA2 was not altered in bleomycin-triggered inflammation process

(**A** and **B**) Histologic examination of bleomycin-induced pulmonary fibrosis in C57BL/6 mice. Lungs were harvested, sectioned, and stained with H&E (A) or with Masson trichrome stain (B) on day 14 and 21 after bleomycin instillation. Microscope magnification: (A) top panels, Σ 50×; (A) bottom panels, Σ 400×; (B) top panels, Σ 200×; (B) bottom panels, Σ 400×. (C and E) Representative immunostaining of p65, p-p65 (**C**), and ANXA2 (**E**) in lungs from bleomycin-treated mice. Positive staining was indicated by arrows. Microscope magnification: (C) Σ 400×; (E) top panels, Σ 200×; (E) bottom panels, Σ 400×. (**D**) IFN-γ, TNF-α, IL-4, IL-6, IL-17, and TGF-β levels were measured in BALF at the indicated time points post bleomycin treatment. n=6-8 per group. (**F**) ANXA2 immunohistochemical staining in pulmonary fibrosis (PF) tissues (n=34) or adjacent normal lung tissues (n=28) from patients. Microscope magnification: Σ 200×. $^*P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001.
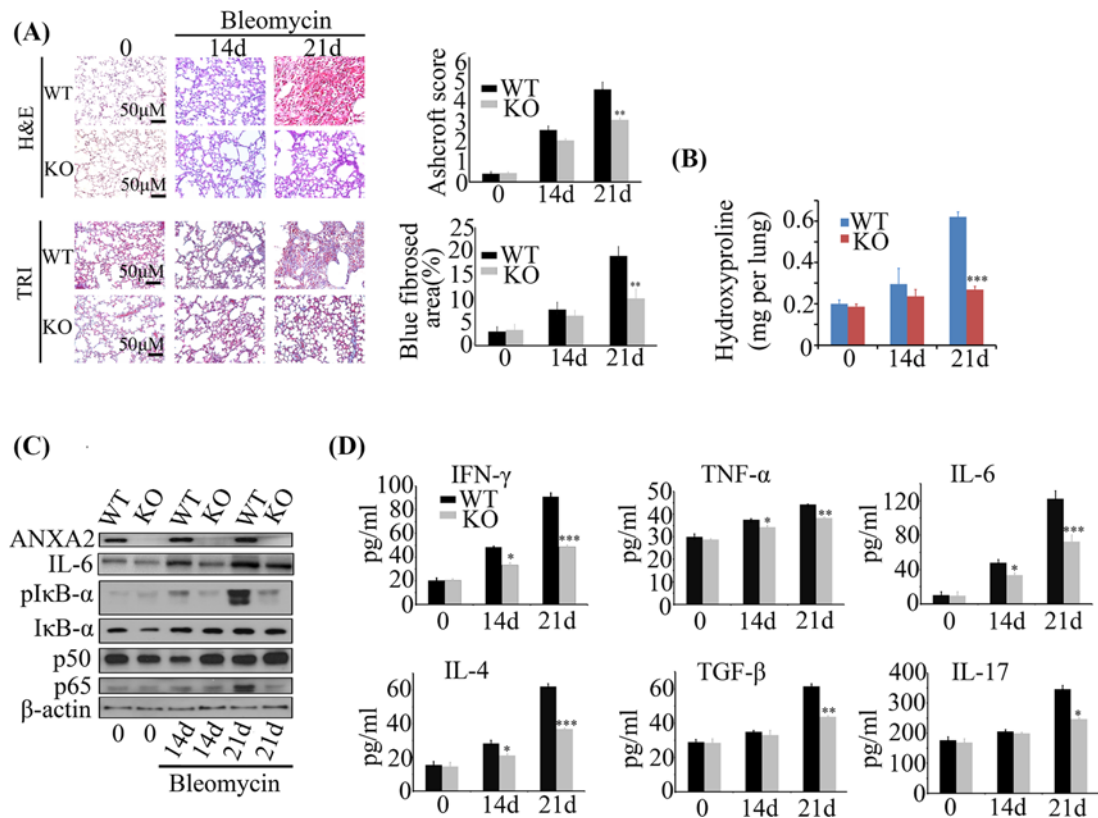
Figure 2. ANXA2 deficiency protects mice from fibrosis and decreases cytokine production

(**A**) Sectioned lungs stained with H&E (top panels) and Masson's trichrome staining (bottom panels). Ashcroft score was determined by H&E staining of sectioned lungs. Fibrotic area was determined by Masson's trichrome staining. $n=4$–6 per group. Microscope magnification: Σ 200×. (**B**) The hydroxyproline contents of whole lung homogenates were analyzed to examine collagen contents. (**C**) Western blot analysis of ANXA2 and key mediators in NF-κB pathway including p65, p50, IκB-α, p-IκB-α, and IL-6 in lungs. β-actin was measured as an internal control. (**D**) IFN-γ, TNF-α, IL-4, IL-6, IL-17, and TGF-β levels were measured in BALF from wild-type and ANXA2$^{-/-}$ (ANXA2 knockout (KO)) mice at the indicated time points post-bleomycin treatment. $n=4$–6 per group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

14 following bleomycin treatment, while the lungs contained dense patches of collagen replaced large areas of lung parenchyma on day 21 (Figure 1B). In addition, consistent with previous studies [2–7], we observed up-regulated p65 and p-p65 in the lung tissues, as well as elevated inflammatory factors, including interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin(IL)-4, IL-6, IL-17, and transforming growth factor beta (TGF-β) in BALF of bleomycin-challenged mice (Figure 1C,D), indicating inflammation was associated with bleomycin-induced pulmonary fibrosis. Furthermore, immunohistochemical staining showed that ANXA2 was localized in various lung cell types by morphological observations, such as the endothelial cells, inflammatory cells, fibroblasts, alveolar, and bronchiolar epithelial cells. However, the expression of ANXA2 was not obviously changed in the lung tissues of bleomycin-challenged mice compared with the control (Figure 1E). Consistent with this, although about 33% patients exhibited elevated ANXA2 expression, no significantly difference was found in the expression of ANXA2 between 34 human pulmonary fibrosis tissues and 28 paired adjacent normal lung tissues (Figure 1F). These results suggested that the expression of ANXA2 was not altered in bleomycin-triggered inflammation process.

ANXA2 deficiency protects mice from pulmonary fibrosis and decreases cytokine production induced by bleomycin

We next investigated whether ANXA2 expression was essential for bleomycin-induced inflammation and lung fibrosis in mice. To test this hypothesis, we exposed WT or ANXA2-deficient (ANXA2$^{-/-}$) mice to bleomycin by intratracheal injection. As shown in Figure 2A, ANXA2$^{-/-}$ mice were protected from bleomycin-induced pulmonary fibrosis compared with WT mice. ANXA2$^{-/-}$ mice revealed reduced interstitial thickening (Figure 2A, top panels),

and reduced subpleural collagen deposition evidenced by Masson trichrome staining (Figure 2A, bottom panels) and hydroxyproline (a marker of collagen) quantification (Figure 2B). To determine whether the pro-fibrotic role of ANXA2 was involved in inflammation induced by bleomycin, we assessed the activation of NF-κB pathway in lung tissues. As shown in Figure 2C, ANXA2 depletion in mice reduced bleomycin-induced phosphorylation of IκBα and expression of p65 and IL-6 (a downstream target of NF-κB pathway). Consistent with this, the levels of inflammatory factors (including IFN-γ, TNF-α, IL-4, IL-6 and IL-17) and TGF-β in BALF of bleomycin-treated mice were significantly reduced in ANXA2$^{-/-}$ mice compared with those in WT mice (Figure 2D). Consistent with our results, Schuliga *et al.* [40] also showed that ANXA2 deletion reduces lung IL-6 production and fibrogenesis. These results suggest that ANXA2 is required for bleomycin-induced inflammation and lung fibrosis development by activating NF-κB signaling.

