Universidade Estadual de Londrina

STEPHANIE BADARÓ GARCIA

EFFECTS OF STANDARD OF CARE DRUGS AND RESOLVIN D2 ON CELLULAR SENESCENCE IN PULMONARY FIBROSIS

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Advisor: Prof. Dr. Waldiceu Ap. Verri Jr.
Co-advisor: Prof. Dr. Cory M. Hogaboam

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To my parents, Cristina and Alberto.

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BADARO-GARCIA, Stephanie. **Effects of Standard of Care Drugs and Resolvin D2 on Cellular Senescence in Pulmonary Fibrosis.** 2022. 93 p. Thesis. (Ph.D. degree in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2022.

ABSTRACT

Cellular senescence is pivotal in idiopathic pulmonary fibrosis (IPF) progression. Still, it is yet unknown the effects of standard-of-care (SOC) drugs nintedanib and pirfenidone on senescence lung fibroblasts. In addition, specialized pro-resolving lipid mediators have been shown to be effective at improving infection clearance and hold strong therapeutic potential in the management of COVID-19. However, its mechanisms of action on proliferative and senescent fibroblasts were not yet explored. In this study, we elucidated the effects of SOC drugs on senescent normal and IPF lung fibroblasts *in vitro* and the effects of Resolvin D2 on proliferative and senescent lung fibroblasts from normal and IPF patients. Colorimetric/fluorimetric assays, qRT-PCR, and western blotting were used to evaluate the effect of SOC drugs on senescent normal and IPF lung fibroblasts. SOC drugs did not induce apoptosis without death ligands in normal or IPF senescent cells. SOC drugs increased caspase-3 activity in the presence of FasL in normal but not in IPF senescent fibroblasts. Conversely, nintedanib enhanced Bcl-2 expression in senescent IPF lung fibroblasts. Moreover, in senescent IPF cells, pirfenidone alone induced MLKL phosphorylation, provoking necroptosis. In addition, fragmented gasdermin D, indicating pyroptosis, was not detected under any condition. In addition, SOC drugs increased transcript levels of fibrotic and senescence markers in senescent IPF fibroblasts, whereas D+Q inhibited all these markers. Finally, D+Q enhanced GDF15 transcript and protein levels in both normal and IPF senescent fibroblasts. Conversely, RvD2 drugs significantly decrease transcript levels of *BIRC5*, *CCR10*, *COL1A1*, *COL3A1*, *FN1*, GDF15, and *WNT16* in senescent IPF fibroblasts. However, further studies are necessary to elucidate the therapeutic implications of RvD2 in IPF fully.

Keywords: idiopathic pulmonary fibrosis; cellular senescence; lung fibroblasts; nintedanib; pirfenidone, resolvin D2.

LIST OF FIGURES

ABBREVIATION LIST AND SYMBOLS

α-SMA	Alpha-smooth muscle actin
ACE	Angiotensin-converting enzyme
ACTA2	Smooth Muscle Actin Alpha 2
AEC	Alveolar epithelial cells
AFC	7-amino-4-trifluoromethyl coumarin
AKT	Ak Strain Transforming
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
ATS	American Thoracic Society
Bcl	B cell lymphoma
BIRC5	Baculoviral IAP repeat-containing 5
CCL	C-C Motif Chemokine Ligand
CCR	C-C Motif Chemokine Receptor
CDKN	Cyclin-Dependent Kinase Inhibitor
cDNA	Complementary DNA
COL	Collagen
CTGF	Connective tissue growth factor
CXCL	Chemokine (C-X-C motif) ligand
D+Q	Dasatinib plus Quercetin
DCR3	Decoy receptor 3
DHA	Docosahexaenoic acid
DKC1	Dyskerin pseudouridine synthase 1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DSS	Dextran sodium sulfate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunoassay
EPA	Eicosapentaenoic acid
EPHA3	EPH Receptor A3
ERS	European Respiratory Society
FasL	Fas Ligand

FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FGFR	Fibroblast Growth Factor Receptor
FITC	Fluorescein isothiocyanate
FN	Fibronectin
GDF	Growth Differentiation Factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GPCR	G protein-coupled receptor
GPR18	G protein-coupled receptor 18
HGF	Hepatocyte growth factor
HMGB1	High Mobility Group Box 1
ICAM-1	Intercellular adhesion molecule 1
IGFBP	Insulin-like growth factor binding proteins
IL	Interleukin
ILD	Interstitial lung disease
IPF	Idiopathic pulmonary fibrosis
LDH	Lactate dehydrogenase
MCP	Monocyte Chemoattractant Protein
MLKL	Mixed Lineage Kinase Domain Like Pseudokinase
MMP	Matrix metalloproteinase
MUC5B	Mucin 5B, oligomeric mucus/gel-forming
NOX-4	NADPH oxidase 4
OD	Optical Density
p-MLKL	Phosphorylated MLKL
PAI-1	Plasminogen activator inhibitor 1
PARN	Poly(A)-specific ribonuclease
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PDGFR	Platelet-derived growth factor
PI3K	Phosphatidylinositol 3-kinase
qRT-PCR	Quantitative Real-Time PCR
RIPK3	Receptor Interacting Serine/Threonine Kinase 3
RNA18S5	RNA, 18S Ribosomal 5

ROS	Reactive oxidative species
RTEL	Regulator of telomere elongation helicase 1
RvD2	Resolvin D2
SA-βgal	Senescence-associated β-galactosidase
SASP	Senescence-associated secretory phenotype
SEM	Standard Error of the Mean
SFTP	Surfactant protein
SOC	Standard of care
SPM	Specialized pro-resolving lipid mediators
SSEA-4	Stage-specific embryonic antigen
TBS	Tris-buffered Saline
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitors of metalloproteases
TNF	Tumor necrosis factor
TOLLIP	Toll interacting protein
tPA	Tissue plasminogen activator
TRAIL	TNF-Related Apoptosis-Inducing Ligand
uPA	Urokinase plasminogen activator
VCAM-1	Vascular cell adhesion molecule 1
VEGFR	Vascular endothelial growth factor receptor
βgal	β-galactosidase

SUMMARY

1. INTRODUCTION

1.1 Idiopathic pulmonary fibrosis

Definition and epidemiology

According to the American Thoracic Society/European Respiratory Society (ATS/ERS), idiopathic pulmonary fibrosis (IPF) is defined as a specific form of chronic, progressive fibrosing interstitial pneumonia of unknown etiology, occurring in older adults and limited to the lungs (RAGHU; COLLARD; EGAN; MARTINEZ *et al.*, 2011). It is described as a dense deposition of extracellular matrix (ECM) in the lung interstitium, causing irreparable destruction of lung structure and function (LIN; XU, 2020). IPF is associated with radiological and histopathological patterns of usual interstitial pneumonia, affecting more men than women, with an estimated median of survival between 2 and 3 years after diagnosis (MARTINEZ; SAFRIN; WEYCKER; STARKO *et al.*, 2005).

The prevalence of IPF in the United States is 20.2 per 100,000 for men and 13.2 per 100,000 for women (LEY; COLLARD, 2013). The IPF adjusted prevalence estimates fluctuated from 2.40 to 2.98 in North America, 0.33 to 2.51 in Europe, and 0.57 to 4.51 in Asia – Pacific countries. Overall, this disease's adjusted global incidence and prevalence are 0.09–1.30 and 0.33–4.51 per 10,000 persons, respectively (MAHER; BENDSTRUP; DRON; LANGLEY *et al.*, 2021).

The United States, South Korea, and Canada present the highest incidences compared with other countries (MAHER; BENDSTRUP; DRON; LANGLEY *et al.*, 2021). Compelling evidence has shown that IPF incidence increases over time, primarily because of more disease awareness and better diagnostic tools. Therefore, there are some explanations for why the incidence and mortality vary across countries, related to under-diagnosis and under-reporting on death certificates, especially in

South America (HUTCHINSON; FOGARTY; HUBBARD; MCKEEVER, 2015). In 2010, the incidence of IPF in Brazil was 4.48 cases/1,000,000 population, while mortality was 12.11 deaths/1,000,000 population. However, the accuracy of the differential diagnosis of interstitial lung diseases and the comprehensiveness of death certificates are not optimal in Brazil (BADDINI-MARTINEZ; PEREIRA, 2015).

