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Secretion of Leukotrienes by Senescent Lung Fibroblasts Promotes Pulmonary Fibrosis

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Conflict of Interest disclosure

Alexis N. Brumwell, Sonnet S. Davis, Julia R. Jackson, Cheresa Calhoun, Fatouma Alimirah, Richard Riu, Ying Wei: No conflict

Christopher D. Wiley, Arvind Ramanathan, and Judith Campisi are inventors on a patent application for eicosanoids as biomarkers of senescence.

Alexis Valdovinos: is an employee at Unity but at the time of her contribution to this work was an employee in Dr. Campisi’s laboratory. There is no conflict of interest with the current manuscript.

Harold A. Chapman: Scientific founder of Pliant Therapeutics, which develops anti-fibrotic drugs. He owns >10,000 shares of stock. There is no conflict of interest with the current manuscript.

Judith Campisi: Scientific founder of Unitiy Biotechnology, which develops senolytic and other drugs to combat aging. She owns >10,000 shares of stock and Unity funds a small basic research project in her lab, which is not covered in this manuscript. She is also named on several planned, pending and awarded patents on the use of small molecules to eradicate senescent cells. There is no conflict of interest with the current manuscript.

Claude Jourdan Le Saux: In the last 12 months, has been consultant at Unity Biotechnology and Gordian. There is no conflict of interest with the current manuscript.
Abstract

Accumulation of senescent cells is associated with the progression of pulmonary fibrosis but mechanisms accounting for this linkage are not well understood. To explore this issue, we investigated whether a class of biologically active profibrotic lipids, the leukotrienes (LT), is part of the senescence-associated secretory phenotype. The analysis of conditioned medium (CM) lipid extracts and gene expression of LT biosynthesis enzymes revealed that senescent cells secreted LT regardless of the origin of the cells or the modality of senescence induction. The synthesis of LT was biphasic and followed by anti-fibrotic prostaglandin (PG) secretion. The LT-rich CM of senescent lung fibroblasts (IMR90) induced pro-fibrotic signaling in naïve fibroblasts, which were abrogated by inhibitors of ALOX5, the principal enzyme in LT biosynthesis. The bleomycin-induced expression of genes encoding LT and PG synthases, level of cysteinyl leukotriene in the bronchoalveolar lavage, and overall fibrosis were reduced upon senescent cells removal either in a genetic mouse model or after senolytic treatment. Quantification of ALOX5⁺ cells in lung explants obtained from Idiopathic Pulmonary Fibrosis (IPF) patients indicated that half of these cells were also senescent (p16INK4a⁺). Unlike human fibroblasts from unused donor lungs made senescent by irradiation, senescent IPF fibroblasts secreted LTs but failed to synthesize PGs. This study demonstrates for the first time that senescent cells secrete functional LTs, significantly contributing to the LTs pool known to cause or exacerbate IPF.
Introduction

The development of fibrosis is a frequent complication of a number of chronic lung diseases including Idiopathic Pulmonary Fibrosis (IPF), and contributes to their morbidity and mortality. Characterized by an irreversible destruction of their lung architecture associated with a progressive accumulation of fibrotic tissue, the median survival for IPF patients is 3-5 years from diagnosis (1). Indeed, no effective therapies are available to stop the progression of fibrotic tissue remodeling despite two FDA-approved therapeutic options (1). The underlying pathobiology is thought to be due to repetitive injuries and the inability of the epithelium to repair. Although once not considered critical to IPF initiation or progression, inflammation, especially macrophage accumulation, has re-emerged as a potentially important contributor to fibrosis progression (2, 3). A recent study showed that severe innate and adaptive inflammatory infiltrates were detected in IPF patients and were predictive of the disease progression (4). In addition to mediators derived from activated epithelial and immune cells, recent data implicate senescent and necroptotic cells as sources of those proinflammatory mediators [5].

For some time, senescence has been thought to contribute to the pathology of IPF without strong supportive data. However, accumulating evidence links the extent of senescent cells in fibrotic tissue with the severity of IPF (5). Independent studies show that markers of cellular senescence (p53, p21\textsuperscript{WAF}, and p16\textsuperscript{Ink4a}) are upregulated in type II alveolar epithelial cells (AECIIs) in IPF lungs within the remodeled fibrotic areas (5-7). The presence of relatively short telomeres which can lead to cellular senescence, was reported in AECIIs and fibroblasts in IPF lungs (8, 9). Recent findings indicate that senescent fibroblasts secrete PDGF receptor ligands supporting their role in fibrosis (10). Furthermore, limiting the number of these senescent cells is beneficial in controlling the extent of remodeling in experimental models of lung injury (11-15). However, the mechanism(s) by which senescent cells contribute to the pathobiology of IPF
remain incompletely understood. Many deleterious effects of senescent cells result from the secretion of myriad biologically active proteins, including inflammatory cytokines and chemokines, matrix metalloproteinases and growth factors (16, 17). This senescence-associated secretory phenotype (SASP) has been characterized primarily in the context of secreted proteins. However, the expression of 5-lipoxygenase (ALOX5) and cyclooxygenase (COX2) are reported in senescent fibroblasts, raising the possibility that biologically active signaling lipids could be derived from senescent cells (18, 19). Eicosanoids are divided in several families, of which the most relevant for fibrosis are the leukotrienes (LT) and prostaglandins (PG). Cyclooxygenase-2 (COX2), the rate limiting enzyme in prostaglandin (PG) biosynthesis, is highly up-regulated during both normal and stress-induced fibroblast senescence (18). However, secretion of the anti-fibrotic prostaglandins has been found to be low in fibrotic models and IPF (20-23). Indeed, IPF-derived cultured fibroblasts failed to increase COX2 protein expression or COX activity in response to stimuli in contrast to normal lung fibroblasts (24). Additionally, the leukotrienes (LTs), a second class of biologically active signaling lipids, can modulate diverse physiological responses, including inflammation and fibrosis (25-27). To expand our knowledge of the SASP and its relevance to fibrosis, here we investigate LT and PG secretion by senescent cells.
Results:

