UNIVERSITY OF CALIFORNIA SAN DIEGO

The Role of Oxidized Phospholipids in Bleomycin-induced Pulmonary Fibrosis

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for the degree Master of Science

In

Biology

By

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The Thesis of Wenxi Tang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California San Diego

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Dedication

To my parents and my grandparents

For their love and support

TABLE OF CONTENTS

LIST OF FIGURES

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Lastly, I would like to thank Professor Joseph Witztum and Dr. Xiaoli Sun for reviewing my thesis draft and providing critical comments and valuable advice.

ABSTRACT OF THE THESIS

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By

Wenxi Tang

Master of Science in Biology

University of California San Diego, 2020

Professor Joseph Witztum, Chair
Professor Nan Hao, Co-Chair

Oxidized phospholipids (OxPLs), which are generated with the presence of oxidative stress, are proven to be pro-inflammatory and pro-fibrogenic in the liver in the context of nonalcoholic steatohepatitis but their roles in pulmonary fibrosis remain unknown. In this study, in particular, I show that OxPLs contribute to a widely used chemotherapy medication, bleomycin, induced pulmonary fibrosis. I found that bleomycin induces the expression of fibrogenic and inflammatory genes and the accumulation of OxPLs in mouse alveolar macrophages. Furthermore, I demonstrated that OxPAPC

induced the expression of fibrogenic and inflammatory genes in mouse alveolar macrophages, which suggest a potential fibrogenic role of OxPLs in the lung. Given that bleomycin acutely induces the accumulation of OxPLs, whereas its induction of the inflammatory and fibrotic genes is chronic, this temporal order of the two events suggests that OxPLs might play a role in bleomycin-induced fibrogenic effects on alveolar macrophages. To further confirm the roles of OxPLs in bleomycin-induced fibrogenic effects, I will take advantage of the E06-scFv transgenic mice, which express an E06-scFv antibody to neutralize OxPLs, to investigate the fibrogenic effects of bleomycin on the primary alveolar macrophages. I also plan to evaluate lung fibrosis in bleomycin administered wildtype and E06-scFv transgenic mice. My study might provide a new direction for therapies that aims to attenuate bleomycin-induced pulmonary fibrosis during chemotherapy.

Introduction

Pulmonary fibrosis means scarring in the lungs, which causes irreversible destruction of lung architecture and affects the normal functions of the lungs. Pulmonary fibrosis exists in over 200 different lung diseases, and one of the most prevalent fibrotic lung diseases is called idiopathic pulmonary fibrosis (IPF), which has unknown etiology and affects approximately 50,000 people every year in the United States (Selman et al., 2001). There are several possible causes of pulmonary fibrosis, including genetic mutations in telomerase or surfactant protein C genes, viral infection, radiotherapy, and chemotherapy (Lawson et al., 2011; Wynn et al., 2011). Noticeably, the pulmonary toxicity caused by chemotherapeutic drugs presents a great challenge for clinicians. It has been reported that up to 10% of patients who receive chemotherapeutic drugs exhibit pulmonary toxicity, and even this percentage is thought to be lower than the accrual number as the condition is underdiagnosed worldwide (Schwaiblmair et al.,2012). Studies have shown that a higher dose used in clinical practice is associated with a higher death rate resulting from pulmonary consequences of chemotherapy, which further limits its clinical use (Limper et al., 2004). Commonly used chemotherapeutic agents that are known to have pulmonary toxicity include bleomycin, mitomycin C and actinomycin D and others. Among these agents, bleomycin has the most prevalent toxic effects in the lungs, with 20% of patients later developing pulmonary diseases, up to 10% of patients developing pulmonary fibrosis, and a 1% death rate due to pulmonary failure (Limper et al., 2004). Due to its prevalent toxicity, bleomycin-induced pulmonary fibrosis is one of the most studied chemotherapy-induced pulmonary diseases, yet the underlying mechanisms are still not well-defined. Because bleomycin has a significant role in clinical

practice and because of its known ability to cause pulmonary fibrosis from previous studies, it is of critical importance to study the mechanisms of bleomycin-induced pulmonary fibrosis.

Bleomycin is an antibiotic produced by Streptomyces Verticillis and is commonly used to treat various types of cancer, such as Hodgkin's lymphoma, non-Hodgkin's lymphoma, testicular cancer, ovarian cancer and cervical cancer (Drugs 2019). It is activated by Fe(II) and oxygen, and the activated bleomycin attacks the DNA and causes both single and double-strand breaks. Studies have shown that bleomycin can induce apoptosis in a dose- and time-dependent manner in various cell types such as pneumocytes and lymphocytes (Tounekti et al., 1993; Yeung et al., 2015). In addition to causing cell damage by breaking DNA strands, bleomycin can potently induce lipid peroxidation by generating extensive "oxidative stress" via free radical mediated mechanisms (Latta et al., 2015).