Diagnosis

IPF patients develop unspecific respiratory symptoms, such as progressive dyspnea, shortness of breath, and non-productive cough, which impairs significantly lower quality of life. For those reasons, the diagnosis is complex. It relies on excluding other causes of known interstitial lung disease (ILD), the presence of a usual interstitial pneumonia pattern, and a combination of high-resolution computed tomography and surgical lung biopsy findings. The proper diagnosis requires a multidisciplinary discussion with clinical experts in interstitial lung diseases (RAGHU; COLLARD; EGAN; MARTINEZ *et al.*, 2011).

Moreover, clubber fingers, a deformation of the nail base that leads to swollen and convex shape of the phalanx, are reported in 30%-50% of patients and associated with poor prognosis (VAN MANEN; VERMEER; MOOR; VRIJENHOEFF *et al.*, 2017). Although its cause is still unclear (NAKAMURA; SUDA, 2015) a correlation was observed between smooth muscle proliferation within regions of fibrotic change in the lung biopsy specimens and club fingers (KANEMATSU; KITAICHI; NISHIMURA; NAGAI *et al.*, 1994).

Risk factors

Even though the etiology is still unknown, several risk factors have been linked to the development of IPF, such as genetic susceptibility, tobacco smoking, aging, environmental (contaminants) and occupational exposures (agriculture and textile manufacturing), comorbidities, and viral infections (PADILLA, 2015).

Over the last years, IPF has been related to mutations or polymorphisms in several genes, such as genes encoding surfactant proteins A and C (*SFTPA1*, *SFTPA2,* and *SFTPC*), genes affecting host defense like mucin 5B, oligomeric mucus/gel-forming (*MUC5B)* and toll interacting protein (*TOLLIP),* genes associated with disrupted telomerase function such as telomerase reverse transcriptase (*TERT*) and telomerase RNA component (*TERC*), dyskerin pseudouridine synthase 1 (*DKC1*), poly(A)-specific ribonuclease (*PARN*) and regulator of telomere elongation helicase 1 (*RTEL*) (KAUR; MATHAI; SCHWARTZ, 2017). Mutations on *SFTPA*, *SFTPC*, *MUC5B, and* telomerase regulation genes are present in 1, 1, 35, and 3% of IPF patients, respectively. The remaining 60% of patients do not present any identified genetic predispositions (WOLTERS; COLLARD; JONES, 2014)

Moreover, the smoking habit is related to telomere shortening and endoplasmatic reticulum stress, on top of stimulating an excessive production of reactive oxidative species (ROS), which ultimately leads to cell death (JORGENSEN; STINSON; SHAN; YANG *et al.*, 2008; PSATHAKIS; MERMIGKIS; PAPATHEODOROU; LOUKIDES *et al.*, 2006). In addition, nicotine is associated with the overproduction of transforming growth factor β (TGF-β), which stimulates the production of collagen-1 and other ECM proteins in fibroblasts, parenchymal and inflammatory cells, playing a pivotal role in fibrosis (JENSEN; NIZAMUTDINOV; GUERRIER; AFROZE *et al.*, 2012).

Furthermore, wood and metal dust have been described to increase the risk of IPF. Metal dust can accumulate in the lung and interfere with immune cells in the lungs (PARK; AHN; KIM, 2021). Exposure to pesticides and occupational history of farming or agriculture are also related to the increased risk of IPF (SACK; RAGHU, 2019).

Aging is a substantial risk factor and an independent prognostic biomarker for progressive IPF. The hallmarks of aging, such as genomic instability, telomere attrition, mitochondrial dysfunction, and altered proteostasis, promote premature and persistent cellular senescence of epithelial cells and fibroblasts in IPF lungs (PARDO; SELMAN, 2021). Over the last years, researchers have been trying to comprehend the relationship between IPF and aging, but it is still unclear.

Pathogenesis of IPF

The constant exposure to micro-injuries caused by infections, smoking, or environmental inhaled toxins damages alveolar epithelial cells (AEC), which become dysfunctional (SGALLA; IOVENE; CALVELLO; ORI *et al.*, 2018). Accumulating studies suggest that AEC injury is the key element of IPF pathogenesis, which results in a deficient reconstitution of the epithelium, followed by the differentiation of resident interstitial fibroblasts into myofibroblasts, provoking fibrosis, excessive matrix formation, and ultimately architectural distortion (WUYTS; AGOSTINI; ANTONIOU; BOUROS *et al.*, 2013).

Most of the discoveries of the mechanisms of the disease come from research on animal models. However, there is no reliable animal model for IPF that reproduces the disease features, mainly because the majority of animal models show a pronounced inflammatory cellular component as a tissue response to injury. The most used model, the bleomycin model, induces patchy fibrosis and airway centric, while in

IPF, the fibrosis starts in subpleural lung regions, and fibroblasts are arranged in the reticulum (Figure 1) (WOLTERS; COLLARD; JONES, 2014).

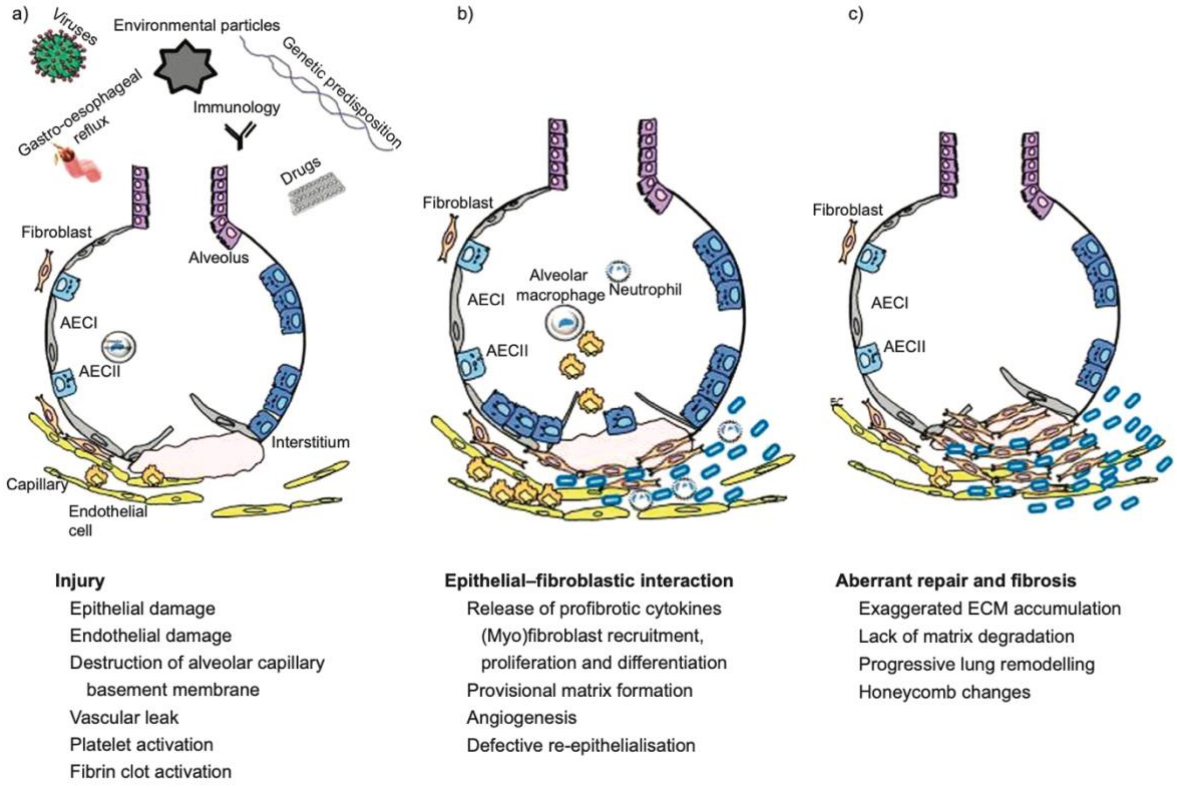

Figure 1. Pathogenesis of IPF. a) The start of fibrosis is triggered by injury and predisposition to the formation of progressive fibrosis. Several agents have been identified to lead to epithelial and endothelial damage, vascular leak, and fibrin clot formation. b) The subsequent events are an atypical repair process characterized by an abnormal re-epithelialization, abundance of myofibroblasts, and the formation of a collagen matrix. c) The consequences of this process proceed to excessive matrix formation leading to architectural deformation and death. ECM: extracellular matrix; AEC: alveolar epithelial cell (WUYTS; AGOSTINI; ANTONIOU; BOUROS *et al.*, 2013).