Senescent fibroblasts secrete leukotrienes as part of their SASP

We first investigated whether a class of biologically active signaling profibrotic lipids, namely the leukotriene (LT) family, are part of the SASP. We interrogated the status of cytosolic phospholipase A2 (cPLA2), the major enzyme that releases arachidonic acid from the plasma membrane (28). cPLA2 is activated by phosphorylation on serine 505 by p38MAPK (29), which is activated in senescent cells (30). As expected, irradiated lung fibroblasts confirmed to be senescent by p21\(^{WAF1}\) expression (Figure 1A) showed activation (phosphorylation) of p38MAPK and cPLA2 (Figure 1B). Because p38MAPK signaling often culminates in increased transcription, we used quantitative PCR to measure the abundance of mRNAs encoding genes that specifically participate in eicosanoid synthesis. Compared to quiescent cells, senescent cells expressed higher levels of several of these genes (Figure 1C), including those encoding LT synthases, including ALOX5 (5-LO), ALOX12, ALOX15, ALOX5AP, LTC4S and LTA4H, and ALOX15B (Figure 1B). To confirm the activation of the biosynthesis of LT in the senescent fibroblasts, increased level of ALOX5 synthesis and activation as showed by a significant increased phosphorylated ALOX5 (6.5-fold increased) were measured in senescent cells compared to quiescent cells (Figure 1D). In addition, a significant 3-fold increase of cysteinyll leukotriene level was measured in conditioned medium (CM) from senescent fibroblasts and significantly inhibited in the presence of the ALOX5 inhibitors BW-B70C (BW) or zileuton (Zil) (Figure 1E). Additionally, in agreement with our RNA data, lipid extracts from senescent cells showed elevated levels of LTB4 (Figure 1F). A time course revealed the complex dynamics that govern the expression of eicosanoids synthesis genes during the senescence response as exemplified by the time course expression of LT synthases (ALOX5 and LTA4H) and prostaglandin (PG) synthases (COX2 or PTGS2 and PTGES). The LT expression was biphasic, showing a large increase, 2 days after irradiation, followed by a decline and a smaller peak of expression 10-20 days following irradiation (Figure 1G). Interestingly, in the late phase of
senescence between 15-20 days following irradiation, PG biosynthesis enzymes including COX2 (PTGS2) were increased (Figure 1H).

**Leukotriene expression is a widespread component of the SASP**

To determine whether the cell origin or the mode of senescence-induction affected the LT biosynthesis activation, we also measured cysteinyi leukotriene levels in human umbilical vein cells (HUVEC) and human liver carcinoma cells (HEPG2). We cultured the cells under standard conditions specific to each cell lines. A 3-fold and 2-fold increase in LT expression was in measured in irradiated HUVEC and HEPG2 cell lines, respectively (Figure 2A).

We induced senescence in IMR90 fibroblasts following different modality: mitochondria dysfunctional associated senescence (31), exposing them to a relatively high dose (10 Gy) of ionizing radiation (γRA [γ-irradiation]), oncogene induced (Ras), and oxidative stress (bleomycin). Each of these modalities was able to promote the secretion of LTs either evaluated by the measurement of cysteinyi leukotriene in the CM (Figure 2B) or the activation of the expression of enzymes involved in the biosynthesis of LT (Figure 2C). Interestingly, these senescent cells also expressed enzymes related to the PG pathway (Figure 2D). Bleomycin-induced senescence was confirmed by the increased expression of p16−ink4a and p21−Waf1 mRNA (Figure 2E). Thus, the secretion of LT appears to be a feature of the SASP independently of the cell type or the modality of senescence induction.

**Senescent fibroblasts promote fibrosis by secreting leukotrienes as part of their SASP**

Since the enzymatic machinery leading to the biosynthesis of LTs and PGs is activated and cysteinyi leukotriene is released in senescent fibroblasts, we sought to test whether the secreted LT and PGs had a functional effect on naïve fibroblasts. We generated conditioned media (CM) from control (Day 0) or senescent cells, 2 (2-day CM) or 20 days (20-day CM) after irradiation to determine whether the secreted LTs and PGs from senescent cells could alter
fibrotic responses. Prior to collecting the CM, the senescent cells were treated with NS-398 to inhibit PG biosynthesis or zileuton (Zil) to prevent LT biosynthesis. Naïve non-senescent IMR-90 fibroblasts were then treated with the CMs and the activation of COL1A2 and αSMA gene expression was assessed as surrogates for the evaluation of fibrotic response (Figure 3A). The upregulation of COL1A2 mRNA level measured after treatment with 2-day CM was prevented with 2-day CM from the senescent cells treated with Zil (2-day + Zil CM). Further, upregulation of COL1A2 mRNA was lower using 20-day CM consistent with declined expression of LT in the 20-day post irradiation SASP. Treatment of the naïve fibroblasts with 2-day or 20-day CM + NS-398 (2-day + NS-398 or 20-day + NS-398 CM) did not affect the upregulation of COL1A2 mRNA expression (Figure 3B). Similarly, the expression of smooth muscle actin (αSMA, Acta2), a marker of myofibroblast differentiation, was upregulated in the presence of the 2-day CM and significantly reduced after treatment with 20-day CM. The downregulation of αSMA mRNA expression was abrogated if the naïve fibroblasts were treated with 20-day + NS-398 CM, suggesting that the decline of αSMA expression was in part controlled by the released of prostaglandins by the senescent cells (Figure 3C). However, αSMA mRNA expression was not affected by CMs +Zil, suggesting the upregulation of αSMA mRNA was independent of LT expression. Of note, TGF-β mRNA levels were not upregulated in irradiated senescence cells and treatment with LT inhibitors did not change its expression (Figure 5D). It is, therefore, unlikely that the pro-fibrotic effects are related to the presence of TGF-β in the SASP.

Since TGF-β is the master regulator of fibrosis, we also treated the naïve fibroblasts with TGF-β and the CMs. The level of COL1A2 mRNA expression was comparable when the naïve fibroblasts were co-stimulated with 2-day CM or with 2-day CM + TGF-β; and inhibition of LT synthesis by Zil prevented the collagen expression induction using 2-day CM + TGF-β + Zil (supplemental Figure 1B). The presence of TGF-β was required to maintain collagen
expression when treated with 20-day CM (supplemental Figure 1B). Treatment with TGF-β alone did not affect αSMA mRNA expression. Twenty-day CM was less effective at inducing αSMA mRNA expression than 2-day CM, but treating senescent cells with 20-day CM + 2-day CM + TGF-β + NS-398 prevented the downregulation of αSMA mRNA expression (supplemental Figure 1C), consistent again with findings that prostaglandins antagonize myofibroblast activation (32, 33).