Once the lung is formed, a fundamental purpose of pulmonary fibrogenesis is the repair and replacement of dead or injured tissue, but if the healing response is dysregulated and/or exaggerated, it will form scars—fibrous tissue made up of collagen--in the lungs. In normal tissue, collagen consists of a random basketweave formation of flexible collagen fibers, but in scars, the collagen is cross-linked and forms fibers that are in the same direction, more rigid, and of an inferior quality to the original tissue (Corr et al., 2013). Fibrosis usually starts with an injury of alveolar epithelial or endothelial cells, and the injured cells in turn release inflammatory mediators, which leads to the recruitment of leukocytes including macrophages, lymphocytes, and other blood elements (Kato et al., 2018). The recruited leukocytes then secrete fibrogenic cytokines

such as transforming growth factor-beta (TGF-β), tumor necrosis factor-alpha (TNF-α), interleukin 1 beta (IL-1β) and platelet-derived growth factor (PDGF), which further lead to migration and differentiation of fibroblast into myofibroblast, which in turn release components of extracellular matrix (Fig. 1; Wynn et al., 2011).

Bleomycin-induced pulmonary fibrosis in mice is a commonly used model to study pulmonary fibrosis, in which fibrosis is induced by intratracheal administration of bleomycin. Studies have shown that mice develop pulmonary fibrosis between 7~28 days after a single dose administration of bleomycin, with elevated expression of proinflammatory and fibrogenic genes in epithelial cells and their products in the bronchoalveolar lavage fluid including TGF-β, PDGF, TNF-α and IL-1β. There is also increased collagen deposition in lung tissue and disrupted alveolar structure (Romero et al., 2015). Levels of reactive oxygen species (ROS), oxidized proteins and oxidized lipids are also significantly elevated in the bleomycin-induced pulmonary fibrosis mouse model, which suggests that bleomycin-induced lipid peroxidation and oxidative stress could potentially play a role in fibrogenesis in the lungs (Cheresh et al., 2013). However, due to the lack of agents that could specifically neutralize OxPLs *in vivo*, the roles of OxPLs *in vivo* in disease models in general and pulmonary fibrosis in particular has not been previously investigated.

Oxidized phospholipids (OxPLs-defined here as phosphocholine (PC)-containing phospholipids) are a major class of oxidized lipid molecules that are generated from lipid peroxidation of polyunsaturated fatty acids containing phospholipids. The OxPLs are very pro-inflammatory and are ubiquitous in many inflammatory and aging-related settings, such as atherosclerosis, nonalcoholic steatohepatitis (NASH), H1N5 and SARS-infected

lungs, Alzheimer's Disease and apoptotic cells (Van Dijk et al., 2012; Imai et al., 2008; Chang et al., 1999; Dei et al., 2002).

Our lab previously cloned a mouse IgM natural antibody, E06, from high cholesterol diet fed *Apoe*$^{-/-}$ mice, which had high titers of IgM antibodies binding to OxLDL (oxidized LDL). E06 specifically binds to the phosphocholine (PC) headgroup of OxPLs, in which the *sn2* fatty acid is oxidized. Remarkably, E06 only binds to the PC of OxPLs and does not bind to the PC of unoxidized, native PC-containing phospholipids (Friedman et al., 2002; Shaw et al., 2000). By binding to the PC headgroup of OxPLs, the E06 antibody blocks the proinflammatory effects of OxPLs, such as induction of IL-1β and TNF-α by macrophages and inhibits the uptake of OxLDL by macrophages (Shaw et al., 2000, Que et al., 2018, Sun et al., 2020). To study the roles of OxPLs *in vivo* in disease conditions, we recently generated a transgenic mouse model that overexpresses a single-chain variable fragment of the E06 antibody (E06-scFv). We found that neutralizing OxPLs with E06-scFv greatly protected mice from inflammation and atherosclerosis (Que et al., 2018). Most recently, we showed that targeting OxPLs using the E06-scFv mice also protected them from manifestations of nonalcoholic steatohepatitis (NASH). Of particular interest, neutralizing OxPLs with the E06-scFv in several different mouse models of NASH greatly reduced liver fibrosis, which was shown by reduced hepatic fibrogenic gene expression and decreased levels of liver hydroxyproline, a measure of collagen content (Sun et al., 2020). These findings suggest an antifibrogenic role of E06. Based on the previous studies, I hypothesize that OxPLs contribute to bleomycin induced lung fibrosis.