Bleomycin induced surface expression of ANXA2 and activation of NF-κB signaling in lung epithelial cells

In addition to possible profibrotic effects in response to cytokines produced by inflammatory cells, the fibrosis progression could be also influenced by the cytokines produced by epithelial cells themselves [7–9]. To address the role of ANXA2 in bleomycin-induced activation of NF-κB pathway in lung epithelial cells, we treated A549 cells with bleomycin for 48 h, and determined cell viability by MTT assay. Our data showed that bleomycin treatment inhibited proliferation of A549 in a dose-dependent manner, and the IC$_{50}$ value of bleomycin against A549 cells was about 100 μg/ml (Supplementary Figure S1A). Next, we examined bleomycin-induced apoptosis in A549 using TUNEL assay, and found that treatment with bleomycin induced limited apoptosis until 160 μg/ml (Supplementary Figure S1B). These results were consistent with a previous study [41], in which bleomycin-treated A549 cells exhibited a high level of growth arrest for 120 h and led to about 20% cell death at concentration of 100 μg/ml. Then, we measured the expression of ANXA2 and p65, as well as the phosphorylation status of p65 in A549 cells treated with different sublethal doses of bleomycin (less than 80 μg/ml) for 24 and 48 h. Bleomycin treatment induces phosphorylation of p65, while no obvious change was observed in the expression of ANXA2 (Figure 3A). Similar results were also observed in normal human bronchial epithelial 16HBE cells (Figure 3B and Supplementary Figure S2).

ANXA2 has different subcellular localization, leading to distinct biological functions [24]. Several recent studies have demonstrated that the surface expression of ANXA2 on macrophage cells and pancreatic cancer cells could induce the activation of NF-κB pathway [27,39,42]. Thus, we have a particular interest to detect whether bleomycin could affect the surface expression of ANXA2 on A549 and 16HBE cells. To this end, A549 and 16HBE cells treated with different doses of bleomycin at different time points were surface-eluted with HBS containing 20 mM EGTA, which is an extracellular calcium chelator to elute cell surface ANXA2 [24,34]. As shown in Figure 3A,B, the level of surface ANXA2 in the EGTA eluate was increased in response to bleomycin treatment, while the cytoplasmic protein, α-tubulin, was not detected in the surface EGTA eluate. In addition, surface staining also confirmed that bleomycin could enhance the expression of ANXA2 on the surface of A549 and 16HBE cells (Figure 3C,D). To validate whether bleomycin binding facilitates the surface translocation of ANXA2, we next performed immunofluorescence analysis to examine the interaction between F-BLM and ANXA2 on the surface of A549 cells. Our data showed that both ANXA2 expression and its colocalization with F-BLM on cell surface were enhanced over time following bleomycin treatment (Figure 3E), suggesting that bleomycin binding induces the surface translocation of ANXA2. These results firmly establish that bleomycin induces surface translocation of ANXA2 and activation of NF-κB pathway.
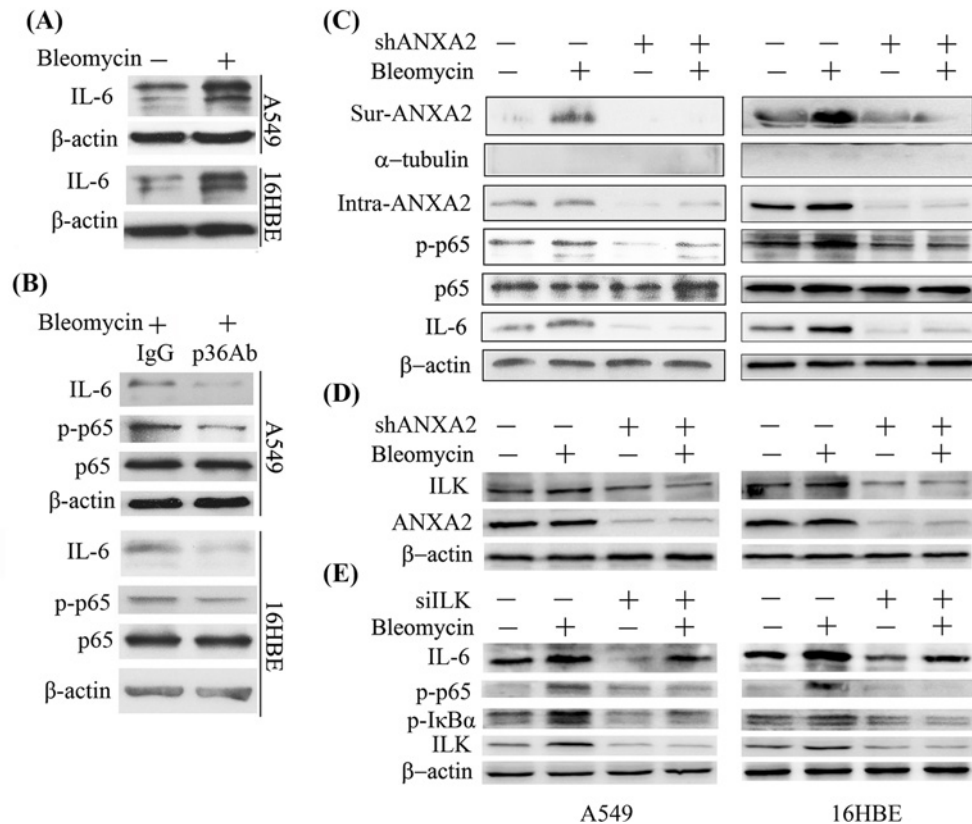
Surface translocation of ANXA2 is essential in bleomycin-induced activation of NF-κB pathway by activating ILK