1.2 Cellular senescence in IPF

Senescence from the Latin word *senex* means growing old (DODIG; CEPELAK; PAVIC, 2019). Firstly reported by Hayflick and Moorehead in 1961, cellular senescence consists of an irreversible cell cycle arrest, sustained viability with resistance to apoptosis, and increased metabolic activity (HAYFLICK; MOORHEAD, 1961).

Cellular senescence is considered one of the nine hallmarks that contribute to

the aging process, which are genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, altered intercellular communication, and stem cell exhaustion (Figure 2)(LOPEZ-OTIN; BLASCO; PARTRIDGE; SERRANO *et al.*, 2013; SCHAFER; WHITE; IIJIMA; HAAK *et al.*, 2017).

Despite the fact senescent cells are not replicating, they are metabolically active and able to perform functions of replication-competent cells from which they came. Senescent cells secrete an array of chemokines, pro-inflammatory cytokines, growth factors, and extracellular matrix proteases, thus comprising the senescence-associated secretory phenotype (SASP)(SALAMA; SADAIE; HOARE; NARITA, 2014). SASP can be classified into three groups: (a) factors that bind to a receptor: soluble molecules that influence which includes interleukins (IL-6, IL-8, IL-1α), chemokines (CXCL1, CXCL2, CCL2, CCL5, CCL16, CCL20, CCL26), and the growth factors hepatocyte growth factor (HGF), fibroblast growth factor (FGF), TGF-β, and granulocyte macrophage-colony stimulating factor (GM-CSF); (b) factors that bind directly: matrix metalloproteases MMP-1, MMP-9 MMP-10, and serine proteases: the tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA); (c) regulatory factors: inhibitors that bind to factors from the previous groups and regulate their activity, such as tissue inhibitors of metalloproteases (TIMP), the plasminogen activator inhibitor (PAI), and insulin-like growth factor binding proteins (IGFBP) (BORODKINA; DERYABIN; GIUKOVA; NIKOLSKY, 2018).

These active molecules can have autocrine or paracrine effects by activating cell-surface receptors and signaling transduction pathways that lead to several diseases, including cancer (COPPE; DESPREZ; KRTOLICA; CAMPISI, 2010). One of the pivotal functions of SASP is the recruitment of innate and adaptative immune cells

to eliminate senescent cells. By releasing SASPs, senescent cells can affect cells and tissues around and at a distance by inducing inflammation, attracting immune cells, spreading senescence to other cells, interfering with stem and progenitor cell function, and impacting endocrine and metabolic function (PRATA; OVSYANNIKOVA; TCHKONIA; KIRKLAND, 2018).

Although there are many cellular senescence hallmarks, the senescence phenotype is diverse, and its mechanisms are not conserved, which makes it a challenge in terms of finding a specific marker (SOTO-GAMEZ; QUAX; DEMARIA, 2019). The senescence-associated β-galactosidase (SA-βgal) was the first marker described as a potent identification of senescent cells. However, this marker is also present in proliferating cells, and due to the lack of a robust senescent marker, researchers rely on checking the presence of two to four markers to confirm the presence of senescent cells, such as the expression of SA-βGal (CAMPISI; D'ADDA DI FAGAGNA, 2007), p16, p21, besides the expression of key SASP factors like IL-6 or IL-1α (FAGET; REN; STEWART, 2019).

Fibroblast senescence is one of the major targets of IPF, but the way cellular senescence contributes to the disease progression is not fully understood. One of the hypotheses is that cellular senescence cause exhaustion of progenitor cells, leading to the weakening in tissue regenerative capacity during aging or upon injury (LIU; LIU, 2020). Altogether, therapeutic drugs that target senescence cells constitute a promising strategy to modulate IPF progression.

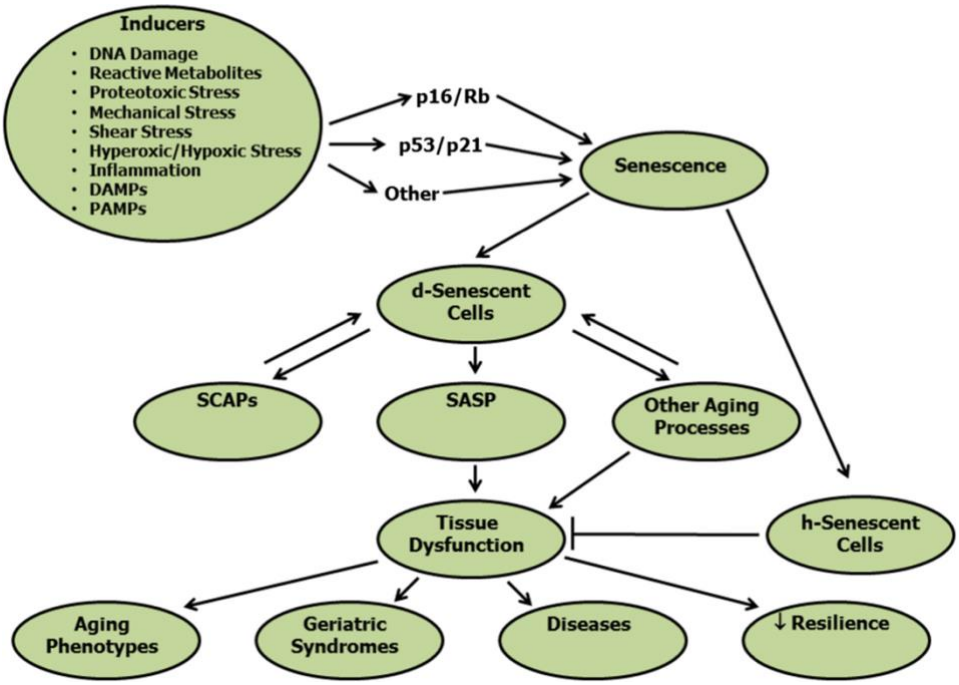

Figure 2. Causes, mechanisms, and consequences of cellular senescence (KIRKLAND; TCHKONIA, 2020).

1.3 Cell death

Theoretically, cellular senescence is a mechanism to prevent malignant transformation, eliminate harmful factors and limit the de proliferation of damaged cells. Besides aging, cellular senescence also plays a role in embryonic development, tissue renovation, wound healing, and tumor suppression (LIN; XU, 2020).

However, the dark side of senescence starts with the fact senescent cells are highly resistant to apoptosis, and they can resist stress better than non-senescent cells in serum-deprived conditions (WANG, 1995). This apoptosis resistance has been demonstrated after stimuli such as UV damage (MARCOTTE; LACELLE; WANG, 2004), serum withdrawal (WANG, 1995), oxidative stress (SANDERS; LIU; ZHANG; HECKER *et al.*, 2013), cytotoxic drugs (TEPPER; SELDIN; MUDRYJ, 2000), and extrinsic apoptosis inducers (SAGIV; BIRAN; YON; SIMON *et al.*, 2013). Several

mechanisms have been described to have an implication in that apoptosis resistance, such as metabolic reprogramming, activation of unfolded proteins, and immune evasion (SOTO-GAMEZ; QUAX; DEMARIA, 2019).