Taking advantage of the E06-scFv transgenic mouse model, our preliminary studies found that neutralizing OxPLs in *vivo* reduced bleomycin induced lung damage and immune cell influx including macrophages and lymphocytes, suggesting that OxPLs might play a role in bleomycin-induced pulmonary fibrosis. Moreover, many studies have demonstrated that a major mechanism of bleomycin-induced pulmonary fibrosis involves alveolar macrophages as one of the major sources of fibrogenic cytokines. It has been shown that bleomycin directly activates human alveolar macrophages to produce TGF-β, TNF-α, and IL-1β (Scheule et al., 1992). Given that bleomycin causes cell damage by inducing extensive oxidative stress, which leads to lipid peroxidation, it is highly likely that OxPLs are generated during bleomycin administration, which could in turn activate alveolar macrophage to cause fibrogenesis in the lung. Therefore, although I hope to study a variety of the pulmonary cell types potentially involved, for my initial studies I will utilize macrophages for my *in vitro* studies.

Although it has been reported that there is increased oxidative stress and increased lipid peroxidation with bleomycin administration, whether bleomycin induces OxPL accumulation and how this contributes to fibrogenesis in the lungs remains unknown. In my studies accomplished to date, I demonstrated that bleomycin elevated the level of OxPLs in alveolar macrophages and induced fibrogenic gene expression. Also, I have shown that OxPAPC—a mixture of different OxPLs-- induced proinflammatory and fibrogenic gene expression in alveolar macrophages, which suggests that OxPLs stimulate the alveolar macrophages to secrete fibrogenic cytokines. To further confirm that OxPLs can mediate bleomycin induced fibrogenic effects in macrophages in vitro and *in vivo*, I plan to take advantage of the E06-scFv transgenic mice and primary cells to

study if targeting OxPLs could protect against bleomycin-induced pulmonary fibrosis. I will isolate primary alveolar macrophages from both wildtype and E06-scFv transgenic mice and treat them with bleomycin to measure the secretion of fibrogenic cytokines and production of OxPLs. I will also instill bleomycin into E06-scFv and control mice under conditions that cause bleomycin-induced pulmonary fibrosis and determine if targeting OxPLs block the fibrogenic effects by evaluating lung histology and collagen deposition. Since I am going to start my Ph.D studies in the UCSD Biomedical Science Graduate program in the coming semester, I will continue this project and further characterize the detailed mechanisms involved in bleomycin-induced lung fibrosis. These studies include the effects of OxPLs on the functions of different cell types in the lung, and the effects of OxPLs on gene expression and signaling transduction.

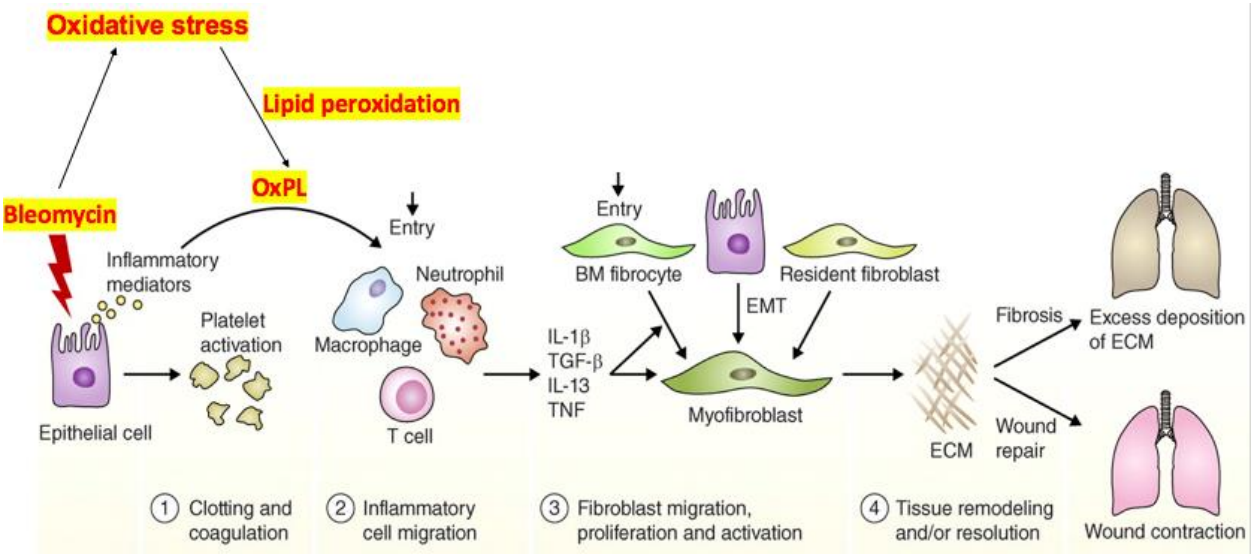


Figure 1. General mechanism of pulmonary fibrosis. Graph modified from Wynn et al., *Journal of Exp Med* 2011.

Results

Bleomycin treatment increases the expression of inflammatory and fibrogenic genes in alveolar macrophages.