NF-κB activation induces the production of pro-inflammatory cytokines in lung epithelial cells, such as TNF-α, IL-6 and IL-8, which could contribute to a sustained inflammatory response in pulmonary fibrosis [43]. We found increased expression of IL-6 in both bleomycin-treated A549 cells and 16HBE cells (Figure 4A). To examine whether bleomycin-induced activation of NF-κB pathway was associated with the surface expression of ANXA2 in lung epithelial cells, A549 cells and 16HBE cells were pre-neutralized with anti-ANXA2 or control (rabbit IgG) antibody before bleomycin treatment. As shown in Figure 4B, functional inhibition of surface ANXA2 attenuated bleomycin-induced phosphorylation of p65 and production of IL-6 in both A549 cells and 16HBE cells, suggesting that ANXA2 surface translocation plays important role in bleomycin-induced activation of NF-κB pathway. Next, we stably transfected A549 and 16HBE cells with shRNA-ANXA2 (shANXA2) to knockdown ANXA2 expression and found that depletion of ANXA2 significantly reduced phosphorylation of p65 and production of IL-6 in A549 and 16HBE cells with or without bleomycin treatment (Figure 4C). In addition to the regulation of inflammatory and innate immune
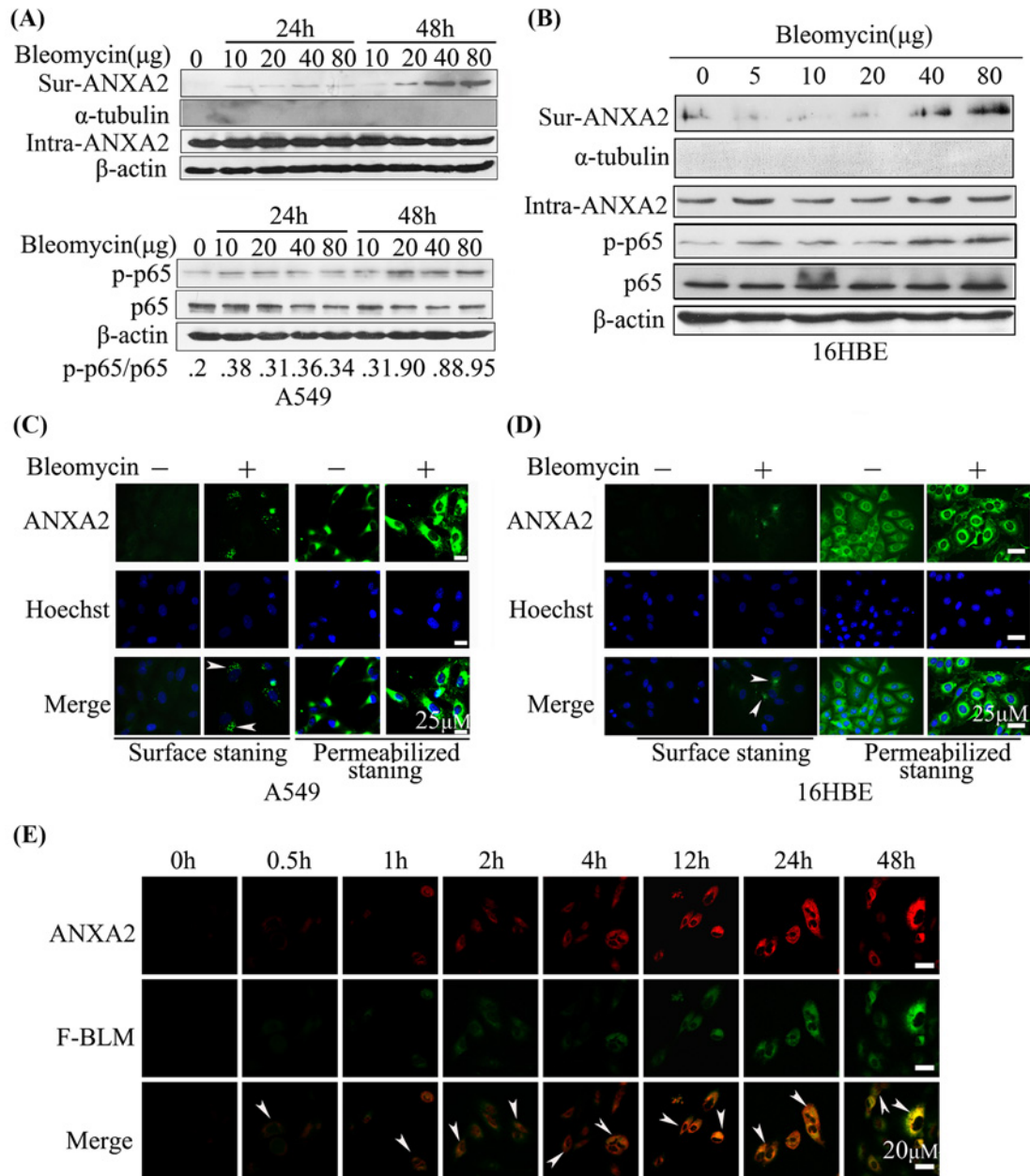
Figure 3. Bleomycin could enhance the surface translocation of ANXA2 and activation of NF-κB pathway in lung cells

(**A**) A549 cells were treated with indicated concentrations of bleomycin (μg/ml) for 24 and 48 h, respectively. Western blot analysis of total expression and surface expression ANXA2 from EGTA elution (see Materials and methods) after bleomycin treatment (top). α-tubulin and β-actin were used as a control. The total p65 and phosphorylation status of p65 treated with or without bleomycin were measured by Western blot analysis (bottom). β-actin was used as an internal control. (**B**) Western blot analysis of total expression ANXA2 and surface expression ANXA2 from EGTA elution, as well as the total p65 and phosphorylation status of p65 in 16HBE cells treated with indicated concentrations of bleomycin (μg/ml) for 48 h. α-tublin or β-actin was used as a control. (**C**) Immunofluorescent detection of ANXA2 on the surface of A549 cells treated with or without 40 μg/ml bleomycin for 48 h. (**D**) Immunofluorescent detection of ANXA2 on the surface of 16HBE cells treated with or without 40 μg/ml bleomycin for 48 h. (**E**) Colocalization of ANXA2 with F-BLM on the surface of A549 cells. Microscope magnification: Σ 400×.

Figure 4. The surface translocation of ANXA2 is essential in bleomycin-induced activation of NF-κB pathway and production of pro-inflammatory cytokines by inducing ILK expression

(**A**) Western blot analysis of IL-6 production treated with or without 40 μg/ml bleomycin for 48 h in both A549 cells and 16HBE cells. (**B**) Bleomycin-treated A549 cells and 16HBE cells were pre-neutralized with 10 μg/ml rabbit-anti-ANXA2 or control IgG antibody, and then the expression of p65 and IL-6, as well as phosphorylation status of p65 were detected by Western blot. (**C**) The total expression of ANXA2, IL-6, and p65, surface expression ANXA2 and the phosphorylation status of p65 in ANXA2-knockdown A549 and 16HBE cells or control cells treated with or without 40 μg/ml bleomycin for 48 h were measured by Western blot analysis. α-tubulin or β-actin was used as a control. (**D**) The total expression of ANXA2 and ILK in ANXA2-knockdown A549 and 16HBE cells or control cells treated with or without 40 μg/ml bleomycin for 48 h were measured by Western blot analysis. β-actin was used as an internal control. (**E**) Cells were transfected with siScramble or siILK for 24 h, and then treated with 40 μg/ml bleomycin for 48 h. The total expression of ILK and IL-6, and the phosphorylation status of p65 and IκBα were measured by Western blot analysis. β-actin was used as an internal control.

responses, NF-κB pathway in cells has been associated with the balance between proliferation and apoptosis, alteration of which has emerged as a significant characteristic of pulmonary fibrosis. Our results also showed that ANXA2 deficiency reduced the proliferative capacity and increased caspase 3 activity in A549 and 16HBE cells upon bleomycin treatment (Supplementary Figures S3 and S4). These results revealed that surface ANXA2 was crucial in bleomycin-induced activation of NF-κB pathway.

Surface ANXA2 has been reported to activate NF-κB pathway by inducing expression of ILK [39], which was involved in bleomycin-induced skin fibrosis [44]. Thus, we have particular interest to detect whether ILK was involved in bleomycin-induced and ANXA2-mediated NF-κB pathway activation. As shown as Figure 4D, bleomycin treatment could increase ILK expression in A549 and 16HBE cells, while ANXA2 knockdown abolished bleomycin-induced ILK expression. Furthermore, interfered ILK expression by specific siRNA counteracted bleomycin-induced accumulation of p-p65, p-IκBα, and IL6 in A549 and 16HBE cells (Figure 4E). These results suggested that ILK plays an important role in bleomycin-induced and ANXA2-mediated activation of NF-κB in A549 and 16HBE cells.
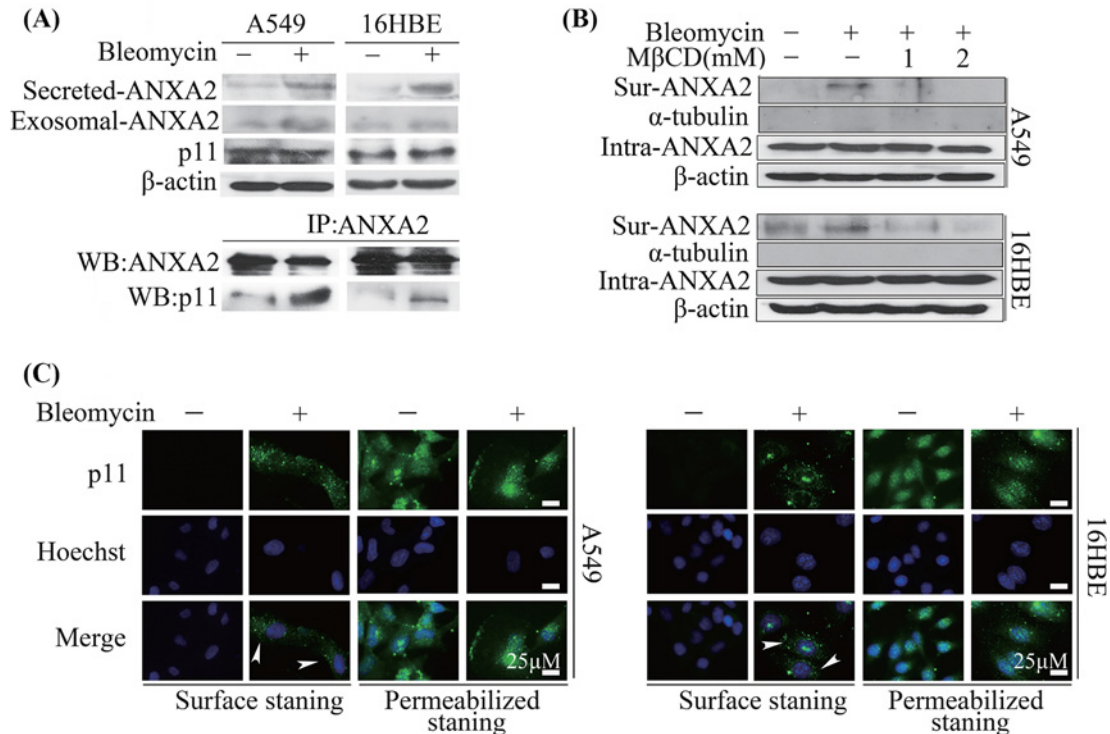
Figure 5. Interaction of ANXA2 with p11 and exosomal secretion was involved in surface expression of ANXA2
(**A**) A549 cells and 16HBE cells were treated with or without 40 μg/ml bleomycin for 48 h. The secreted and exosomal ANXA2 in recovered culture supernatants, as well as the expression of p11 in total cell lysates were detected by Western blot analysis (top). Total lysates were immunoprecipitated with ANXA2 antibody, and immunoblotted with ANXA2 antibody or p11 antibody (bottom). (**B**) Cells were pretreated with inhibitors MβCD for 1 h, and then treated with or without 40 μg/ml bleomycin for 48 h. The surface ANXA2 from the EGTA eluates was detected by Western blot analysis. (**C**) Immunofluorescent detection of p11 on the surface of A549 cells and 16HBE cells treated with or without 40 μg/ml bleomycin for 48 h. Microscope magnification: Σ 400×.