Because LTs trigger cellular signaling through membrane-bound G-protein coupled receptors (GPCRs), we then profiled known LT receptors in naïve quiescent fibroblasts to determine which type was the most likely to trigger the pro-fibrotic responses in naïve fibroblasts. We first detected the mRNA expression of several LT receptors (Supplemental Figure 1A). Among all the ones we tested, the LT B4 receptors B1 and B2 (BLT1, BLT2), the G-protein receptors 99 and 17 (GPR99, GPR17), the adenosine diphosphate (ADP)–reactive purinergic (P2Y<sub>12</sub>) receptor, and the cysteinyl leukotriene receptors 1 and 2 (CYSLTR1, CYSLTR2) were expressed. This suggests that the naïve fibroblasts were capable to responding to multiple members of the LT family. To investigate the requirement of any of the expressed receptors in mediating profibrotic effects induced by the SASP LTs, we used pharmacological antagonists LT receptors LY255283 (a selective, competitive BLT<sub>2</sub> receptor antagonist) and Montelukast sodium (CysLT<sub>1</sub> and GPR17 antagonist). We generated CM from control (Day 0) or senescent cells, 10 days after irradiation and treated naïve fibroblasts for 48hrs with CM and LT receptor antagonists (Figure 4A). The activation of COL1A2 gene expression was assessed (Figure 4B). Treatment with vehicles alone didn’t affect the level of expression of COL1A2 gene as expected. Decreased COL1A2 gene expression was only measured when the cells were treated with 10-day CM and Montelukast sodium, in agreement with reports that Montelukast antagonizes bleomycin-induced lung fibrosis in mice (34).
To assess whether senescence-associated LTs influences the expression of other SASP factors, senescent fibroblasts were continually treated with the ALOX5 inhibitors, Zil or BW-B70C. After 10 days, as expected, the level of expression of pro inflammatory factors known to be part of the SASP (IL8, CXCL1, IL6, IL1b, IL1a, CCL2, and VEGF) were upregulated in irradiated senescent cells compared to controls. Treatment with the ALOX5 inhibitors did not affect or moderately decreased the expression level of these genes, suggesting that the LTs secreted by senescent cells have little to no effect on the expression of other pro-inflammatory factors of the SASP (Figure 5A). Similarly, induction of pro-fibrotic factors such as SERPINE-1, TIMP1, COL1A2, and FIBRONECTIN (FN1) mRNA were unchanged by the presence leukotriene inhibitors (Figure 5B). Furthermore, mRNA expression of receptors for LTB4 (LTBR2) and cysteiny1 leukotrienes (CYSLTR2) was lost from senescent fibroblasts (Figure 5C), suggesting that leukotrienes produced by senescent cells are most likely to have paracrine effects rather than autocrine ones.

We also investigated whether senescence-associated LTs promoted senescence in naïve fibroblasts by assessing p16 mRNA expression in these cells after 48 hrs of treatment with CM collected from 10-day post irradiated fibroblasts (supplemental Figure 1D). No change in expression was measured and therefore our data would not support a paracrine effect for LT in the induction of senescence.

Taken together, these data support a model in which early leukotriene synthesis by senescent cells stimulates collagen synthesis and fibrosis, but not senescence, by a paracrine mechanism, whereas the later prostaglandin synthesis by senescent cells antagonizes myofibroblast differentiation and collagen expression. In addition, among the senescence-associated LTs, our data indicate that naïve fibroblasts could respond to any member of the LT family based on the expression of various receptors. However, based on treatment with antagonists, it is more likely
that the cysteiny1 leukotrienes LTC4 and LTD4 contribute to the profibrotic effect rather than the non-cysteiny1 leukotriene LTB4.

**Senescent cells release leukotrienes in the lungs and contribute to fibrosis**

To determine whether senescence-associated LT and PG biosynthesis occurs *in vivo*, and to be consistent with our *in vitro* data using lung fibroblasts, we asked whether senescent cells were responsible for LT-driven disorders in the lungs. LTs were previously shown to significantly contribute to fibrotic responses in the lungs (21, 35-37). Using the classical bleomycin-induced fibrosis model, wild-type C57Bl6/J mice were subsequently treated with vehicle or ABT-263, a BCL2/BCL-XL/BCL-W inhibitor that selectively eliminates senescent cells in some tissues (38, 39). *p16^{ink4a}* and *p21^{Waf1}* RNA levels increased 14 days after bleomycin injury and were significantly reduced by ABT-263 (Figure 5A).

To assess whether the eicosanoid biosynthesis was affected by the senolytic treatment with ABT-263, we first measured the activation of cPLA2 by determining the level of expression of its phosphorylated form by western blot. We measured a significant 1.5-fold expression increased in the bleomycin-injured mice that was reduced by ABT-263 (Figure 5B). Subsequently, the level of mRNA expression of enzymes, *ALOX5*, *LTC4S*, *PTGS2*, *PTGDS*, AND *PTGES*, involved in eicosanoid biosynthesis was also measured. Their respective mRNA levels were increased in the lungs of bleomycin-injured mice, and elimination of senescent cells with ABT-263 lowered these levels to those of control animals (Figure 5C). Consequently, bleomycin increased the levels of both cysteiny1 leukotrienes and prostaglandin E2 in the bronchoalveolar lavage fluid (BALF) 21 days after injury, and ABT-263 reduced both levels (Figures 5D). The removal of senescent cells was also significantly associated with an attenuation of collagen content assessed by histological staining with picrosirius red (Figure 5E) and measurement of
hydroxyproline (Figure 5F), 21 days after injury. Furthermore, ABT-263 treatment attenuated the increase in collagen mRNA (Col3a1 and Col4a1, Supplemental Figure 2).

To confirm that the attenuation of cysteinyll leukotriene levels in BAL after ABT-263 treatment was the result of deletion of p16\textsuperscript{ink4a+} cells, we undertook two separated approaches. First, as one of the major cell populations secreting LTs, CD45+ cells were isolated from bleomycin-injured lungs 14 days after injection, and treated with ABT-263 for 48 hrs. Our data indicate that the level of expression of LT or PG biosynthesis enzymes in CD45+ cells was not directly affected by ABT-263 treatment (Figure 7A). In addition, the percentage of CD45+ cells in the lungs 14 days after bleomycin injury were not affected by ABT-263 (Figure 7B). Second, p16\textsuperscript{ink4a-}3MR mice were injured with bleomycin then treated with ganciclovir (GCV). p16\textsuperscript{ink4a-}3MR mice contain a transgene that permits the detection and selective killing of p16\textsuperscript{ink4a+}-positive senescent cells by administering an otherwise innocuous drug (GCV) (10). Our data indicate that deletion of p16\textsuperscript{ink4a+} cells resulted in reduced collagen deposition as well as reduced level of cysteinyll leukotriene in the BAL of the GCV treated injured p16\textsuperscript{ink4a-}3MR to a similar level as the level after ABT-263 treatment (Figure 7C, 7D, and 7E).