Nowadays, there are many well-established pro-survival regulators, including the B-cell lymphoma-2 (Bcl-2) family, p53 pathway, phosphoinositide 3-kinase (PI3K)/AKT pathway, and heat shock protein 90 (HSP-90) (PAEZ-RIBES; GONZALEZ-GUALDA; DOHERTY; MUNOZ-ESPIN, 2019). The most explored mechanism attributed to an apoptotic-resistant phenotype of senescent cells in IPF is the changes in the levels of Bcl-2 family proteins (PARIMON; HOHMANN; YAO, 2021). Bcl-2 promotes the inhibition of apoptosis by regulating mitochondrial cytochrome c release from mitochondria. However, the mechanism by which cytochrome c is released from mitochondria still needs to be elucidated (YANG; LIU; BHALLA; KIM *et al.*, 1997).

1.4 Standard care drugs

Currently, there is no cure for IPF (CHANDA; OTOUPALOVA; SMITH; VOLCKAERT *et al.*, 2019). In 2014, two drugs were approved by *U.S. Food and Drug Administration* (FDA) for IPF treatment: Ofev® (nintedanib) and Esbriet® (pirfenidone). These antifibrotic agents have been shown to slow the decline of forced vital capacity, avert exacerbations, and slow IPF progression (KISHABA, 2019). Lung transplantation is a common choice among patients with advanced IPF due to the progressive course of the disease and occurrence of severe exacerbations (KISTLER; NALYSNYK; ROTELLA; ESSER, 2014).

Nintedanib (Ofev®; Boehringer Ingelheim), also known as BIBF1120, is a potent intracellular tyrosine kinase inhibitor that targets platelet-derived growth factor (PDGFR) receptor-α and -β, fibroblast growth factor receptor (FGFR) -1–3, and

vascular endothelial growth factor receptor (VEGFR)-1–3 (WOLLIN; DISTLER; REDENTE; RICHES *et al.*, 2019). It was originally designed as an anti-angiogenic drug for cancer. However, the fact that IPF and cancer present remarkable similarities and tyrosine kinase receptors have been shown to play a role in lung fibrosis, the inhibition of those receptors seemed a good approach to slow the progression of IPF.

In bleomycin-induced pulmonary fibrosis study, rats showed a reduction of collagen deposition and inhibition of pro-fibrotic gene expression when Nintedanib was administered before or after the fibrotic phase of the disease (CHAUDHARY; ROTH; HILBERG; MULLER-QUERNHEIM *et al.*, 2007). In the INBUILD trial, IPF patients treated with 150 mg of nintedanib twice daily showed a reduced rate of decline in the forced vital capacity (FLAHERTY; WELLS; COTTIN; DEVARAJ *et al.*, 2019). The adverse effects of Nintedanib include diarrhea, nausea, abdominal pain, vomiting, liver enzyme elevation, decreased appetite, headache, weight loss, and hypertension (GLASSBERG, 2019).

The mechanism of action of Nintedanib consists of occupation of the intracellular ATP-binding pocket of tyrosine kinases (PDGFRs, FGFRs, and VEGFRs), leading to their autophosphorylation and downstream signaling cascades. Inhibition by nintedanib ultimately leads to reduced proliferation, migration, and survival of fibroblasts and, potentially, also attenuated angiogenesis in the lung (Figure 3) (WOLLIN; WEX; PAUTSCH; SCHNAPP *et al.*, 2015).

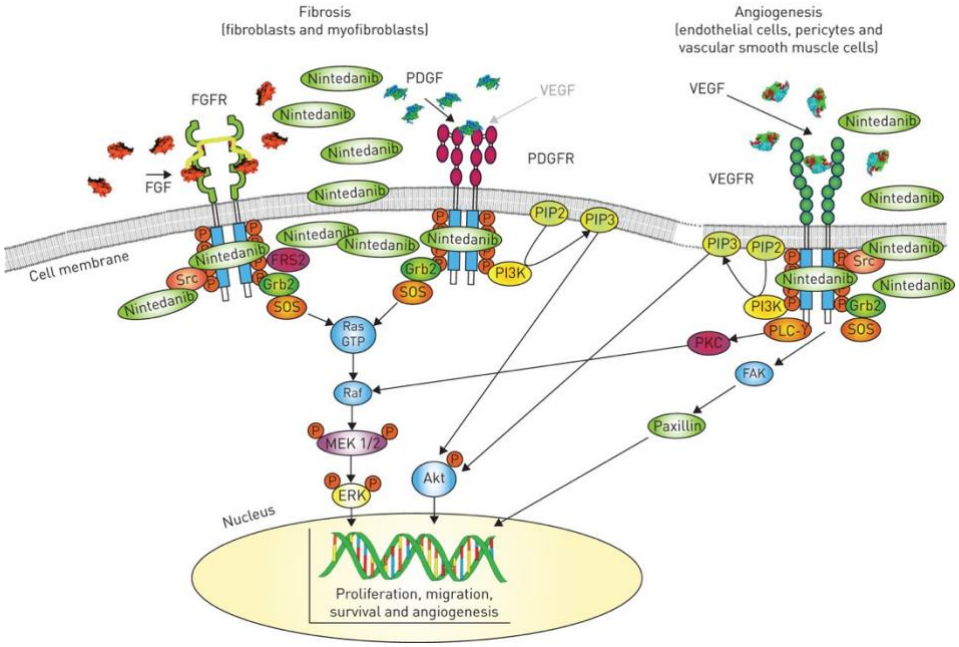

Figure 3. Pharmacological effects of nintedanib and downstream signaling pathways. Nintedanib binds to the intracellular ATP binding pocket of fibroblast growth factor receptors (FGFRs), platelet-derived growth factor receptors (PDGFRs), and vascular endothelial growth factor receptors (VEGFRs), resulting in blockage of the autophosphorylation of these receptors and the downstream signaling cascades. Nintedanib was shown to inhibit PDGFR phosphorylation and subsequent protein kinase B (Akt) and extracellular signal-regulated kinase (ERK)1/2 phosphorylation in lung tissue from mice. FAK: focal adhesion kinase; FGF: fibroblast growth factor; FRS2: FGFR substrate 2; Grb2: growth factor receptor-bound protein 2; MEK1/2: mitogen-activated protein kinase 1/2; PDGF: platelet-derived growth factor; PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase; PIP2/3: phosphatidylinositol-2/3-phosphate; PKC: protein kinase C; PLC-γ: phospholipase C-γ; SOS: son of sevenless, a guanine nucleotide exchange factor that acts on the Ras GTPases. (WOLLIN; WEX; PAUTSCH; SCHNAPP *et al.*, 2015)

Pirfenidone (Esbriet®; Roche/Genentech USA, Inc) is an oral antifibrotic which inhibits collagen synthesis and fibroblast proliferation, consequently reducing fibrotic progression. It was also reported to reduce markers of oxidative stress and attenuate TGF-β signaling pathways (KOLB; BONELLA; WOLLIN, 2017). In a multicentre, double-blind, placebo-controlled, randomized phase III clinical trial, Pirfenidone has been shown to preserve vital capacity and improve progression-free survival time (TANIGUCHI; EBINA; KONDOH; OGURA *et al.*, 2010).