Interaction of ANXA2 with p11 and exosomal secretion is involved in surface expression of ANXA2

Since ANXA2 lacks a signal peptide for trafficking to the cellular plasma membrane through the classical ER-Golgi route, we presumed that the surface expression of ANXA2 upon bleomycin stimulation may be associated with exosomal secretion [24–26]. Indeed, we found that the level of ANXA2 was markedly increased in the cultured supernatant and exosome from both bleomycin-stimulated A549 cells and 16HBE cells (Figure 5A). To further examine whether bleomycin-induced surface expression of ANXA2 is associated with exosomal secretion, we pretreated cells with MβCD which inhibits exosome formation by disrupting cholesterol-rich lipid raft domains. Our result showed that MβCD treatment inhibited bleomycin-induced surface translocation of ANXA2 in both A549 cells and 16HBE cells (Figure 5B), suggesting that bleomycin-induced surface translocation of ANXA2 was dependent on exosomal secretion.

In addition, surface ANXA2 are known to form a molecular complex by interacting with its natural ligand p11/S100A10, which may be involved in its surface translocation and is required for maintaining biological function of ANXA2 [24,45,46]. To reveal the role of p11 in bleomycin-induced surface expression of ANXA2, we first examined whether bleomycin could affect the expression of p11. As shown in Figure 5A,C, although intracellular expression of p11 remained unchanged, surface expression of p11 was increased upon bleomycin treatment in A549 cells and 16HBE cells. The N-termini of ANXA2 can bind to p11, which was involved in exosome-induced surface expression of ANXA2 [24]. Therefore, we detected the level of cytoplasmic ANXA2/p11 complex and noted a significant increase in the interaction between ANXA2 and p11 in total cell lysates of A549 and 16HBE cells upon bleomycin treatment (Figure 5A), showing that bleomycin-induced surface translocation of ANXA2 was associated with enhanced interaction between ANXA2 and p11.
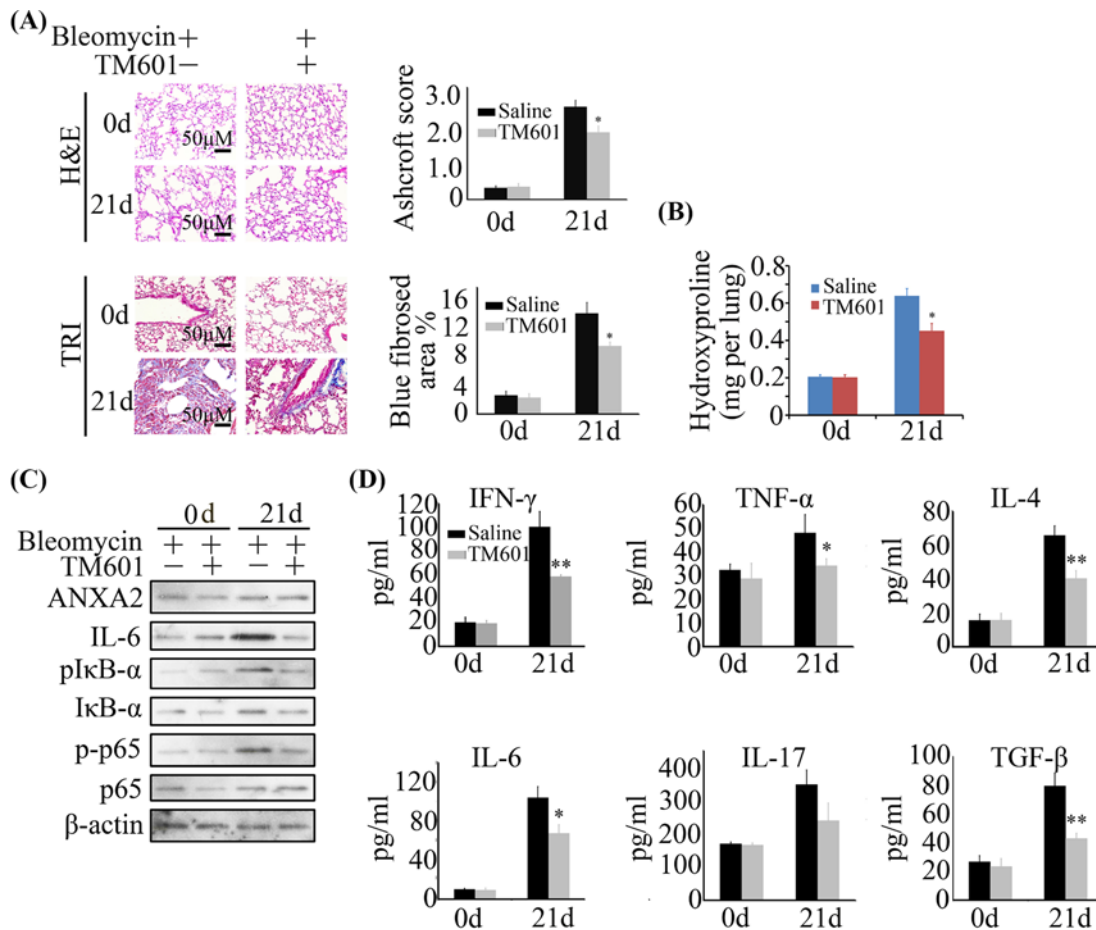
Figure 6. Inhibition of membrane ANXA2 by TM601 attenuates bleomycin-induced fibrosis and inflammation *in vivo*

Mice were challenged with bleomycin, and then treated with saline or TM601 (10 mg/kg, every third day) for 21 days. Mice were examined on day 21. (**A**) Sectioned lungs stained with H&E (top) and Masson's trichrome staining (bottom). Ashcroft score was determined by H&E staining of sectioned lungs. Fibrotic area was determined by Masson's trichrome staining. Microscope magnification: Σ 200×. (**B**) The hydroxyproline contents of whole lung homogenates were analyzed to examine collagen contents. (**C**) The expression of ANXA2 and key mediators in NF-κB pathway including p-p65, p65, IκB-α, p-IκB-α, and IL-6 in lungs were measured by Western blot analysis. β-actin was measured as an internal control. (**D**) IFN-γ, TNF-α, IL-4, IL-6, IL-17, and TGF-β levels were measured in BALF from bleomycin-challenged mice at the indicated time points post-saline or TM601 treatment. $n=$4–6 per group. $^{*}P<$0.05, $^{**}P<$0.01.