These data indicate that removal of senescent cells is associated with both reduced eicosanoid biosynthesis and collagen deposition, consistent with the hypothesis that the senescent cells are an underlying driver of bleomycin-induced pulmonary fibrosis due to the secretion of LTs as part of their SASP or by inducing LT expression in other cells via a paracrine effect.

An interesting feature of the bleomycin-induced pulmonary fibrosis mouse model is that it resolves over time (40, 41), suggesting that either senescent cells are eventually cleared or the fibrosis-promoting properties of senescent cells change over time. We therefore analyzed p16\textsuperscript{ink4a} and collagen (COL1A2) mRNA levels over a 42-day period following bleomycin-induced
injury. Notably, although $p16^{ink4a}$ expression increased over 14 days post-injury, peaked 30 days after injury and remained elevated through day 42 (Figure 7F, green line), collagen expression peaked at 14 days after injury but thereafter progressively declined (Figure 7E, orange line). These data suggest that the fibrosis-inducing properties of senescent cells change over time. Because we observed an early rise in LT synthases expression (Figure 1F), followed by a later rise in PG synthases expression (Figure 1G) in cultured cells, we test whether a similar pattern develops in vivo. Indeed, bleomycin injury caused an early spike in whole lung ALOX5 expression (Figure 7G), followed by a progressive rise in the expression of PTGDS, which synthesizes PGD2 (Figure 7G), consistent with a switch in senescent cell phenotype from pro-fibrotic to anti-fibrotic.

**Senescent IPF fibroblasts synthesize leukotrienes but fail to produce prostaglandins**

Previous reports indicate that fibroblasts from IPF patients fail to elevate prostaglandin synthesis (42), or fail to respond to prostaglandin treatment (43). To determine whether senescent IPF fibroblasts synthesize LTs and PGs in a similar fashion to senescent normal lung fibroblasts, we compared mRNA levels of ALOX5, PTGS2, PTGDS, and PTGES in control and radiation-induced senescent fibroblasts isolated from normal and IPF lungs. As expected at baseline, IPF lung fibroblasts expressed high level of ALOX5 mRNA compared to normal lung fibroblasts. However, even though ALOX5 mRNA expression increased upon irradiation-induced senescence in both cell types, IPF fibroblasts still expressed significantly higher level of ALOX5 mRNA (Figure 8A). Compared to non-irradiated cells, senescent normal lung fibroblasts increased expression of mRNA of the PG synthases PTGS2 and PTGD. This induction of PG biosynthesis enzymes expression is also measured in senescent IPF fibroblasts but to a much lesser extend (Figure 8A). These data suggest that IPF patients respond differently to senescence-inducing stimuli with regard to PG synthesis. Senescent cells as defined as $p16^{ink4a}$ cells, ALOX5$^+$ cells, and dual positive cells are detected in IPF lung in greater number
than in normal lungs (Figure 8B, C). Overall, the $p16^{ink4a+}$ cells represented an average 30% of total cells in the IPF lungs, of which 50% expressed ALOX5 (Figure 8C). Because several cell types, mainly type II epithelial cells and fibroblasts, have been previously reported to undergo senescence in IPF lungs (44), we co-stained ALOX5+ cells with marker for type II epithelial cells (surfactant protein C) and for fibroblasts (vimentin). Very few ALOX5+ cells, less than 3%, were detected in the SPC+ population (Figure 8D,E). However, $23.3 \pm 5.7\%$ of ALOX5+ cells also co-stained for vimentin (Figure 8D, E). Despite this staining, we did not detect the expression of ALOX5 in myofibroblast cells as defined as $\alpha$-SMA+ (data not shown), indicating that the producers of leukotrienes are a distinct population of lung fibroblasts. Together, our experiments point to senescent lung fibroblasts as a key source of profibrotic leukotrienes during lung fibrosis.

**Discussion**

The contributing role of senescent cells to physiological or pathological processes is mainly attributed to the release of secreted factors grouped as senescence-associated secretory phenotype or SASP. Thus far, the SASP has been defined as a complex set of secreted proteins that are variable depending on the cell type and driver of senescence (10, 16, 45). Here we show that senescent cells also synthesize a number of eicosanoids and in particular LTs. The expression of LTs appears to be ubiquitous regardless of the modality of senescence induction and to some extent of the cell types when tested in vitro. However, the pattern of eicosanoid expression, as reflected by dynamic changes in the mRNA levels of ALOX5 and COX2, is a time-dependent process (Fig 1). It is commonly accepted that the phenotype of senescent cells is highly heterogeneous depending upon specific stresses and cell types (46). More recently, the transcriptomic signature of fibroblasts, melanocytes, and keratinocytes were obtained 4, 10 and 20 days after ionizing radiation. Hernandez-Segura et al. demonstrated that
among the 55 genes of the senescence core signature that they have previously identified, 13 genes were differentially regulated at every time point and in every cell type (46). In this study, the expression of biosynthetic enzymes of the LTs and PGs is clearly time-dependent. This suggest that the regulation of the expression of SASP factors is very tight. These findings emphasize the need to include time as a variable when studying the relevance and the role of senescent cells in any pathology. As LTs are released, they moderately modify the expression of some but not all SASP elements. In parallel, LT receptors expression declined over time, suggesting an autocrine control from SASP factors. Overall, dynamic changes in eicosanoid expression over time superimposes another layer of complexity on the evolution of the SASP. This complexity may explain some of the disparate findings on the physiological impact of senescence on normal and pathological processes, including the controversial role of senescent cells in fibrosis. Senescent cells appear to have a negative impact in lung fibrosis (Fig 5; (5, 7, 13-15, 47)) in contrast with a positive effect in CCL4-induced liver fibrosis or in wound healing (10, 48). And this complexity underscores the challenge in developing senolytics as therapeutic agents.

In a recent model of skin wound healing, senescent fibroblasts were detected 3-6 days after injury. Interestingly, these senescent fibroblasts were found to be critical to normal wound healing as their deletion via the p16-3MR transgenic system delayed wound repair (1). In other contexts, however, the induction of a SASP could be expected to promote tissue injury. In healthy older individuals, for example, protease secretion by senescent cells is believed to facilitate the enlargement of airway spaces in a mechanism similar to tobacco-related emphysema (49). These temporal changes of the SASP could also explain the deceptive contradiction in the role of senescent cells in wound healing and fibrosis. It has been already demonstrated that the removal of senescent cells in various animal models using genetic mouse models or pharmacology intervention alleviates the development of fibrosis (13, 38, 39, 47).
As senolytic therapeutic approaches emerge, it is important to better understand these dynamic and temporal changes of the SASP as the role of the senescent cells may change during the course of the disease.