Its mechanism of action is still unknown. However, several mechanisms have

been proposed, such as the regulation of TGF-β1, connective tissue growth factor (CTGF), platelet-derived growth factors (PDGF), and tumor necrosis factor-alpha (TNF-α). Recently, it was suggested as a novel hypothetical treatment for COVID-19, relying on the hypothesis that it could, among other effects, downregulate angiotensin-converting enzyme (ACE) receptors' expression and decrease inflammation (Figure 4) (SEIFIRAD, 2020)

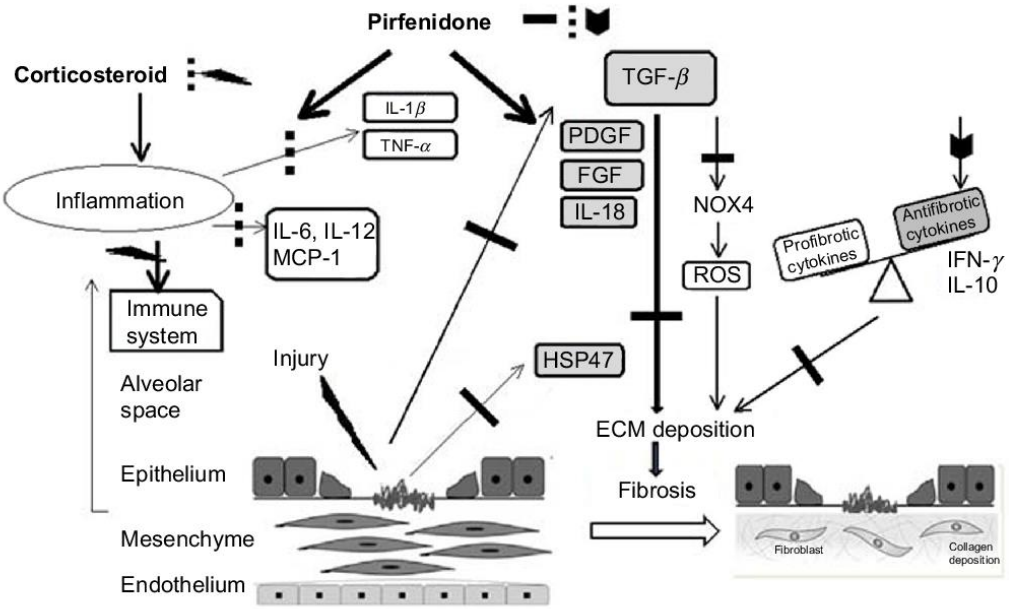

Figure 4. Pharmacological effects of pirfenidone (TAKEDA; TSUJINO; KIJIMA; KUMANOGOH, 2014)

1.5 Dasatinib and Quercetin

Dasatinib (Sprycel; Bristol-Myers Squibb) is an inhibitor of tyrosine kinases and targets BCR-ABL, SRC family kinases, c-KIT, and PDGFR-β (KANTARJIAN; JABBOUR; GRIMLEY; KIRKPATRICK, 2006). In 2016, the FDA approved the use of this drug for the treatment of adults with Philadelphia chromosome-positive chronic myeloid leukemia or acute lymphoblastic leukemia.

Quercetin belongs to the class of flavonoids, and it is present in fruits and

vegetables such as apples, berries, onions, and capers (DAVIS; MURPHY; CARMICHAEL, 2009). This flavonoid presents anti-carcinogenic, anti-inflammatory, and antiviral activities (LI; YAO; HAN; YANG *et al.*, 2016) and has been described to exert anti-fibrogenic and anti-inflammatory effects on bleomycin-induced pulmonary fibrogenesis (BOOTS; VEITH; ALBRECHT; BARTHOLOME *et al.*, 2020).

Together, the cocktail dasatinib plus quercetin (D+Q) constitutes the first senolytic described, able to alleviate several age-related disorders in mice (JUSTICE; NAMBIAR; TCHKONIA; LEBRASSEUR *et al.*, 2019). In addition, D+Q reduces bleomycin-induced pulmonary fibrosis, causing improved pulmonary function, body composition, and physical function (SCHAFER; WHITE; IIJIMA; HAAK *et al.*, 2017). Xu and coworkers have demonstrated that D+Q causes not only selective elimination of senescent cells but also the reduction of naturally occurring senescent cells and proinflammatory cytokines in human adipose tissue (XU; PIRTSKHALAVA; FARR; WEIGAND *et al.*, 2018).

1.6 Resolvin D2

Resolvin D2 (7S,16R,17S-trihydroxy-4Z,8E,10Z,12E,14E,19Z-docosahexaenoic acid, RvD2) (Figure 5) is a specialized pro-resolving mediator (SPM) physiologically produced by the oxygenation of docosahexaenoic acid (DHA) through the action of 15-lipoxygenase and enzymes 5-lipoxygenase (SERHAN; PETASIS, 2011), which plays an important endogenous role in anti-inflammatory and pro-resolution processes (SERHAN; CHIANG; VAN DYKE, 2008). RvD2 binds to its specific cell surface GPCR (G protein-coupled receptor), GPR18 (G protein-coupled receptor 18), expressed on leukocytes, including neutrophils, monocytes, and macrophages. The RvD2-GPR18 axis thus stimulates phagocytic functions and the resolution of bacterial infections, promoting the protection of affected organs (CHIANG;

DALLI; COLAS; SERHAN, 2015).

RvD2 reduces the production of pro-inflammatory cytokines such as TNF-α and IL-1β and the expression of adhesion molecules vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) in dextran sodium sulfate (DSS)-induced colitis (BENTO, A. F.; CLAUDINO, R. F.; DUTRA, R. C.; MARCON, R. *et al.*, 2011). Klein et al. also demonstrated the effect of RvD2 on the inhibition of pro-inflammatory cytokines using a reserpine-induced fibromyalgia model (KLEIN; SPEROTTO; MACIEL; LEITE *et al.*, 2014). In bleomycin-induced pulmonary fibrosis, 17(R)-Resolvin D1, another SPM, had shown a decrease in IL-1β, TGF-β, connective tissue growth factor (CTGF) and type I collagen mRNA expression, attenuating pulmonary fibrosis by promoting resolution of neutrophilic inflammation in mice (RAGHU; CHEN; HOU; YEH *et al.*, 2016). However, RvD2 effects in IPF were not yet explored. Bearing that in mind and considering the ineffectiveness of current therapies and the anti-inflammatory potential of RvD2, this may represent a new therapeutic alternative for the treatment of IPF. Thus, in the present work, we sought to evaluate the effect of RvD2 in proliferative and senescent fibroblasts from normal and IPF patients.

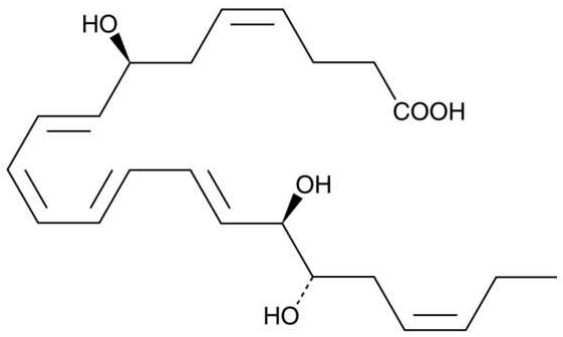

Figure 5. Chemical structure of RvD2 (NIH PubChem, 2018)

2. OBJETIVES

General Objetive

Paper 1: Effects of anti-fibrotic standard-of-care drugs and a senolytic cocktail on senescent human lung fibroblasts

Evaluate the effects of the two standard of care drugs, nintedanib and pirfenidone, and the senolytic cocktail, D+Q, on senescent lung fibroblasts from normal and IPF patients.

Paper 2: Effects of specialized pro-resolving lipid mediator Resolvin D2 on proliferative and senescent human lung fibroblasts

Evaluate the effect and mechanism of action of the specialized pro-resolving mediator Resolvin D2 in proliferative and senescent lung fibroblasts from normal and IPF patients.

Specific Objectives

Paper 1: Effects of anti-fibrotic standard-of-care drugs on senescent human lung fibroblasts

- Evaluate the effects of Nintedanib, Pirfenidone, and D+Q on the cell death by viability, the release of lactate dehydrogenase, and caspase-3 release in senescent lung fibroblasts from control and IPF patients;
- Evaluate the senescence status of senescent fibroblasts after the treatment with SOC drugs and D+Q by assessing β-galactosidase and mRNA expression of *CDKN1A*, *CDKN2A,* and *WNT16.*
- Assess the effects of SOC drugs on *ACTA2*, *CCR10*, *COL1A1*, *COL3A1*, *EPHA3*, *FN1*, and *GDF15* mRNA expression in normal and IPF senescent lung

fibroblasts;

- Evaluate the levels of IL-6, IL-8, MCP-1, GDF-15, WNT16, and collagen-1 by ELISA after the treatment with SOC drugs and D+Q.
- Evaluate the effect of SOC drugs on necroptosis and pyroptosis.