Inhibition of membrane ANXA2 by TM601 attenuates bleomycin-induced fibrosis *in vivo*

To further elucidate the role of membrane ANXA2 in bleomycin-induced fibrosis *in vivo*, we treated bleomycin-challenged mice with saline or TM601, a peptide which can bind and inhibit membrane ANXA2. We found that treatment with TM601 reduced bleomycin-induced tissue destruction and collagen deposition in mice compared with controls (Figure 6A,B). Activation of the NF-κB pathway in lung tissues induced by bleomycin was also significantly dampened by TM601, as evidenced by reduced expression of IL-6 as well as phosphorylation levels of IκBα and p65 (Figure 6C). Furthermore, the secreted levels of inflammatory factors (including IFN-γ, TNF-α, IL-4, IL-6, and IL-17) and TGF-β in BALF of bleomycin-treated mice were also decreased in response to TM601 treatment (Figure 6D). These results indicate that membrane ANXA2 indeed played an important role in bleomycin-induced inflammatory responses and pulmonary fibrosis development.

Discussion

IPF is a serious disease in which the lung tissue becomes thickened, stiff and scarred due to plural stimuli [6]. Complex and redundant pathways are involved in this process, such as inflammation, overexpression of profibrotic cytokines, autophagy, oxidant/antioxidant imbalances, EMT of lung epithelial cells, abnormal repair, and dysregulated angiogenesis [3–6,47]. However, the precise molecular mechanisms of IPF remain to be fully revealed, and thus the optimal therapeutic treatment has not yet been developed [6–9]. In previous study, we found that ANXA2 was a special target of bleomycin and contributed to the development of pulmonary fibrosis by blocking TFEB-induced autophagy [30]. ANXA2 is required for multiple biological functions such as signal transduction, inflammation, EMT, oxidative balance, angiogenesis, cell proliferation and apoptosis, airway epithelial wound repair *etc.*, all of which are also believed to be important pathologic mechanisms of pulmonary fibrosis [20,21,37,48–55]. Aberrant expression of ANXA2 has been linked to fibrotic diseases, including liver fibrosis, renal fibrosis, cystic fibrosis, and pulmonary fibrosis [19,40,56–59]. In the present study, we found that bleomycin specifically binds to ANXA2, which promotes the interaction between ANXA2 and p11, resulting in translocation of ANXA2 from cytoplasm to cellular surface through exosomal secretion. Surface translocation of ANXA2 could activate NF-κB pathway for the production of pro-inflammatory cytokines by inducing ILK expression in lung epithelial cells. Both ANXA2 knockdown and functional inhibition of surface ANXA2 can ameliorate fibrotic and inflammatory response in bleomycin-challenged mice. In addition, different from Korfei's and Schuliga's study, which found the down-regulation of ANXA2 expression in IPF lung samples (*n=9* and *n=5*, respectively) versus controls (both *n=5*) by Western blot analysis, we observed no significant difference in the expression of ANXA2 between 34 human pulmonary fibrosis tissues and 28 paired adjacent normal lung tissues by immunohistochemistry although about 33% patients with elevated ANXA2 expression. This might be due to the highly heterogeneous clinical course of IPF.

A recent study has shown that the expression of ANXA2 represents the highest correlation with inflammatory activity stage in human fibrotic liver and a rat bile duct ligation (BDL) model [60]. Furthermore, previous studies have shown that exposure to fibrogenic agents, such as bleomycin and silica particles, can activate the NF-κB pathway and elicit the production of inflammatory cytokines in lung epithelial cells, which is associated with the development of pulmonary inflammation [61,62]. In lung epithelial cells, we found that bleomycin binding could enhance surface translocation of ANXA2. Surface expression of ANXA2 is recently known to be involved in the activation of NF-κB pathway which is critical for inflammatory processes [5,38]. For example, Swisher *et al.* found that on the surface of human and murine macrophages, ANXA2 tetramer could elicit activation of NF-κB through TLR4, resulting in inflammatory cytokine production as well as chemokine production [27,42]. While Gong *et al.* [63] found that interaction between alternatively spliced segment of Tenascin-C and surface ANXA2 activate the NF-κB pathway, resulting in gemcitabine resistance in pancreatic cancer cells. Lin *et al.* [39] found that tissue plasminogen activator (tPA) activates the NF-κB pathway in macrophages through a signaling pathway involving surface ANXA2/CD11b-mediated ILK expression. Therefore, we first determined the role of surface ANXA2 in bleomycin-induced inflammatory response in lung epithelial cells. We found that bleomycin-induced activation of NF-κB and production of pro-inflammatory cytokine IL-6 were significantly attenuated by anti-ANXA2 antibody in A549 and 16HBE cells. Consistent with the *in vitro* results, inhibition of surface ANXA2 with TM601 can also attenuate fibrotic and inflammatory responses in bleomycin-challenged mice (Figure 6). Consistently, Schuliga *et al.* [40] also found that ANXA2 deletion attenuated inflammation and fibrotic features in bleomycin-challenged mice. However, because of the different modeling methods (intranasal instillation compare with intratracheal instillation), no significant difference in lung hydroxyproline (collagen) content was observed in bleomycin-treated WT and ANXA2$^{-/-}$ mice in this previous study [40]. These results demonstrated that bleomycin binding-induced ANXA2 surface translocation could contribute to bleomycin-induced pulmonary fibrosis through regulating inflammatory process. Interestingly, we also found bleomycin-induced and ANXA2-mediated activation of NF-κB pathway was, at least partially, dependent on ILK expression, which is widely linked to fibrosis process including skin, kidney, liver, and lung fibrosis [44,64]. In addition, Brichory *et al.* found that lung cancer patients that exhibited autoantibodies to annexins I and II had significantly higher IL-6 serum levels, while treatment of A549 cells with IL-6 resulted in an increase in membrane-bound annexin A1 and A2 [36,65]. These studies suggest that surface ANXA2 induced production of IL-6 might further enhance surface translocation of ANXA2 in lung epithelial cells.

A wide range of stimuli factors, such as IFN-γ, tyrosine phosphorylation, heat stress and thrombin, have been reported to induce surface translocation of ANXA2. However, ANXA2 devoid of a signal peptide cannot be transported to cell surface through the classical ER-Golgi pathway [22–24,36,45,46]. Exosomes present in multivesicular bodies (MVBs) which are intracellular transport vesicles independent of the ER/Golgi system, and membrane lipids at the inner leaflet of plasma membranes by which proteins physically traverse the plasma membrane, have been

discussed as playing a role in surface translocation of ANXA2 [24,36]. Consistent with the results obtained from IFN-γ-treated A549 cells by Fang *et al.*, we observed that bleomycin-induced surface translocation of ANXA2 was dependent on exosomal secretion associated with enhanced interaction between ANXA2 and p11, suggesting that external stimuli-induced surface translocation of ANXA2 may mainly depend on intracellular transport vesicles. In addition, autophagy was also involved in exosome transport. Autophagosome could fuse with MVBs to form amphisome, which will finally fuse with lysosome to degrade damaged proteins and organelles [66]. If autophagy is inhibited, exosome secretion will be enhanced and act as an alternative way to excrete harmful/damaged proteins and RNAs, and thereby preserve cell viability [67]. In our previous study, we found bleomycin could impede autophagy flux by binding ANXA2 [30], which may play a role in surface translocation of ANXA2 by enhancing exosome secretion.