LTs and PGs have been implicated in the pathobiology of pulmonary fibrosis for over two decades (27). LTs synthesis via 5-lipoxygenase (ALOX5) is constitutively activated in IPF (50), and genetic deletion of ALOX5 attenuates development of fibrosis in a murine model of lung injury (35). Conversely, inhibition of PG synthesis via either genetic deletion or inhibition of cyclooxygenase 2 (COX2) exacerbates the development of pulmonary fibrosis in mice (22), and PGE2 and COX2 levels decline in patients with IPF (24, 51, 52). Notably, genetic deletion of ALOX5 not only results in loss of leukotriene synthesis following lung damage, but also results in elevation of PGE2 levels (35). The source of the LTs has been believed to be mainly macrophages, however in the recent years other cells have emerge as “inflammatory cells” and among them the senescence cells. Quantification of the p16\(^{ink4a}\)/ALOX5 double positive cells in IPF tissues as roughly 50% of all the ALOX5 cells and their morphological distinction from macrophages indicates a significant contribution of senescent structural cells to the eicosanoid profile of IPF lungs (Fig 7). Here, we demonstrate that removal of senescent cells using ABT-263 that targets three of the essential pro-survival players, BCL-2, BCL-W and BCL-XL, is, not only, associated with reduced fibrosis, but also suppression of the leukotriene biosynthesis enzyme expression and secretion in the BAL of cysteiny1 leukotrienes. It remains to be determined if senolytics would have similar effects in IPF patients.

Evidence presented in our data (Figs 2-6) indicate senescent cells may contribute to the development of fibrosis due to the secretion of LTs. Naïve fibroblasts expressed a wide variety of LT receptors. However, our data indicate that the pro-fibrotic effects of the LT-enriched SASP may be associated with the presence of cysteiny1 leukotrienes in the SASP and the expression of CysTL1/L2 on the surface of the responding cells. This receptor/ligand combination observed
here is not really surprising as the role of LTC4 and LTD4 as well as the CysTL1/L2 receptors in the regulation of collagen expression by fibroblasts is well-established and characterized (36, 53). Senescence-associated LT synthesis promoted the expression of pro-fibrotic genes implicated in pulmonary fibrosis in naïve fibroblasts (Fig 3). However, over time, the composition of the LTs and PGs in the SASP evolve from being profibrotic to anti-fibrotic in vitro (Fig 3 and 4). This evolution in vitro is mirrored by the pattern of eicosanoid expression over time in vivo in the bleomycin model, a timing that corresponds well with the ultimate resolution of the fibrosis in the lungs. If these associations are causal, then there are implications for IPF. Our data suggest that IPF progression might result in part from an altered senescence response – one that promotes fibrosis via LT release but does not resolve because of the inability of senescent IPF fibroblasts to express COX2 and secrete anti-fibrotic PGs. The inability of IPF senescent fibroblasts to secrete prostaglandins could explain at least in part why the maintenance of senescent cells in the lungs is associated with increased fibrosis whereas in other organs such as the skin or liver, the senescent cells eventually express prostaglandins and attenuate fibrosis.
Materials and Methods:

**In vitro** studies

Human fetal lung fibroblasts (IMR-90, American Tissue Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% FBS and penicillin/streptomycin. Human umbilical vein cells (HUVEC, ATCC) and human liver carcinoma cells (HEPG2, ATCC) were cultured based on manufacturer’s instructions. Normal and IPF fibroblasts were explanted from tissue provided by UCSF Interstitial Lung Disease Blood and Tissue Repository. Quiescence was induced after the cells reached confluence by replacing culture media with media containing 0.2% FBS. Cells were cultured at 3% O₂, and were mycoplasma free. Inhibition of eicosanoid biosynthesis was performed by adding to the serum free media, DMSO (vehicle control), NS-398 (1 μg/mL, Cayman Chemical, Ann Arbor, MI), BW-B70C (10 μM, Cayman Chemical), or zileuton (50 μM, Cayman Chemical). Inhibition of leukotriene activity was performed by supplementing CM with LY 255283 (50 nM, Tocris) or Montelukast sodium (25 nM, Tocris).

**Senescence induction:** Ionizing Radiation: Senescence was induced by 10 Gy X-irradiation. Non-senescent controls (proliferating or quiescent) were placed in the irradiator for an identical period of time but without irradiation (Mock controls). Mitochondrial dysfunction associated senescence (MiDAS): MiDAS was induced by depleting mitochondrial DNA in the presence of 100 ng/mL Ethidium Bromide 100 ug/ml sodium pyruvate, and 50 ug/ml uridine for 2 months, followed by removal of pyruvate, as described in (31). Bleomycin-induced senescence: Fibroblasts were treated with bleomycin (50 μg/ml, Calbiochem, Burlington, MA) for 3 hr. Bleo treatments were administered at atmospheric oxygen. After 3 hours, cells were washed and media was replaced with bleomycin-free media, and cultured at 3% O₂ for 8 days before analysis. Oncogene-induced senescence was induced using lentiviral-mediated overexpression of HRASV12, as described (31).
**Generation of conditioned media:** Conditioned media (CM) were generated by culturing cells in serum-free DMEM supplemented with penicillin/streptomycin and eicosanoid inhibitors for 24 h before harvest either 2 or 20 days after irradiation. Day 0 cells were sham irradiated. Where indicated, CM were supplemented with 5 ng/mL TGF-β or carrier (BSA). 200,000 cell equivalents/ml of CM +/- TGF-β was used to stimulate serum-starved naïve non-senescent IMR-90 fibroblasts for 24 h prior to isolation of RNA.

**Gene expression analysis:** RNA was extracted from cells or tissues using commercially available kits (Isolate II - Bioline for cells; Direct-zol - Zymo for tissues) according to the manufacturer’s instructions. cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher) according to the manufacturer’s instructions. Quantitative PCR was performed on a LightCycler 480 II (Roche) using primers and probes designed for the Universal Probe Library and https://pga.mgh.harvard.edu/primerbank/ (see list on supplemental material and methods).