PAPER 2: EFFECTS OF SPECIALIZED PRO-RESOLVING LIPID MEDIATOR RESOLVIN D2 ON PROLIFERATIVE AND SENESCENT HUMAN LUNG FIBROBLASTS:

- Evaluate the effect of RvD2 on cell death by measuring LDH, cell viability, and caspase-3 ;
- Evaluate the effect of RvD2 on cell invasion of proliferative fibroblasts from patients with idiopathic pulmonary fibrosis and normal patients;
- Evaluate mRNA expression of *ACTA2*, *BIRC5*, *CCR10*, *CDKN1A*, *CDKN2A*, *COL1A1*, *COL3A1*, DCR3, *EPHA3*, *FN1*, *GDF15*, *GPR18, IL-1β, IL-6, IL-33, MCP-1, MMP9, NOX4, PAI1, ST2* and *WNT16* on senescent lung fibroblasts from control and IPF patients*;*
- *Assess the l*evels of IL-6, IL-8, MCP-1, GDF-15, WNT16, and collagen-1 by ELISA on senescent lung fibroblasts from control and IPF patients;
- *Assess* GPR18 protein expression by western blotting on senescent lung fibroblasts from control and IPF patients.

3. ARTICLE I FOR PUBLICATION

The present study was performed in Hogaboam laboratory at Cedars-Sinai Medical Center, Los Angeles, EUA in collaboration with Laboratório de Dor, Inflamação, Neuropatia e Câncer, from Universidade Estadual de Londrina, Brazil. The results are described in the article entitled “Standard of care drugs do not modulate activity of senescent primary human lung fibroblasts,” to be submitted to *Nature Scientific Reports.*

Standard of care drugs do not modulate activity of senescent primary human lung fibroblasts

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Abstract

Cellular senescence contributes to progression in idiopathic pulmonary fibrosis (IPF), but it is not apparent whether the standard-of-care (SOC) drugs, nintedanib and pirfenidone, have senolytic properties. In this study we found that SOC drugs did not induce apoptosis in the absence of death ligands in normal or IPF senescent lung fibroblasts. Nintedanib increased caspase-3 activity in the presence of Fas Ligand in normal but not in IPF senescent fibroblasts. Conversely, nintedanib enhanced B cell lymphoma 2 expression in senescent IPF lung fibroblasts. Moreover, in senescent IPF cells, pirfenidone induced mixed lineage kinase domain-like pseudokinase phosphorylation, provoking necroptosis. In addition, SOC drugs increased transcript levels of fibrotic and senescence markers in senescent IPF fibroblasts, whereas D+Q inhibited all these markers. Finally, D+Q enhanced growth differentiation factor 15 (GDF15) transcript and protein levels in both normal and IPF senescent fibroblasts. SOC drugs failed to trigger apoptosis in senescent primary human lung fibroblasts, possibly due to enhanced Bcl-2 levels and the activation of the necroptosis pathway. SOC drugs elevated fibrotic and senescence markers in IPF lung fibroblasts. Together, these data demonstrated that IPF SOC drugs do not target senescent IPF fibroblasts.

Keywords: idiopathic pulmonary fibrosis; cellular senescence; lung fibroblasts; nintedanib; pirfenidone.

Introduction

Idiopathic pulmonary fibrosis (IPF) is chronic and progressive idiopathic interstitial lung disease, which is characterized by histopathologic and/or radiologic findings of usual interstitial pneumonia(MARTINEZ; COLLARD; PARDO; RAGHU *et al.*, 2017; RAGHU; CHEN; HOU; YEH *et al.*, 2016). IPF is an age-related disease that commonly manifests in individuals older than 50 years, and its median survival is approximately 3 to 5 years after diagnosis(RAGHU; CHEN; HOU; YEH *et al.*, 2016). The disease course is heterogeneous and includes dyspnea, worsening lung function, and impaired quality of life(KING; PARDO; SELMAN, 2011; MAHER; KREUTER; LEDERER; BROWN *et al.*, 2019).

Several risk factors have been described to enhance the risk of IPF, including genetics, gender, aging, comorbidities, smoking, environmental and occupation exposure. Nonetheless, aging is the most prominent factor, and recent studies have highlighted the contribution of senescence cells in IPF(ZAMAN; LEE, 2018). Cellular senescence is one of the hallmarks of aging, characterized by cell cycle arrest and secretion of an array of chemokines, pro-inflammatory cytokines, growth factors, and extracellular matrix proteases, thus comprising a secretome referred to as the senescence-associated secretory phenotype (SASP)(SALAMA; SADAIE; HOARE; NARITA, 2014). Although many cellular senescence hallmarks have been described, the senescence phenotype is diverse, and its mechanisms are not conserved among various cell types(SOTO-GAMEZ; QUAX; DEMARIA, 2019). Accumulating evidence demonstrates that senescent cells play a deleterious role in IPF, and it has been shown that the removal of senescent cells increases the life span in animal models(BAKER; CHILDS; DURIK; WIJERS *et al.*, 2016). However, the way senescent cells exacerbate the disease remains unclear.

Lung fibroblasts are essential in wound healing in response to lung injury(KENDALL; FEGHALI-BOSTWICK, 2014). Upon lung epithelium damage, activated fibroblasts differentiate into myofibroblasts and migrate to the injury site, producing extracellular matrix (ECM) components promoting tissue repair(PARIMON; HOHMANN; YAO, 2021). On the one hand, accumulated fibroblasts become senescent and reduce their ECM deposition, limiting the fibrotic process. On the other hand, evidence has shown that targeting senescent fibroblasts reduces pulmonary fibrosis in mice models. That stated, the identification of mechanisms to remove senescent cells would have a remarkable impact on the quality of life and burden of IPF.

Nintedanib (Ofev, Boehringer Ingelheim) and pirfenidone (Esbriet, InterMune) are two drugs approved for the treatment of IPF(KARIMI-SHAH; CHOWDHURY, 2015). Nintedanib, a tyrosine kinase inhibitor, blocks the effects of platelet-derived growth factor, fibroblast growth factor, and vascular endothelial growth factor receptor and inhibits signaling pathways in the proliferation, migration, and differentiation of lung fibroblasts(HOSTETTLER; ZHONG; PAPAKONSTANTINOU; KARAKIULAKIS *et al.*, 2014; RAGHU; SELMAN, 2015). Whereas pirfenidone (whose precise mechanism of action remains unclear) exerts anti-fibrotic, antioxidant, and anti-inflammatory effects to reduce lung collagen synthesis and deposition in bleomycin animal models. Although these standard-of-care (SOC) drugs are anti-fibrotic neither drug truly modify disease nor significantly improve the quality of life of IPF patients(KATO; SHIN; PALUMBO; PAPAGEORGIOU *et al.*, 2021). Senolytics are drugs that can selectively induce senescent cells apoptosis(KELLOGG; KELLOGG; MUSI; NAMBIAR, 2021). Dasatinib plus Quercetin (D+Q), a tyrosine kinase inhibitor and a flavonoid with antioxidant properties, respectively, constitutes the first combination of senolytics

described (SCHAFER; WHITE; IIJIMA; HAAK *et al.*, 2017). D+Q ameliorated bleomycin-induced pulmonary fibrosis and improved pulmonary and physical function(SCHAFER; WHITE; IIJIMA; HAAK *et al.*, 2017).

Taken together, the relevance of senescence in IPF and the fact SOC drugs are being broadly used, herein we evaluate whether SOC drugs exert senolytic or senomorphic effects and compare them to D+Q on apoptosis-resistant senescent primary human lung fibroblasts and we evaluated the types of cell death type induced after SOC drugs treatment of these lung cells.