In conclusion, we have shown that surface ANXA2 translocation plays an important role in bleomycin-induced pulmonary fibrosis by activating ILK/NF-κB pathway-mediated inflammatory responses. Both ANXA2 deficiency and surface ANXA2 inhibition could ameliorate fibrotic and inflammatory response in bleomycin-treated mice. These findings reveal that ANXA2 is a potential target for the treatment of pulmonary fibrosis.

Clinical perspectives

- This preclinical study aims to further reveal the role of ANXA2 in bleomycin-induced pulmonary fibrosis, which may help optimize the clinical use of bleomycin in cancer therapy and enable better understanding of the pathologic mechanisms of pulmonary fibrosis.

- Cell-surface translocation of Annexin A2 is essential in bleomycin-induced activation of NF-κB pathway and production of pro-inflammatory cytokines, which is a critical event in pulmonary fibrosis.

- ANXA2-deficient (ANXA2$^{-/-}$) mice treated with bleomycin exhibits reduced pulmonary fibrosis along with decreased cytokine production, while the surface ANXA2 inhibitor TM601, could also ameliorate fibrotic and inflammatory response in bleomycin-treated mice, suggesting that ANXA2 is a potential target for the treatment of pulmonary fibrosis.

Ethics approval and consent to participate

The research has been carried out in accordance with the World Medical Association Declaration of Helsinki. Human tissue ethics approval was obtained from the Institutional Ethics Committee of Sichuan University. All animal studies were reviewed and approved by the Institutional Animal Care and Treatment Committee of Sichuan University, and the University of North Dakota Institutional Animal Care and Use Committee. All institutional and national guidelines for the care and use of laboratory animals were followed.

Author contribution

Conception and design: Y.L., C.H., T.Z.; Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.L., X.L., Y.L., Z.Z., X.F., J.Z. (Especially, Z.Z., X.F., and J.Z. are new added authors in the revised manuscript, and their main contributions are collected human IPF tissues from Hospital and analysis the ANXA2 expression in these tissues.); Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.L., K.W., X.L., C.H., T.Z.; Writing, review, and/or revision of the manuscript: Y.L., K.W., T.Z.; Study supervision: C.H., T.Z.

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Abbreviations

ANXA2 (p36), annexin A2; ANXA2$^{-/-}$, ANXA2-deficient; BALF, bronchoalveolar lavage fluid; BDL, bile duct ligation; ER, endoplasmic reticulum; HBS, hepes-buffered saline; IFN-γ, interferon-γ; IL, interleukin; ILK, integrin-linked kinase; IPF, Idiopathic pulmonary fibrosis; KO, knockout; MβCD, methyl-β-cyclodextrin; MVB, multivesicular bodies; NF-κB, nuclear factor-κB; PBS, phosphate buffered saline; PBST, phosphate buffered saline with Tween-20; siRNA, small interfering RNA; TFEB, transcription factor EB; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor-α; tPA, tissue plasminogen activator; WT, wild-type.

References

1 Day, R.M., Suzuki, Y.J., Lum, J.M., White, A.C. and Fanburg, B.L. (2002) Bleomycin upregulates expression of gamma-glutamylcysteine synthetase in pulmonary artery endothelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **282**, L1349–1357, https://doi.org/10.1152/ajplung.00338.2001

2 Oury, T.D., Thakker, K., Menache, M., Chang, L.Y., Crapo, J.D. and Day, B.J. (2001) Attenuation of bleomycin-induced pulmonary fibrosis by a catalytic antioxidant metalloporphyrin. *Am. J. Respir. Cell Mol. Biol.* **25**, 164–169, https://doi.org/10.1165/ajrcmb.25.2.4235

3 Lin, X., Sime, P.J., Xu, H., Williams, M.A., LaRussa, L., Georas, S.N. et al. (2011) Yin yang 1 is a novel regulator of pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* **183**, 1689–1697, https://doi.org/10.1164/rccm.201002-0232OC

4 Zhao, L., Wang, X., Chang, Q., Xu, J., Huang, Y., Guo, Q. et al. (2010) Neferine, a bisbenzylisoquinline alkaloid attenuates bleomycin-induced pulmonary fibrosis. *Eur. J. Pharmacol.* **627**, 304–312, https://doi.org/10.1016/j.ejphar.2009.11.007

5 Russo, R.C., Garcia, C.C., Barcelos, L.S., Rachid, M.A., Guabiraba, R., Roffe, E. et al. (2011) Phosphoinositide 3-kinase gamma plays a critical role in bleomycin-induced pulmonary inflammation and fibrosis in mice. *J. Leukoc. Biol.* **89**, 269–282, https://doi.org/10.1189/jlb.0610346

6 Kliment, C.R. and Oury, T.D. (2010) Oxidative stress, extracellular matrix targets, and idiopathic pulmonary fibrosis. *Free Radical Biol. Med.* **49**, 707–717, https://doi.org/10.1016/j.freeradbiomed.2010.04.036

7 Wynn, T.A. (2011) Integrating mechanisms of pulmonary fibrosis. *J. Exp. Med.* **208**, 1339–1350, https://doi.org/10.1084/jem.20110551

8 Todd, N.W., Luzina, I.G. and Atamas, S.P. (2012) Molecular and cellular mechanisms of pulmonary fibrosis. *Fibrogenesis Tissue Repair* **5**, 11, https://doi.org/10.1186/1755-1536-5-11

9 Fernandez, I.E. and Eickelberg, O. (2012) New cellular and molecular mechanisms of lung injury and fibrosis in idiopathic pulmonary fibrosis. *Lancet* **380**, 680–688, https://doi.org/10.1016/S0140-6736(12)61144-1

10 Pullamsetti, S.S., Savai, R., Dumitrascu, R., Dahal, B.K., Wilhelm, J., Konigshoff, M. et al. (2011) The role of dimethylarginine dimethylaminohydrolase in idiopathic pulmonary fibrosis. *Sci. Transl. Med.* **3**, 87ra53, https://doi.org/10.1126/scitranslmed.3001725

11 Yasuoka, H., Zhou, Z., Pilewski, J.M., Oury, T.D., Choi, A.M. and Feghali-Bostwick, C.A. (2006) Insulin-like growth factor-binding protein-5 induces pulmonary fibrosis and triggers mononuclear cellular infiltration. *Am. J. Pathol.* **169**, 1633–1642, https://doi.org/10.2353/ajpath.2006.060501

12 Bringardner, B.D., Baran, C.P., Eubank, T.D. and Marsh, C.B. (2008) The role of inflammation in the pathogenesis of idiopathic pulmonary fibrosis. *Antioxid. Redox Signal.* **10**, 287–301

13 Balestro, E., Calabrese, F., Turato, G., Lunardi, F., Bazzan, E., Marulli, G. et al. (2016) Immune Inflammation and Disease Progression in Idiopathic Pulmonary Fibrosis. *PLoS ONE* **11**, e0154516, https://doi.org/10.1371/journal.pone.0154516

14 Fujimoto, H., D'Alessandro-Gabazza, C.N., Palanki, M.S., Erdman, P.E., Takagi, T., Gabazza, E.C. et al. (2007) Inhibition of nuclear factor-kappaB in T cells suppresses lung fibrosis. *Am. J. Respir. Crit. Care Med.* **176**, 1251–1260, https://doi.org/10.1164/rccm.200609-1288OC

15 Monastyrskaya, K., Babiychuk, E.B. and Draeger, A. (2009) The annexins: spatial and temporal coordination of signaling events during cellular stress. *Cell. Mol. Life Sci.* **66**, 2623–2642, https://doi.org/10.1007/s00018-009-0027-1

16 Rescher, U. and Gerke, V. (2004) Annexins–unique membrane binding proteins with diverse functions. *J. Cell Sci.* **117**, 2631–2639, https://doi.org/10.1242/jcs.01245

17 Kim, J. and Hajjar, K.A. (2002) Annexin II: a plasminogen-plasminogen activator co-receptor. *Front. Biosci.* **7**, d341–348, https://doi.org/10.2741/kim