To assess if bleomycin has any effect on pro-fibrogenic gene expression in alveolar macrophages, I first treated MH-S, immortalized mouse alveolar macrophages, with bleomycin in a dose and time-dependent manner. I measured mRNA levels of the prominent collagen gene, *col3α1,* in MH-S cells at 6 hours and 13 hours after bleomycin treatment with doses of 10ug/ml and 100ug/ml. The doses used were chosen in accordance with the clinical dose (Blum et al., 1973). Gene expression analysis showed that there was an observable trend of increased expression of *col3α1* with 10ug/ml of bleomycin for both 6hr and 13hr treatment (Fig. 2A-B). A lack of statistical significance might be due to a small number of biological replicates (n=3). Although 100 ug/ml showed the highest induction at 6 hrs, the level of gene expression at 13 hrs was decreased raising concern about toxicity, and therefore 10ug/ml bleomycin treatment might represent a better dose to study for the following cell culture experiments. MH-S cells were then treated with 10ug/ml of bleomycin for 6hrs, and the expressions of *Col1α1*, *Col3α1*, *Ccr2*, *Ccr5* and *Tgfb1* were measured by qPCR. Bleomycin treated cells showed an increasing trend but this was not statistically significant (Fig. 2C). These data suggest that bleomycin does not directly induce fibrogenic gene expression in macrophages under conditions studied. Therefore, I decided to treat the cells with bleomycin for a longer time to see if there is any chronic or secondary induction of the expression of these genes. To this end, MH-S cells were treated with 10ug/ml of bleomycin for 13hrs. Interestingly, a significant increase in mRNA levels of *Col1α1*, *Col3α1* and *Ccr5* was now observed,

which suggests that bleomycin significantly increases the expression of fibrogenic genes and inflammatory response in MH-S cells with more prolonged stimulation (Fig. 2D). The observations that a 6-hour treatment did not induce significant increases in inflammatory and fibrogenic gene expression but a 13-hour treatment did suggest that bleomycin might not directly induce fibrosis, but might increase gene expression through a secondary effect. I therefore speculate that OxPLs might have to be first induced to promote a fibrogenic response in response to bleomycin.

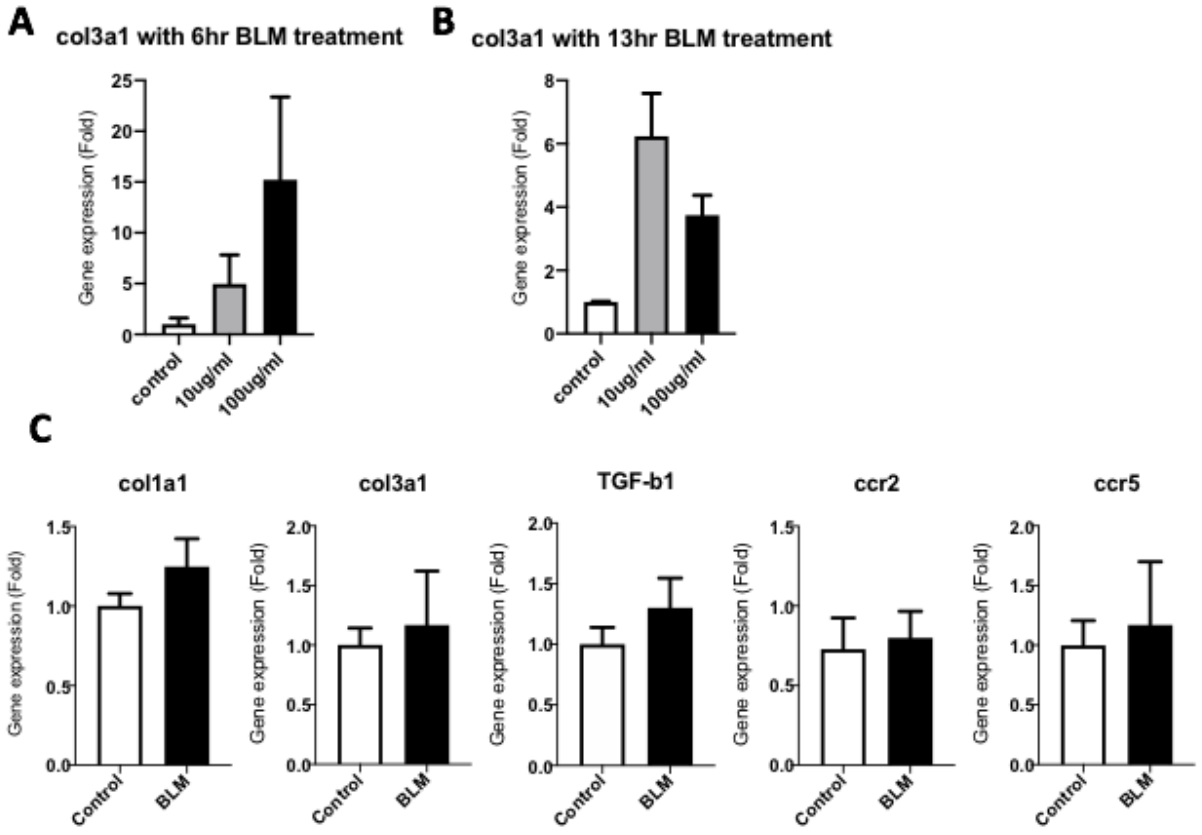
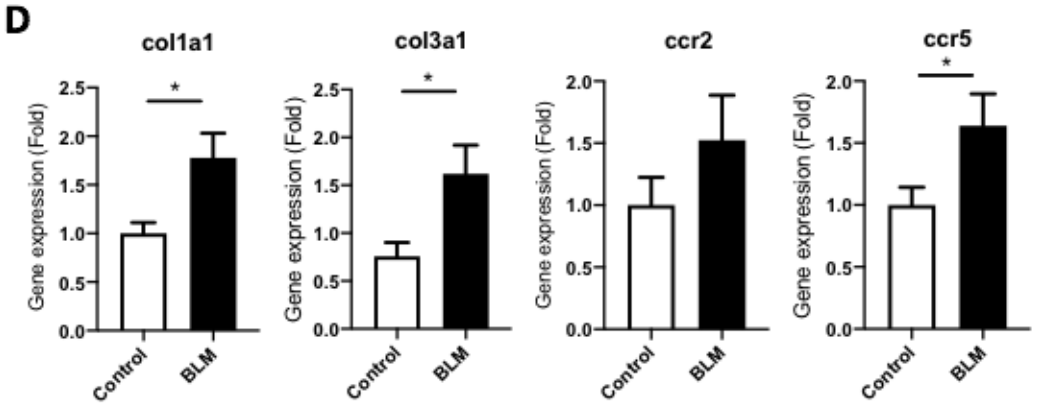