**Level of expression of protein:** Detection of the activation of cPLA2, p38, and ALOX5 by immunoblot: Cells were lysed in 5% SDS in 10 mM Tris, pH 7.4, and protein content determined by BCA assay. 20 μg protein was separated by electrophoresis and transferred to PVDF membranes. Membranes were blocked in TBST + 5% BSA, incubated overnight with primary antibody, washed in TBST, incubated with HRP-conjugated secondary antibody for 30 min, and visualized by chemiluminescence. Densitometry was quantified using NIH ImageJ software. List of antibodies is cPLA2 polyclonal antibody (#2832), phospho-cPLA2 polyclonal antibody (#2831), p38 polyclonal antibody (#9212), ALOX5 monoclonal antibody (C49G1, #3289), phospho-ALOX5 polyclonal antibody (#3748), and were purchased from Cell signaling.
Phospho-p38 (#p190-1802), B-actin monoclonal antibody (#A5442, clone AC-15), and Tubulin (#ab6046) polyclonal antibodies were purchased from PhosphoSolutions, Sigma Aldrich, and Abcam, respectively.

**IL-6 ELISA.** 3x10⁴ cells in 12-well plates were treated as indicated, and cultured in 0.5–1 ml serum-free DMEM for 24 hr. CM were collected and clarified at 2,000x g for 10 min. Supernatants were transferred to a tube; cells were trypsinized and counted. CM (2.5 µl) were analyzed by bead-based ELISAs (AlphaLISA, Perkin-Elmer) as instructed by the manufacturer and normalized to cell number.

**Detection of leukotriene:** Cysteinyl leukotriene level was measured in the CM or BALF by ELISA (Cysteinyl Leukotriene, Cayman Chemical and Amersham Leukotriene C4/D4/E4 Biotrak, GE Healthcare) according to the manufacturer's instructions. The concentration of cysteinyl leukotriene in the CM was then normalized to the cell number. Leukotriene B4 levels were assessed in cell lysate by mass spectrometry as previously described (54).

**In vivo studies**
To induce pulmonary fibrosis, 8-10-week old male C57BL/6J or p16^{Ink4a}-3MR mice were given 2U/kg body weight of PBS (control group) or bleomycin (injured group) intratracheally. Bleomycin-treated animals showing less than 10% weight loss over the first 14 days were excluded from the study. Injured C57BL/6J animals were treated with ABT-263 (50mg/kg in 10% EtOH, 20% PEG-400, and 70% Phosal 40), or vehicle control for 7 d starting one-week post injury. Injured p16^{Ink4a}-3MR mice received daily one intra peritoneal injection of ganciclovir (25mg/kg/day in PBS) for 10 days, starting 10 days after bleomycin injury. Animals were euthanized either 14 or 21 days after bleomycin challenge. To collect brochoalveolar lavage fluid (BALF), 1 ml of PBS was injected intratracheally and approximately 0.8 ml was retrieved.
The BALF was centrifuged at 500 x g for 10 min. Lungs were collected and distributed as follows: left lung was used for hydroxyproline measurement or was embedded (paraffin or OCT); other lobes were used for RNA/protein extraction.

Assessment of pulmonary fibrosis. The extent of fibrosis indices in lungs was assessed with established markers (55). Collagen deposition was quantified using hydroxyproline measurements and picrosirius red staining and quantification. A) Hydroxyproline content was measured as described (55). The left lobe of the lung was homogenized briefly in 1 ml of water and incubated in 50% trichloroactic acid for 20 min. Samples were then hydrolyzed with 6N HCl at 110° C for 24 h, reconstituted in 2 ml of water and agitated at room temperature for 2 h. Samples were then mixed in equal parts chloramine T (1.4% in 0.5 M sodium acetate and 10% isopropanol) and Ehrlich’s solution (1M p-dimethylaminobenzaldehyde in 70% isopropanol and 30% perchloric acid) and heated at 65° C for 15 min. Absorbance was then measured at 550 nm. Standard curves were generated for each experiment using a hydroxyproline standard (trans-4-hydro-1-proline). Results are expressed as mg hydroxyproline/mL tissue extract. Five μm thick paraffin sections were layered on silane-coated slides, and stained with picrosirius red (PSR) to quantify parenchymal collagen deposition. The level of staining was assessed with ImagePro software v6.2 (Media Cybernetics, Inc. Bethesda, MD, United States) and expressed as a percentage of the total area of the image analyzed. To minimize variation, samples were processed under identical conditions. Five slides of the entire left lung per animal were quantified by an operator blinded to the treatments.

Isolation of CD45+ by flow cytometry. Mouse lung macrophages were isolated from PBS- or bleomycin- injured lungs 14 days after injection as previously described (56). Single cell preparations were incubated for 1 hr at 4°C with APC-Cy7 rat anti-mouse CD45 antibody
(1:1:00, Biolegend, #103116) in sort buffer (DMEM without phenol red plus 2% FBS (Gibco)). Cell were resuspended in sort buffer plus Sytox Blue (1:1000 Thermo S34857) and sorting was performed on BD FACS Aria cytometers. CD45+ were then cultured in DMEM+10% fetal bovine serum for 48 hr in presence of DMSO (vehicle control) or 10mM ABT-263. RNA extraction and qPCR were performed as previously described.

**Human tissues**

*Detection of senescent cells expressing ALOX5, SPC, aSMA, and p16\(^{ink4a}\) in human lung tissue:* As previously described (56), seven-μm thick lung cryosections were cut and incubated with 0.1% sodium borohydride (Sigma-Aldrich) in PBS to reduce aldehyde-induced background fluorescence for 3×10-min intervals, and subsequently blocked and stained in PBS plus 1% BSA (Affymetrix), 5% nonimmune horse serum, 0.1% Triton X-100, and 0.02% sodium azide. Subsequently, tissue sections were stained with rabbit anti-human ALOX5 (ab169788 abcam), goat anti-human SPC (clone C19, Santa Cruz), mouse anti-human aSMA (clone 1A4, Sigma), and mouse anti-human \(p16^{ink4a}\) (725-4793 Ventana) and. Mosaic pictures were taken randomly and over 2,000 cells (DAPI+ cells) per section were counted.

*Isolation of lung fibroblasts.* Distal lung tissue was obtained. Using sterile techniques, tissue was dissected into fragments of approximately 1 mm\(^2\). The tissue fragments were then incubated for 96 hr with DMEM+10% fetal bovine serum+ 1X antibiotic/antimycotic. Fibroblasts crawl out of the tissue fragments and attach to the plate. Pulmonary fibroblasts were then plated into 6-well plates, irradiated as previously described to induce senescence. Ten days after irradiation, total RNA was extracted and mRNA expression of LT and PG biosynthesis enzymes was analyzed by qPCR as previously described.
**Statistical analysis.** One-way ANOVA analyses were used for multiple comparisons. Comparisons between groups were performed using the 2-tailed student t-test, Mann-Whitney U test (for comparison of data sets with non-equivalent variances). Heat maps use p<0.05 for all entities. All data are presented as mean +/- SEM. All analyses were performed in GraphPad Prism 7.0. P value < 0.05 was considered statistically significant.