18 Damazo, A.S., Sampaio, A.L., Nakata, C.M., Flower, R.J., Perretti, M. and Oliani, S.M. (2011) Endogenous annexin A1 counter-regulates bleomycin-induced lung fibrosis. *BMC Immunol.* **12**, 59, https://doi.org/10.1186/1471-2172-12-59

19 Zhang, L., Peng, X., Zhang, Z., Feng, Y., Jia, X., Shi, Y. et al. (2010) Subcellular proteome analysis unraveled annexin A2 related to immune liver fibrosis. *J. Cell. Biochem.* **110**, 219–228

20 Bao, H., Jiang, M., Zhu, M., Sheng, F., Ruan, J. and Ruan, C. (2009) Overexpression of Annexin II affects the proliferation, apoptosis, invasion and production of proangiogenic factors in multiple myeloma. *Int. J. Hematol.* **90**, 177–185, https://doi.org/10.1007/s12185-009-0356-8

21 Huang, Y., Jin, Y., Yan, C.H., Yu, Y., Bai, J., Chen, F. et al. (2008) Involvement of Annexin A2 in p53 induced apoptosis in lung cancer. *Mol. Cell. Biochem.* **309**, 117–123, https://doi.org/10.1007/s11010-007-9649-5

22 Hastie, C., Masters, J.R., Moss, S.E. and Naaby-Hansen, S. (2008) Interferon-gamma reduces cell surface expression of annexin 2 and suppresses the invasive capacity of prostate cancer cells. *J. Biol. Chem.* **283**, 12595–12603, https://doi.org/10.1074/jbc.M800189200

23 Lokman, N.A., Ween, M.P., Oehler, M.K. and Ricciardelli, C. (2011) The role of annexin A2 in tumorigenesis and cancer progression. *Cancer Microenviron.* **4**, 199–208, https://doi.org/10.1007/s12307-011-0064-9

24 Fang, Y.T., Lin, C.F., Wang, C.Y., Anderson, R. and Lin, Y.S. (2012) Interferon-gamma stimulates p11-dependent surface expression of annexin A2 in lung epithelial cells to enhance phagocytosis. *J. Cell. Physiol.* **227**, 2775–2787, https://doi.org/10.1002/jcp.23026

25 Siever, D.A. and Erickson, H.P. (1997) Extracellular annexin II. *Int. J. Biochem. Cell Biol.* **29**, 1219–1223, https://doi.org/10.1016/S1357-2725(97)00057-5

26 Valapala, M. and Vishwanatha, J.K. (2011) Lipid raft endocytosis and exosomal transport facilitate extracellular trafficking of annexin A2. *J. Biol. Chem.* **286**, 30911–30925, https://doi.org/10.1074/jbc.M111.271155

27 Swisher, J.F., Burton, N., Bacot, S.M., Vogel, S.N. and Feldman, G.M. (2010) Annexin A2 tetramer activates human and murine macrophages through TLR4. *Blood* **115**, 549–558, https://doi.org/10.1182/blood-2009-06-226944

28 Liu, J. and Vishwanatha, J.K. (2007) Regulation of nucleo-cytoplasmic shuttling of human annexin A2: a proposed mechanism. *Mol. Cell. Biochem.* **303**, 211–220, https://doi.org/10.1007/s11010-007-9477-7

29 Law, A.L., Ling, Q., Hajjar, K.A., Futter, C.E., Greenwood, J., Adamson, P. et al. (2009) Annexin A2 regulates phagocytosis of photoreceptor outer segments in the mouse retina. *Mol. Biol. Cell* **20**, 3896–3904, https://doi.org/10.1091/mbc.e08-12-1204

30 Wang, K., Zhang, T., Lei, Y., Li, X., Jiang, J., Lan, J. et al. (2018) Identification of ANXA2 (annexin A2) as a specific bleomycin target to induce pulmonary fibrosis by impeding TFEB-mediated autophagic flux. *Autophagy* **14**, 269–282, https://doi.org/10.1080/15548627.2017.1409405

31 Zhou, S., Yi, T., Liu, R., Bian, C., Qi, X., He, X. et al. (2012) Proteomics identification of annexin A2 as a key mediator in the metastasis and proangiogenesis of endometrial cells in human adenomyosis. *Mol. Cell. Proteomics* **11**, M112 017988, https://doi.org/10.1074/mcp.M112.017988

32 Kikuchi, N., Ishii, Y., Morishima, Y., Yageta, Y., Haraguchi, N., Itoh, K. et al. (2010) Nrf2 protects against pulmonary fibrosis by regulating the lung oxidant level and Th1/Th2 balance. *Respir. Res.* **11**, 31, https://doi.org/10.1186/1465-9921-11-31

33 Ashcroft, T., Simpson, J.M. and Timbrell, V. (1988) Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *J. Clin. Pathol.* **41**, 467–470, https://doi.org/10.1136/jcp.41.4.467

34 Hajjar, K.A., Guevara, C.A., Lev, E., Dowling, K. and Chacko, J. (1996) Interaction of the fibrinolytic receptor, annexin II, with the endothelial cell surface. Essential role of endonexin repeat 2. *J. Biological Chem.* **271**, 21652–21659, https://doi.org/10.1074/jbc.271.35.21652

35 Wang, K., Liu, R., Li, J., Mao, J., Lei, Y., Wu, J. et al. (2011) Quercetin induces protective autophagy in gastric cancer cells: involvement of Akt-mTOR- and hypoxia-induced factor 1alpha-mediated signaling. *Autophagy* **7**, 966–978, https://doi.org/10.4161/auto.7.9.15863

36 Fang, Y.T., Lin, C.F., Liao, P.C., Kuo, Y.M., Wang, S., Yeh, T.M. et al. (2010) Annexin A2 on lung epithelial cell surface is recognized by severe acute respiratory syndrome-associated coronavirus spike domain 2 antibodies. *Mol. Immunol.* **47**, 1000–1009, https://doi.org/10.1016/j.molimm.2009.11.019

37 Sarkar, S., Swiercz, R., Kantara, C., Hajjar, K.A. and Singh, P. (2011) Annexin A2 mediates up-regulation of NF-kappaB, beta-catenin, and stem cell in response to progastrin in mice and HEK-293 cells. *Gastroenterology* **140**, 583–595 e584, https://doi.org/10.1053/j.gastro.2010.08.054

38 Inayama, M., Nishioka, Y., Azuma, M., Muto, S., Aono, Y., Makino, H. et al. (2006) A novel IkappaB kinase-beta inhibitor ameliorates bleomycin-induced pulmonary fibrosis in mice. *Am. J. Respir. Crit. Care Med.* **173**, 1016–1022, https://doi.org/10.1164/rccm.200506-947OC

39 Lin, L., Wu, C. and Hu, K. (2012) Tissue plasminogen activator activates NF-kappaB through a pathway involving annexin A2/CD11b and integrin-linked kinase. *J. Am. Soc. Nephrol.* **23**, 1329–1338, https://doi.org/10.1681/ASN.2011111123

40 Schuliga, M., Jaffar, J., Berhan, A., Langenbach, S., Harris, T., Waters, D. et al. (2017) Annexin A2 contributes to lung injury and fibrosis by augmenting factor Xa fibrogenic activity. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **312**, L772–L782, https://doi.org/10.1152/ajplung.00553.2016

41 Aoshiba, K., Tsuji, T. and Nagai, A. (2003) Bleomycin induces cellular senescence in alveolar epithelial cells. *Eur. Respir. J.* **22**, 436–443, https://doi.org/10.1183/09031936.03.00011903

42 Swisher, J.F., Khatri, U. and Feldman, G.M. (2007) Annexin A2 is a soluble mediator of macrophage activation. *J. Leukoc. Biol.* **82**, 1174–1184, https://doi.org/10.1189/jlb.0307154

43 Chang, B., Amemura-Maekawa, J., Kura, F., Kawamura, I. and Watanabe, H. (2004) Expression of IL-6 and TNF-alpha in human alveolar epithelial cells is induced by invading, but not by adhering, Legionella pneumophila. *Microb. Pathog.* **37**, 295–302, https://doi.org/10.1016/j.micpath.2004.10.002