Figure 2. Bleomycin increases expression of inflammatory and fibrogenic genes in mouse alveolar macrophages. (A-B) mRNA levels for *Col3a1* in mouse alveolar macrophages (MH-S cell line) was measured by RT-qPCR after 6-hour and 13-hour bleomycin treatment respectively, both with doses of 10ug/ml and 100ug/ml (n=3). (C) mRNA levels of *Col1a1*, *Col3a1*, *Ccr2*, *Ccr5* and *Tgfb1* in mouse alveolar macrophages (MH-S cell line) were measured by RT-qPCR after 6-hour bleomycin treatment with a dose of 10ug/ml (n=6). (D) mRNA levels of *Col1a1*, *Col3a1* and *Ccr5* in mouse alveolar macrophages (MH-S cell line) were measured by RT-qPCR after 13-hour bleomycin treatment with a dose of 10ug/ml (n=6). Data are expressed as mean ± SEM. The statistical significance was assessed with two-tail student's t test. **P*<0.05.

Figure 2 Continued



Bleomycin increases OxPL level in murine alveolar macrophages.

To investigate whether OxPLs play a role in bleomycin-induced pulmonary fibrosis, it's important to first determine if OxPLs are generated as a result of bleomycin administration. There is a precedent for this concept in that activation of macrophages with TLR4 agonists results in generation of OxPLs (Popat et al., 2017). MH-S cells were treated with 10ug/ml bleomycin and harvested after 6hrs for measurement of OxPLs produced by the cells. A cell lysate was prepared after washing the cells with Dulbecco's phosphate-buffered saline (DPBS), and the relative content of OxPLs was measured using a competitive ELISA (Fig. 3). The data showed a significantly elevated level of OxPLs in the cell lysate of the bleomycin treatment group compared to the control group, demonstrating that bleomycin causes alveolar macrophages to generate (or accumulate) OxPLs *in vitro*.

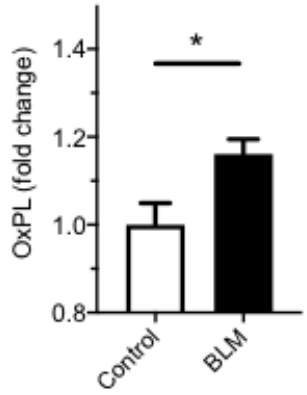


Figure 3. Bleomycin increases OxPL level in mouse alveolar macrophages. Mouse alveolar macrophages were treated with 20ug/ml bleomycin for 6hrs. Media was removed and cell lysate was collected. The level of immunodetectable OxPLs was measured by a competitive ELISA (n=6). Data are expressed as mean ± SEM. The statistical significance was assessed with two-tail student's t test. **P*<0.05.