**Study approval.** All human tissue samples and primary fibroblasts from IPF and normal subjects were obtained from UCSF Interstitial Lung Disease Blood and Tissue Repository. They are classified as Non-identifiable Otherwise Discarded Human Tissues. Animal experiments were conducted using a protocol approved by the Institutional Animal Care and Use Committee of the Buck Institute for Research on Aging and UCSF.
**Author contribution**

Christopher D. Wiley: responsible for the study concept related to senescence and execution and interpretation of culture and leukotriene studies, cowrote the manuscript

Sonnet S. Davis: measured LT and PG levels in all samples

Julia R. Jackson: conducted p16<sup>ink4a</sup>-3MR study and hydroxyproline assay

Alexis Valdivinos: conducted in vivo experiments

Cheresa Calhoun: performed picrosirius red staining

Alexis N. Brumwell: conducted flow sorting, immunochemistry assays, and macrophage in vitro assays

Carlos A. Castellanos: assisted in the in vitro assays

Richard Rui: performed in vitro assays with LT antagonists and collect cell count data

Ying Wei: performed western blots

Harold A. Chapman: study design, data interpretation and cowriting of the manuscript

Arvind Ramanathan: supervised and interpret data from SSD

Claude J. Le Saux: responsible for study concept related to pulmonary fibrosis, conducted assays related to human samples, interpretation of in vivo studies, cowrote the manuscript

Judith Campisi: study design, data interpretation and cowriting of the manuscript
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Figures and Figure Legends
Figure 1. Senescent cells synthesize eicosanoids in a time-dependent manner.

Senescence was induced in human lung fibroblasts (IMR 90) using irradiation (10 Gy). Total RNA was isolated from mock irradiated, quiescent (QUI, cell cultured in 0.2%serum/DMEM) and irradiated cells after ten days of culture, reverse transcribed, and analyzed by quantitative (q)PCR. Signal was normalized to tubulin mRNA. A) Increased p21\textsuperscript{WAF1} mRNA level confirmed the induction of senescence in irradiated cells compared to mock irradiated ones. B) Proteins were extracted from quiescent (QUI) and irradiated senescent (SEN(IR)) cells and analyzed by Western blot for cPLA2 (phosphorylated on serine 505 or total cPLA2), p38MAPK (phosphorylated on threonine 180 or total), and tubulin (control). C) Panel of expression of genes encoding leukotriene synthesis enzymes. D) Lysates from quiescent (QUI) and 10 days post irradiation senescent IMR90 fibroblasts were blotted for ALOX5 (total and phosphorylated on serine 271). Quantification of Western blot bands were first normalized to B-actin and activation of ALOX5 is reported as the ratio p-ALOX5/ALOX5. E) After ionizing radiation, fibroblasts were treated with DMSO (vehicle) or the ALOX5 inhibitors zileuton (Zil, 50 µM) or BW-B70C (BW, 10µM) for ten consecutive days and conditioned medium (CM) was collected.
Levels of cysteiny1 leukotriene secreted in CM was measured by ELISA. F) Intracellular level of leukotriene B4 measured by ELISA. G) Time course expression of ALOX5 and LTA4H mRNA. H) Time course expression of PTGS2 or COX2 and PTGES mRNA. Data are presented as mean +/- SEM of at least 3 replicates. Statistical analyses were performed using Student t-test (A, C, D) or One-Way ANOVA (D) or Individual two-tailed unpaired student's t-test (E, F). ** = p-value $\leq 0.01$; *** = p-value $\leq 0.001$; **** = p$<0.0001$. 
Figure 2. Leukotriene secretion is a common feature of senescent cells. A) HUVEC and HEPG2 cells were cultured in DMEM + 0.2% serum (quiescence, QUI) or irradiated (10 Gy, SEN(IR)). The secretion of leukotrienes was assessed measuring the level of cysteinyl leukotriene by ELISA in the conditioned medium 10 days after irradiation. B) Level of cysteinyl leukotriene from IMR90 fibroblasts in which senescence was promoted by diverse inducers (MiDAS: mitochondrial dysfunction-associated senescence; irradiation: SEN(IR); Ras oncogene-induced: SEN (RAS) and compared to the level from quiescent (QUI) cells. C-E) IMR90 were treated with 50 μg/ml bleomycin for 3 hours. Total RNA was isolated, reverse transcribed, and analyzed by qPCR. Signal was normalized to tubulin mRNA. Activation of eicosanoid biosynthesis was assessed by measuring the level of expression of genes encoding leukotriene (C) and prostaglandin (D) biosynthesis enzymes. Senescence was assessed by measurement of increased expression p16^{INK4a} and p21^{WAF1} mRNA level (E). Data are presented as mean +/- SEM of at least 3 replicates. Statistical analyses were performed using Student t-test (A, C, D, E) or One-Way ANOVA test (B); * = p-value ≤ 0.05; ** = p-value ≤ 0.01; *** = p-value ≤ 0.001; **** = p-value ≤ 0.0001.
Figure 3: Leukotriene released by senescent cells induce pro-fibrotic responses from naïve fibroblasts in vitro. A) Irradiated human lung fibroblasts (IMR 90) were treated with the ALOX5 inhibitor zileutin (Zi, 50 μM) or the PTGS inhibitor NS-398 (1 μg/mL) for 24 hours prior to the conditioned medium (CM) collection. CM was collected 2 and 20 days after radiation and applied to non-senescent IMR90 fibroblasts for 48 hours. B-C) Total RNA was isolated, reverse transcribed, and analyzed by quantitative (q)PCR. Signal was normalized to tubulin mRNA. The profibrotic responses were assessed by the expression of COL1A2 (B) and α-SMA mRNA (C). Data are presented as mean +/- SEM of at least 3 replicates. Statistical analyses were performed using One-Way ANOVA test. * symbol represents the statistical differences calculated by time point. the different time of collection. NS = no statistical difference; * =p-value ≤ 0.05; ** =p-value ≤ 0.01.
Figure 4: Pharmacological Inhibition of the cysteinyl leukotrienes reduces the profibrotic effect of senescent cells on naïve fibroblasts in vitro. A) Schematic representation of conditioned media studies. The conditioned medium (CM) of senescent human lung fibroblasts (IMR 90) was collected 10 days after irradiation (10 Gy IR), LT antagonists LY255283 (LY, 50 nM) and Montelukast sodium (ML, 25 nM) or vehicle were added to the CM, and applied to non-senescent IMR90 fibroblasts for 48 hours. Total RNA was isolated, reverse transcribed, and analyzed by quantitative (q)PCR. Signal was normalized to β-actin mRNA. The inhibitory effect of LT antagonists was assessed by the expression of COL1A2 mRNA. B) Relative levels of COL1A2 mRNA in cells treated with CM supplemented with either LY (left) or ML (right). Data are presented as mean +/- SEM of at least 4 replicates. Statistical analyses were performed using unpaired Student t-test; * = p-value ≤ 0.05.
Figure 5: Inhibition of ALOX5 activity modulates the expression of pro-inflammatory SASP factors. Irradiated human lung fibroblasts (IMR 90) were treated with the ALOX5 inhibitor BW-B70C (BW, 10µM) for 10 days. Total RNA was isolated, reverse transcribed, and analyzed by quantitative (q)PCR. Signal was normalized to tubulin mRNA. Heatmap representation of A) the level of expression mRNA levels of pro-inflammatory SASP components and B) the level of expression mRNA levels of pro-fibrotic SASP components. C) Decreased mRNA expression of genes encoding LT receptors in senescent cells ten days after irradiation. Data are presented as mean +/- SEM of at least 3 replicates. Statistical analyses of A and B were performed using two-way ANOVA, and significance was determined for C by two-tailed student’s t-tests with Welch’s correction. ** =p-value ≤ 0.01; **** =p-value ≤ 0.0001.
Figure 6. Senescence-associated leukotriene synthesis promotes pulmonary fibrosis.