44 Radovanac, K., Morgner, J., Schulz, J.N., Blumbach, K., Patterson, C., Geiger, T. et al. (2013) Stabilization of integrin-linked kinase by the Hsp90-CHIP axis impacts cellular force generation, migration and the fibrotic response. *EMBO J.* **32**, 1409–1424, https://doi.org/10.1038/emboj.2013.90

45 Deora, A.B., Kreitzer, G., Jacovina, A.T. and Hajjar, K.A. (2004) An annexin 2 phosphorylation switch mediates p11-dependent translocation of annexin 2 to the cell surface. *J. Biol. Chem.* **279**, 43411–43418, https://doi.org/10.1074/jbc.M408078200

46 Peterson, E.A., Sutherland, M.R., Nesheim, M.E. and Pryzdial, E.L. (2003) Thrombin induces endothelial cell-surface exposure of the plasminogen receptor annexin 2. *J. Cell Sci.* **116**, 2399–2408, https://doi.org/10.1242/jcs.00434

47 Willis, B.C., duBois, R.M. and Borok, Z. (2006) Epithelial origin of myofibroblasts during fibrosis in the lung. *Proc. Am. Thorac. Soc.* **3**, 377–382, https://doi.org/10.1513/pats.200601-004TK

48 de Graauw, M., Tijdens, I., Smeets, M.B., Hensbergen, P.J., Deelder, A.M. and van de Water, B. (2008) Annexin A2 phosphorylation mediates cell scattering and branching morphogenesis via cofilin activation. *Mol. Cell. Biol.* **28**, 1029–1040, https://doi.org/10.1128/MCB.01247-07

49 Ling, Q., Jacovina, A.T., Deora, A., Febbraio, M., Simantov, R., Silverstein, R.L. et al. (2004) Annexin II regulates fibrin homeostasis and neoangiogenesis in vivo. *J. Clin. Invest.* **113**, 38–48, https://doi.org/10.1172/JCI19684

50 Lima e Silva, R., Shen, J., Gong, Y.Y., Seidel, C.P., Hackett, S.F., Kesavan, K. et al. (2010) Agents that bind annexin A2 suppress ocular neovascularization. *J. Cell. Physiol.* **225**, 855–864, https://doi.org/10.1002/jcp.22296

51 Madureira, P.A., Hill, R., Miller, V.A., Giacomantonio, C., Lee, P.W. and Waisman, D.M. (2011) Annexin A2 is a novel cellular redox regulatory protein involved in tumorigenesis. *Oncotarget* **2**, 1075–1093, https://doi.org/10.18632/oncotarget.375

52 Tanaka, T., Akatsuka, S., Ozeki, M., Shirase, T., Hiai, H. and Toyokuni, S. (2004) Redox regulation of annexin 2 and its implications for oxidative stress-induced renal carcinogenesis and metastasis. *Oncogene* **23**, 3980–3989, https://doi.org/10.1038/sj.onc.1207555

53 Krishnan, S., Deora, A.B., Annes, J.P., Osoria, J., Rifkin, D.B. and Hajjar, K.A. (2004) Annexin II-mediated plasmin generation activates TGF-beta3 during epithelial-mesenchymal transformation in the developing avian heart. *Dev. Biol.* **265**, 140–154, https://doi.org/10.1016/j.ydbio.2003.08.026

54 Sharma, M.R., Koltowski, L., Ownbey, R.T., Tuszynski, G.P. and Sharma, M.C. (2006) Angiogenesis-associated protein annexin II in breast cancer: selective expression in invasive breast cancer and contribution to tumor invasion and progression. *Exp. Mol. Pathol.* **81**, 146–156, https://doi.org/10.1016/j.yexmp.2006.03.003

55 Miragliotta, V., Lefebvre-Lavoie, J., Lussier, J.G. and Theoret, C.L. (2008) Equine ANXA2 and MMP1 expression analyses in an experimental model of normal and pathological wound repair. *J. Dermatol. Sci.* **51**, 103–112, https://doi.org/10.1016/j.jdermsci.2008.03.008

56 Perco, P., Pleban, C., Kainz, A., Lukas, A., Mayer, G., Mayer, B. et al. (2006) Protein biomarkers associated with acute renal failure and chronic kidney disease. *Eur. J. Clin. Invest.* **36**, 753–763, https://doi.org/10.1111/j.1365-2362.2006.01729.x

57 Zhang, L., Jia, X., Feng, Y., Peng, X., Zhang, Z., Zhou, W. et al. (2011) Plasma membrane proteome analysis of the early effect of alcohol on liver: implications for alcoholic liver disease. *Acta Biochim. Biophys. Sin. (Shanghai)* **43**, 19–29, https://doi.org/10.1093/abbs/gmq108

58 Borthwick, L.A., McGaw, J., Conner, G., Taylor, C.J., Gerke, V., Mehta, A. et al. (2007) The formation of the cAMP/protein kinase A-dependent annexin 2-S100A10 complex with cystic fibrosis conductance regulator protein (CFTR) regulates CFTR channel function. *Mol. Biol. Cell* **18**, 3388–3397, https://doi.org/10.1091/mbc.e07-02-0126

59 Korfei, M., Schmitt, S., Ruppert, C., Henneke, I., Markart, P., Loeh, B. et al. (2011) Comparative proteomic analysis of lung tissue from patients with idiopathic pulmonary fibrosis (IPF) and lung transplant donor lungs. *J. Proteome Res.* **10**, 2185–2205, https://doi.org/10.1021/pr1009355

60 Staten, N.R., Welsh, E.A., Sidik, K., McDonald, S.A., Dufield, D.R., Maqsodi, B. et al. (2012) Multiplex transcriptional analysis of paraffin-embedded liver needle biopsy from patients with liver fibrosis. *Fibrogenesis Tissue Repair* **5**, 21, https://doi.org/10.1186/1755-1536-5-21

61 Hubbard, A.K., Timblin, C.R., Shukla, A., Rincon, M. and Mossman, B.T. (2002) Activation of NF-kappaB-dependent gene expression by silica in lungs of luciferase reporter mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **282**, L968–975, https://doi.org/10.1152/ajplung.00327.2001

62 Aoshiba, K., Zhou, F., Tsuji, T. and Nagai, A. (2012) DNA damage as a molecular link in the pathogenesis of COPD in smokers. *Eur. Respir. J.* **39**, 1368–1376, https://doi.org/10.1183/09031936.00050211

63 Gong, X.G., Lv, Y.F., Li, X.Q., Xu, F.G. and Ma, Q.Y. (2010) Gemcitabine resistance induced by interaction between alternatively spliced segment of tenascin-C and annexin A2 in pancreatic cancer cells. *Biol. Pharm. Bull.* **33**, 1261–1267

64 Kavvadas, P., Kypreou, K.P., Protopapadakis, E., Prodromidi, E., Sideras, P. and Charonis, A.S. (2010) Integrin-linked kinase (ILK) in pulmonary fibrosis. *Virchows Arch.* **457**, 563–575, https://doi.org/10.1007/s00428-010-0976-7

65 Brichory, F.M., Misek, D.E., Yim, A.M., Krause, M.C., Giordano, T.J., Beer, D.G. et al. (2001) An immune response manifested by the common occurrence of annexins I and II autoantibodies and high circulating levels of IL-6 in lung cancer. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 9824–9829, https://doi.org/10.1073/pnas.171320598

66 Fader, C.M. and Colombo, M.I. (2009) Autophagy and multivesicular bodies: two closely related partners. *Cell Death Differ.* **16**, 70–78, https://doi.org/10.1038/cdd.2008.168

67 Baixauli, F., Lopez-Otin, C. and Mittelbrunn, M. (2014) Exosomes and autophagy: coordinated mechanisms for the maintenance of cellular fitness. *Front. Immunol.* **5**, 403, https://doi.org/10.3389/fimmu.2014.00403