OxPAPC increases the expression of inflammatory and fibrogenic genes in alveolar macrophages.

The preliminary experiments above suggest that bleomycin treatment of macrophages caused an accumulation of OxPLs. I next asked whether in turn the OxPLs could induce fibrogenic gene expression in alveolar macrophages. To determine if OxPLs have fibrogenic effects on alveolar macrophages, I treated MH-S cells with OxPAPC, a mixture of OxPLs generated by air oxidation of the parent unoxidized PAPC. MH-S cells were incubated with OxPAPC for 24hrs and then I collected the cells for RNA extraction. mRNA levels were measured by RT-qPCR, and the data showed significant increase in both expression of *Col1a1, Col3a1* and *Ccr5*, which suggest that OxPLs potentially have a fibrogenic effect on alveolar macrophages (Fig. 4). This experiment was disrupted by the COVID-19 pandemic, but I will continue to measure gene expression in treated and control cells for *Ccr2* and *Tgfb1* by RT-qPCR. In further experiments, I will also treat the cells with DMPC—(dimyristyl phosphocholine) a closely related saturated phospholipid that cannot be oxidized and will serve as a control for the OxPAPC phospholipid addition.

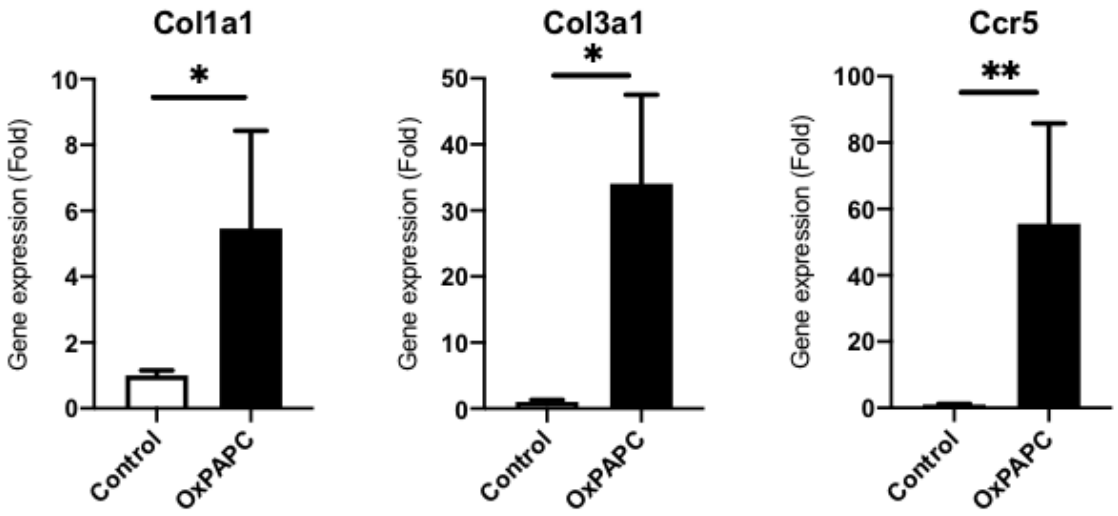


Figure 4. OxPAPC increases expression of inflammatory and fibrogenic genes in mouse alveolar macrophages. mRNA levels of *Col3a1* and *Ccr5* in mouse alveolar macrophages (MH-S cell line) were measured by RT-qPCR after 24-hour OxPAPC treatment with a dose of 50ug/ml (n=6). Data are expressed as mean ± SEM. The statistical significance was assessed with two-tail student's t test. **P*<0.05.

The genotype of the mice was confirmed by genotyping.

E06-scFv mice on the C57BL/6 background was generated by crossing E06-scFv mice on the *Ldlr*-/- and C57BL/6 wildtype mice. Genotyping was performed throughout the breeding process. E06-scFv transgene and wildtype *Ldlr* was checked for each generation (Fig. 5A-B)