Wild type C57BL/6 mice received a single intratracheal injection of PBS (vehicle control) or bleomycin (Bleo, 1.9U/kg). Mice received from day 7 to day 14 vehicle (Veh) or 50 mg/kg/day ABT-263 by gavage. A) Level of p16\(^{INK4a}\) and p21\(^{WAF1}\) mRNA levels normalized to tubulin mRNA. B) Activation of cytosolic phospholipase A2 (cPLA2) was determined by calculating the ratio of the level of expression phosphorylated cPLA2 to the level of expression of total cPLA2. Representative pictures of western blot of lung lysates collected day 14 after the bleomycin injury for the expression of the phosphorylated form of cPLA2, cPLA2-S505P, and non-phosphorylated form cPLA2. n = 3 lysates per group. C) Level of expression of mRNA of gene encoding enzymes of the leukotriene (ALOX5 and LTC4S) and the prostaglandins (PTGDS, PTGS2, and PTGES) biosynthesis pathways was measured by qPCR normalized to tubulin mRNA, in samples collected 14 days after bleomycin injury. Data are presented as dot plot graphs or Heat map. D) Lipids were extracted from broncho-alveolar lavage fluid (BALF) collected 14 days after bleomycin injury and treatment with vehicle or ABT-263. BALF lipid content was analyzed for cysteinyl leukotrienes and for PGE2 by ELISA. E) Representative
pictures of picrosirius red staining of lungs collected 21 days after bleomycin injury and treatment with vehicle or ABT-263. F) Hydroxyproline levels obtained using the right lung lobes of mice, 21 days after bleomycin injury and treatment with vehicle or ABT-263. Unless stated otherwise, lung and BALF from at least 5 PBS-treated mice, 5 bleomycin + vehicle-treated mice, and 3 bleomycin + ABT-263 mice were analyzed. Statistical analyses were performed using One-Way ANOVA test. * means p-value ≤ 0.05; ** means p-value ≤ 0.01.
Figure 7. Temporal changes in eicosanoid biosynthesis reveal pro- and anti-fibrotic activities of senescent cells. A) Isolated CD45+ cells from 14-day bleomycin-injured lungs were treated with DMSO and 10µM ABT-263 for 48 hr. Analysis of mRNA expression of LT and PG biosynthesis enzymes was performed by qPCR. B) Analysis by flow cytometry of % of CD45+ cells isolated from 14-day bleomycin-injured lungs treated ABT-263 or vehicle for 7 days. C-D) p16^INK4a^−3MR mice received a single intratracheal injection of PBS (vehicle control) or bleomycin (Bleo, 1.9U/kg). B) Level of p16^INK4a^ mRNA levels normalized to tubulin mRNA. C) Hydroxyproline levels obtained using the right lung lobes of mice, 21 days after bleomycin injury. D) Lipids were extracted from broncho-alveolar lavage fluid (BALF) collected 21 days after bleomycin injury. BALF lipid content was analyzed for cysteinyl leukotrienes by ELISA. E-F) Whole lung tissues were collected 14, 21, and 42 days after PBS or bleomycin intratracheal injection for RNA extraction (7 mice per group) and analyzed by qPCR. E) mRNA levels of p16^INK4a^ (p16, green line) and collagen (COL1A2, orange line), normalized to tubulin mRNA. F) mRNA levels of ALOX5 (blue line) and PTGDS (green line) normalized to tubulin mRNA.
Statistical analyses were performed using Student t-test (A, B) or one-Way ANOVA (C, D, E) or Individual two-tailed unpaired student's t-test (E, F). ** =p-value ≤ 0.01; **** =p-value ≤ 0.001.
Figure 8. Human senescent fibroblasts express ALOX5 but not COX2 in IPF lungs. A) Primary fibroblasts isolated from normal and IPF lungs (n=2) were induced to senescence by ionizing radiation (10 Gy, IR). Total RNA was collected 10 days later, reverse transcribed and analyzed by qPCR for expression levels of ALOX5, PTGS2, PTGDS and PTGES mRNA, normalized to L19 mRNA. Data are presented as means ± SEM for 3 independent experiments. B) Representative pictures of immuno-detection of senescent cells (p16\(^{\text{ink4a+}}\)) co-expressing ALOX5 in normal and IPF lungs. C) Cell count of senescent p16\(^{\text{ink4a+}}\) cells and ALOX5+ cells detected by immunofluorescence in normal and IPF lungs. D) Representative pictures of immuno-detection of Type II epithelial cells (SPC\(^+\)) and fibroblasts (Vimentin\(^+\)) co-expressing ALOX5 in normal and IPF lungs. E) Cell count of senescent SPC\(^+\) cells, Vimentin\(^+\) cells, and ALOX5+ cells detected by immunofluorescence in normal and IPF lungs. n = 2 independent subjects per group. Each dot represents number of cells per field and bar graph represents means ± SEM. Statistical analysis was performed using one-way ANOVA Holm-Sidak’s multiple comparison test.
comparisons test (A) or unpaired Student t-test (C, E). * means p-value ≤ 0.05; *** means p-value ≤ 0.001; **** means p-value ≤ 0.0001.