Results

Lung fibroblasts isolated from normal and IPF patients exhibited a senescent phenotype after underwent replicative senescent

To confirm the status of the lung fibroblasts before we proceed with the next experiments, we performed co-staining of SA-β-gal and the DNA damage marker γ-H2Ax, a SA-β-Gal staining and a RT-PCR for the senescence markers *CDKN1A*, *CDKN1B*, *CDKN2A* and *WNT16.* The elevated expression of p21 (CDKN1A), an important marker of cellular senescence, was observed in senescent when compared to proliferating lung fibroblast in both normal and IPF cells (Figure 1b). Additionally, we observed that senescent lung fibroblasts exhibited increased SA-β-gal and γ-H2Ax fluorescence compared to proliferating lung fibroblasts (Figure 1c-d). In addition, SA-β-Gal staining also confirmed the abundance of SA-β-Gal in senescent cells but not in proliferating lung fibroblasts (Figure 1e). Moreover, we detected the phenotypic difference between proliferating (elongated and spindle-shaped) and senescent lung fibroblasts (enlarged and irregularly shaped). Together these data confirmed the status of normal and IPF fibroblasts as senescent, which allowed that we proceed to the next experiments.

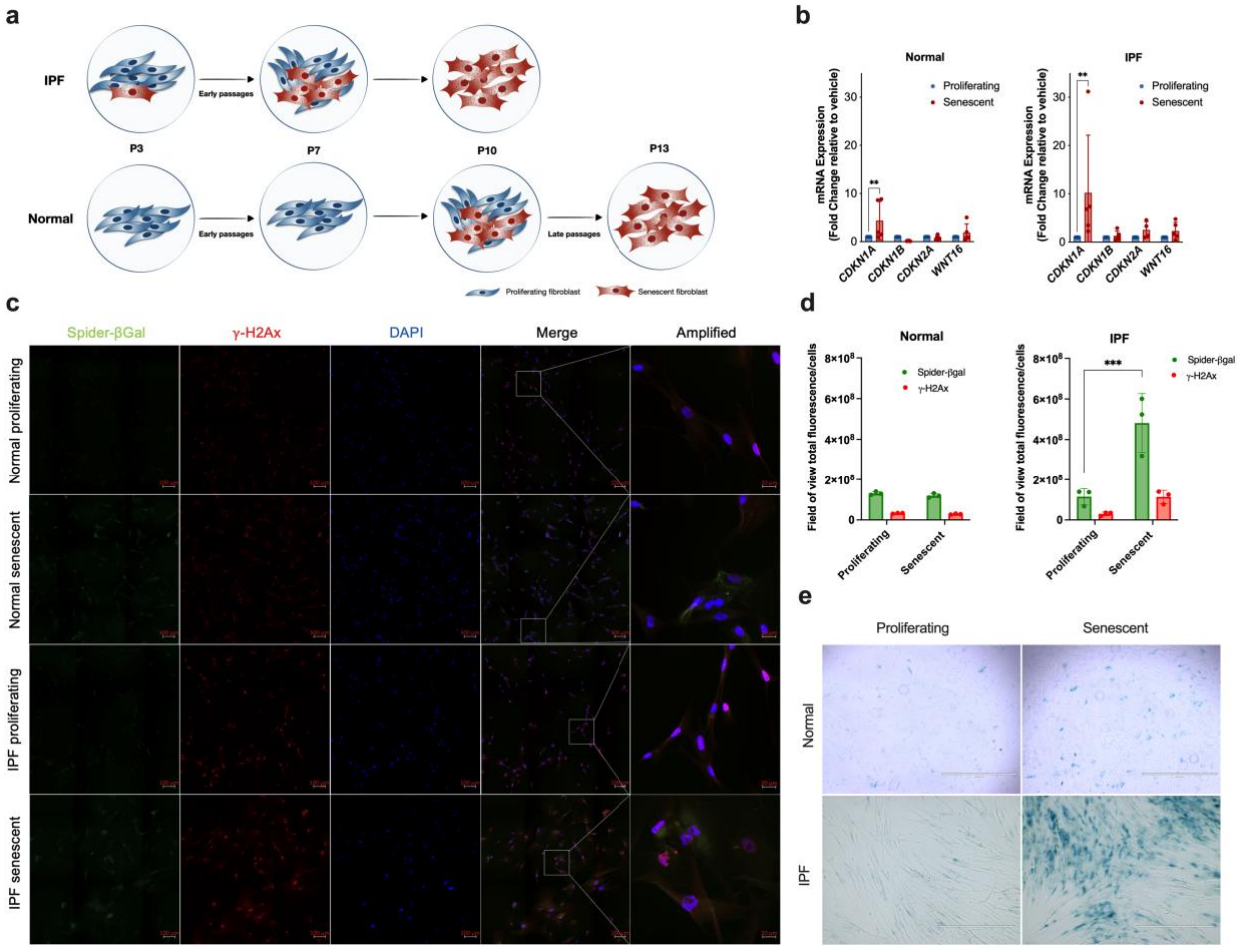

Figure 1. Lung fibroblasts isolated from normal and IPF patients exhibited a senescent phenotype after underwent through replicative senescence. Schematic presentation of normal and IPF lung fibroblasts transition from early to late passages (a). mRNA expression of senescence associated genes *CDKN1A*, *CDKN1B*, *CDKN2A*, and *WNT16* in proliferating and senescent lung fibroblasts from normal or IPF patients (b). Representative images of SA-β-gal and the DNA damage marker γ-H2Ax co-staining is pronounced in senescent lung fibroblasts from normal or IPF patients (c). The scale bars indicate 100 μM (Spider-β-gal, γ-H2Ax, DAPI and merge) and 20 μM for amplified image. Quantification of SA-β-gal and γ-H2Ax total fluorescence/number of cells from proliferating and senescent lung fibroblasts from normal or IPF patients (d). SA-β-galactosidase staining is detected in senescent lung fibroblasts from normal and IPF patients but not in proliferating lung fibroblasts (e). Data are presented as mean ± SD (n = 3 or 5 per group). *p<0.05, **p<0.01 and ***p<0.001 as indicated by the bars.

SOC drugs do not alter cell viability, LDH leakage, or caspase-3 release

Next, we explored the hypothesis that SOC drugs modulate apoptosis in senescent normal and IPF lung fibroblasts in the presence or absence of a proapoptotic stimuli. We treated these cells with vehicle (DMSO) 0.05%, nintedanib (300nM), pirfenidone (2.5mM), or D+Q (20μM /15μM) for 24h, followed by the incubation with 100 ng/mL recombinant FasL protein (Super FasL). Neither nintedanib nor pirfenidone showed an effect on cell viability (2a) or LDH leakage (2b), on normal or IPF senescent lung fibroblasts. However, nintedanib increased caspase-3 activity when combined with the cell death ligand Fas only on normal cells (Figure 2c). Given these findings, we next sought to evaluate the influence of SOC drugs and D+Q on the apoptosis regulator Bcl-2. We saw an increase in Bcl-2 protein levels after the treatment with nintedanib (Figure 2d-e), and the same effect was not observed in cells treated with pirfenidone or D+Q. Collectively, these data demonstrate that SOC drugs do not trigger apoptosis in senescent lung fibroblasts.