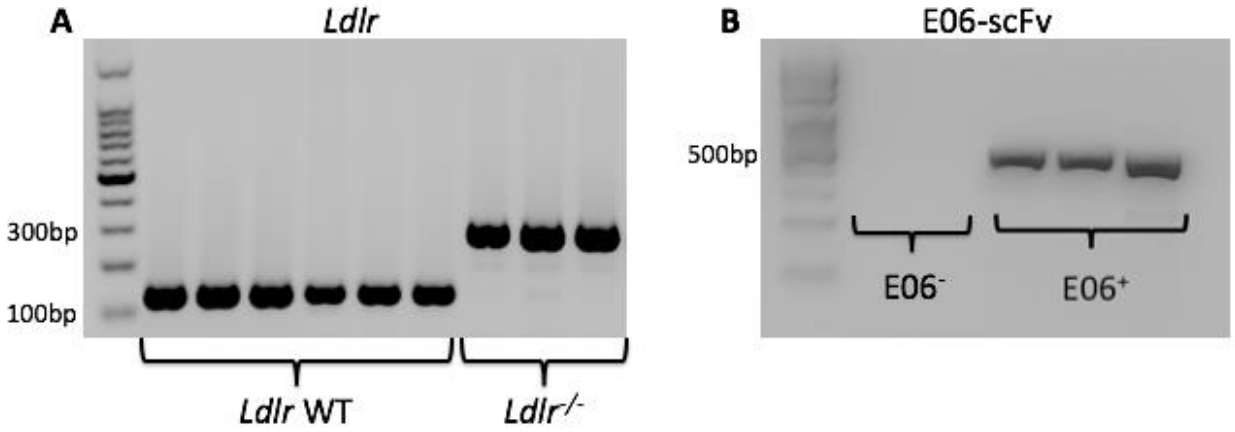


Figure 5. Genotyping of E06-scFv transgenic mice. (A) Detection of wildtype *Ldlr* in mice shown by DNA gel electrophoresis in lane 1-6. (B) Detection of E06-scFv transgene in mice shown by DNA gel electrophoresis in lane 3, 4 and 5.

Discussion

In this study, I showed that bleomycin induced significant mRNA expression of proinflammatory and fibrogenic genes including *col1a1*, *col3a1* and *Ccr5* in mouse alveolar macrophages with 13-hour treatment but not 6-hour treatment. Then I performed ELISA to measure the relative level of immunodetectable OxPLs induced by bleomycin in mouse alveolar macrophage, and the results suggest that the OxPL level was elevated after 6-hour bleomycin treatment. The timeline order of bleomycin-induced OxPLs and afterward fibrogenic gene expression suggest that OxPLs might potentially be an intermediate mediator of the fibrogenic effects of bleomycin. To further test this proposed pathway, I tested whether OxPLs induce fibrogenic gene expression directly in mouse alveolar macrophages, and the data indicate that OxPLs treatment elevated mRNA expression of fibrogenic and inflammatory genes. The accumulation of OxPLs in bleomycin-treated alveolar macrophages and the activation of fibrogenic genes by OxPLs together suggest several possible scenarios: First, bleomycin might directly activate macrophage gene expression. Second: that OxPLs, which are known to directly activate macrophage inflammatory gene expression can also mediate macrophage profibrogenic gene expression, and Third: that bleomycin might first induce the production of OxPLs by macrophages, which then further contribute to fibrogenic gene expression in an autocrine, feed-forward loop, thus amplifying the net effect of bleomycin on macrophages.

In vitro, I found that bleomycin induces the expression of fibrogenic genes and OxPL accumulation. After I finish testing OxPLs' effects on fibrogenic gene expression, it's important to study whether neutralizing OxPLs might attenuate the fibrogenic effects of bleomycin. This will be accomplished in 2 ways: First, I will stimulate

macrophages in vitro with both bleomycin and OxPAPC in the absence or presence of added IgM E06 antibody. Second, I will isolate primary alveolar macrophages from both the wildtype and E06-scFv mice, and treat them with bleomycin and OxPAPC. The expression of fibrogenic genes will be evaluated. Note that the primary alveolar macrophages of E06-scFv mice should express and secret the E06-scFv antibody as the promoter of the E06-scFv construct is the apolipoprotein E promoter, which is highly expressed in macrophages. Most importantly, the roles of OxPLs in bleomycin induced lung fibrosis *in vivo* will be tested on the wildtype and E06-scFv mice. I plan to use E06-scFv transgenic mice to study if targeting OxPLs *in vivo* could protect the mice from bleomycin-induced pulmonary fibrosis. I have already established my own E06-scFV transgenic colonies along with littermates of wildtype mice. Once the COVID-19 situation is under control, I could immediately start my *in vivo* experiments. I plan to generate the disease model by intratracheal administration of bleomycin to both E06-scFv transgenic and wildtype mice. Generally, fibrosis will develop from day 7 to day 28, and level of fibrosis will be elevated by Sirius Red staining and hydroxyproline measurement in the mouse lungs. Based on the results stated above and our lab's recent finding that targeting OxPLs in vivo reduces liver fibrosis (Sun et al., 2020)., I speculate that it will also be protective in the lung.

In addition to alveolar macrophage, which was used in this study, other cell types in the lungs could also contribute to fibrogenesis in the lungs, such as epithelial cells and endothelial cells. For instance, a study showed that in lung endothelial cells isolated from bleomycin-instilled mice, expression of fibrogenic cytokines including TGF-β, TNF-α, and PDGF was significantly elevated at day 7 after the installation (Kato et al., 2018). In this

study, I focus on the roles of macrophages in response to bleomycin treatment because macrophages are documented to be one of the main drivers of fibrogenesis due to their important roles in tissue repair and regeneration (Lech et al., 2013). Moreover, studies from our lab have shown that OxPLs could activate macrophages through multiple mechanisms such as stimulating TLR 2 and TLR 4 (Que et al., 2018; Lee et al., 2012). Therefore, macrophages are an important cell type likely to be involved in the fibrogenic pathways induced by OxPLs. However in the future, I plan to collaborate with the laboratory of Dr. Christopher Glass at UCSD to sort different cell types from the lungs and conduct mRNA sequencing to assess if bleomycin and OxPLs have different effects on gene expression of other cell types. They have recently established a system to sort different cell types in the lungs, which I could adopt into my studies.

This study might provide a new insight into the mechanisms of bleomycin induced lung fibrosis. It potentially offered a new direction in combination usage of E06 antibody with bleomycin to attenuate bleomycin-induced pulmonary fibrosis during chemotherapy. Moreover, understanding one model of fibrosis will potentially provide insight into the roles of OxPLs in other disease models. For instance, it has been shown that OxPLs accumulate in H5N1and SARS infected lungs, and around 20% of patients affected with SARS develop long term lung fibrosis (Imai et al., 2008; Hui et al., 2005). Targeting OxPLs might be a novel direction to develop therapies for other types of pulmonary fibrosis in addition to bleomycin-induced pulmonary fibrosis.

Materials and methods

Chemicals and reagents: Bleomycin sulfate was purchased from Sigma-Aldrich, and was dissolved in sterile water before used. OxPAPC (oxidized 1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine) was obtained from Avanti Polar Lipid.

Cell culture: MH-S **(**mouse alveolar macrophage cell line) and A549 (mouse alveolar epithelial cell line) were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum (FBS). All cells were incubated at 37°C.

Animals: E06-scFv mice on the *Ldlr*$^{-/-}$ and C57BL/6 background (Sun et al, 2020), available from my mentor's laboratory, were crossed with wildtype C57BL/6 mice to generate E06-scFv mice. All mice were housed at the UCSD pathogen-free animal facility, and all animal experiments were performed under the Guide for Care and Use of Laboratory Animals of the National Institute of Health. The protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of UCSD.

Quantification of fibrogenic gene expression by quantitative Real-Time PCR: Bleomycin and OxPAPC were incubated with MH-S cells in dose and time-dependent manner. Bleomycin- and OxPAPC-treated cells were harvested and lysed at various time points. Total RNA was extracted from cell lysate using Nucleospin RNA columns (740955, Clontech), and reverse-transcribed into cDNA using RNA and cDNA EcoDry (639543, Clontech) following the manufacturer's protocol. cDNA was mixed with 2X KAPA SYBR FAST Universal qPCR kit (KK4602, KAPA Biosystems), and primers of interest from

Integrated DNA Technologies (IDT). RT-PCR was performed with a Rotor Gene Q machine (Qiagen). All gene expression data of RT-PCR shown is normalized to housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and shown as a fold change with the control normalized to 1.

Measurement of OxPLs in cell lysate by enzyme-linked immunosorbent assay (ELISA): A competitive chemiluminescent ELISA was used to measure the relative levels of OxPLs in the cell lysate of MH-S (alveolar macrophages). 3ug/ml of E06-IgM antibody (330001 Avanti Polar Lipids Inc) was precoated in the 96-well microplates overnight at 4°C, and the plates were washed 3 times with PBS. Then 1% TBS/BSA was used for blocking for 1hr at room temperature. Cell lysate (1:3) was then added into the wells and incubated for 1hr at room temperature, followed by addition of 3ug/ml PC-KLH (PC-1013-5, LGC Biosearch Technologies) for 1hr at room temperature. Alkaline phosphatase-conjugated anti-KLH antibody (source 600-405-466 Rockland Inc) was added for 1hr at room temperature to the plates to bind to the bound PC-KLH, then Lumi-Phos 530 was added to detect the anti-KLH antibody on the plates. Each incubation before anti-KLH was added was followed by 3 washes with TBS, and anti-KLH was washed 4 times with TBS. Finally, data were collected as relative light units (RLU) collected over 100 ms (RLU/100ms) with a Synergy HTX Multi-Mode Chemiluminescent Plate Reader (BioTek, VT). A standard curve of PC-KLH without cell lysate was run in parallel for calculation of relative concentration of immunodetectable OxPLs in the cell lysate samples. Triplicates were used for all samples.

Quantification and Statistical Analysis: All data are shown as mean ± SEM. Numbers of replicates are indicated in figure legends. When comparing two sets of data, two tailed t-test was used to determine if there is any statistical significance between them and $P<0.05$ was considered statistically significant for all tests.

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