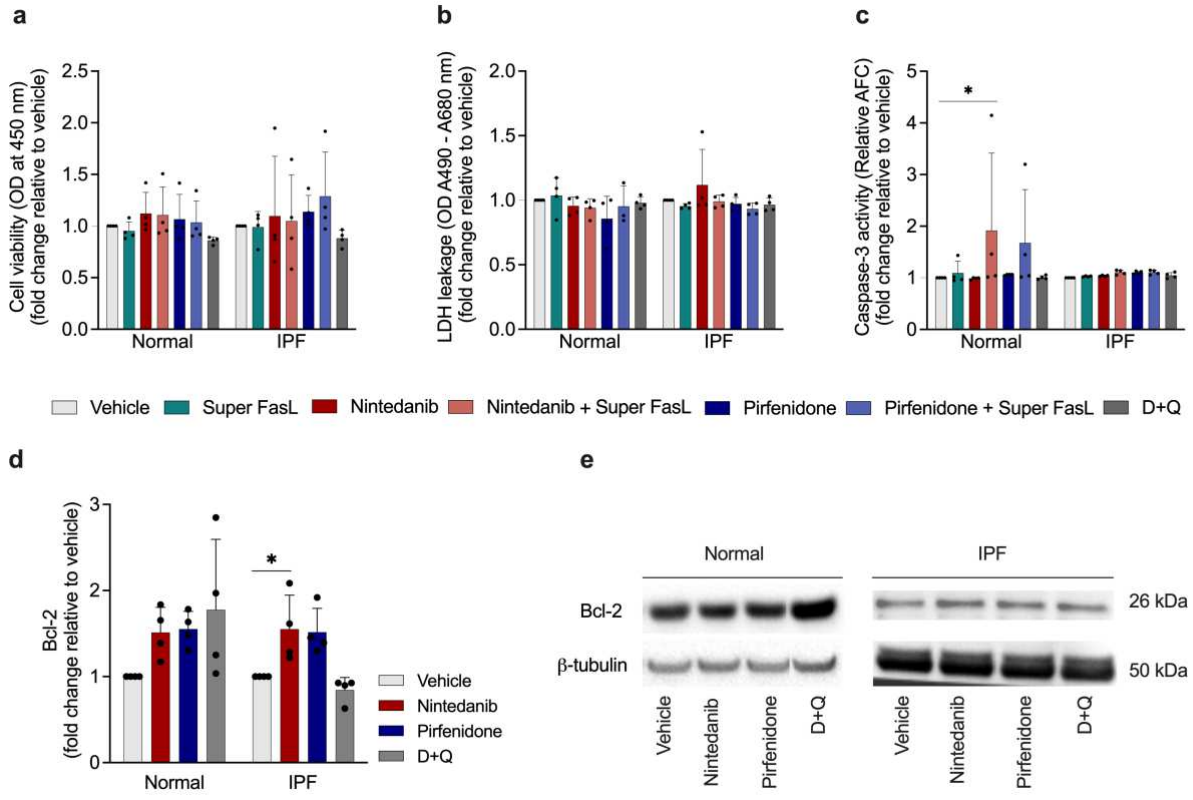

Figure 2. SOC drugs did not trigger apoptosis while nintedanib enhances b-cell lymphoma 2 (Bcl-2) protein levels. Cell viability, lactate dehydrogenase (LDH) release and caspase-3 activity by senescent lung fibroblasts from normal and IPF patients after 24-hour treatment with vehicle (DMSO 0.05%), nintedanib (300 nM), pirfenidone (2.5 mM), or D+Q (20 μM/15μM) followed by 3h of Super Fas Ligand (Super FasL, 100 ng/ml) (a-c). Western blot quantification of Bcl-2 protein levels (d) in senescent normal and IPF fibroblasts lysates. Representative western blot of Bcl-2 protein levels (e). Data are presented as mean ± SD (n = 3 or 4 per group). *P values were calculated using one-way ANOVA followed by Tukey's test.* *p<0.05 as indicated by the bars.

Expression of marker of senescence in normal and IPF senescent fibroblasts after treatment with standard of care drugs and D+Q

Normal and IPF lung fibroblasts were cultured through serial passage rounds until cells obtained the senescence phenotype, as previously described(HOHMANN; HABIEL; COELHO; VERRI *et al.*, 2019). One of the most used methods to evaluate cellular senescence is the detection of β-Gal activity(DE MERA-RODRIGUEZ; ALVAREZ-HERNAN; GANAN; MARTIN-PARTIDO *et al.*, 2021). We assessed the influence of SOC drugs on senescent cells by measuring Spider-βGal intensity, however, we did not find any significant difference after the treatment with SOC drugs or D+Q (Figure 3a). We next determined the expression of *CDKN1A* (p21) and *CDKN2A* (p16) used as markers of cellular senescence(TOMINAGA, 2015). We observed a reduction of *CDKN2A* mRNA expression only in normal lung fibroblasts after the treatment with nintedanib (Figure 3b-c). In addition, we evaluated GDF15 gene expression, which it has been described as one of the components of SASP(ZHANG; JIANG; NOURAIE; ROTH *et al.*, 2019). Interestingly, we observed that

GDF15 was upregulated after D+Q treatment in IPF fibroblasts, which was not observed in any of the other treatments (Figure 3d). Moreover, Wnt16 emerged as a new marker of senescence, regulating p53 activity and phosphatidylinositol 3-kinase (PI3K)/Ak Strain Transforming (AKT) pathway(BINET; YTHIER; ROBLES; COLLADO *et al.*, 2009). We did not observe any significant difference in *WNT16* mRNA expression among the treated groups (Figure 3e).

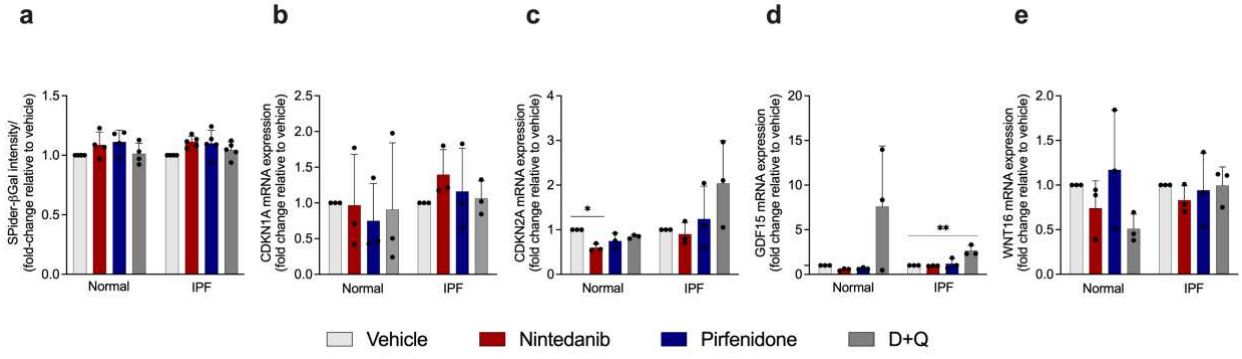

Figure 3. SOC drugs do not alter the senescent phenotype of senescence lung fibroblasts. Senescent fibroblasts isolated from the lungs of normal patients or IPF patients were plated simultaneously at time 0 and treated with vehicle (DMSO 0.05%) nintedanib (300 nM), pirfenidone (2.5 mM), or D+Q (20 μM/15 μM) for 24h. Beta-galactosidase intensity (a), *CDKN1A* (b), *CDKN2A* (c), *GDF15* (d), and *WNT16* (e) transcripts were first normalized to the housekeeping gene *18S*. Data are presented as mean ± SD (n = 3-5 per group). *P values were calculated using one-way ANOVA followed by Tukey's test.* *P<0.05, **P<0.001 as indicated by the bars.

Influence of SOC and D+Q on senescent lung fibroblasts

We next examined whether SOC drugs and D+Q influenced the expression of *ACTA2*, *CCR10*, *EPHA3, COL3A1*, and *FN1*. The treatment with D+Q was able to reduce *ACTA2* mRNA expression only in normal senescent lung fibroblast, while the other drugs did not show an effect on *ACTA2* expression (Figure 4a). We did not

identify a significant change in *CCR10* mRNA expression after the treatment (Figure 4b). We observed a reduced *EPHA3*, a mesenchymal marker, after the treatment with nintedanib and D+Q in normal senescent lung fibroblasts, while the same effect was not observed in IPF cells with any treatment (Figure 4c). Moreover, all drugs reduced *COL3A1* mRNA expression in normal senescent lung fibroblast. However, the same effect was not observed in IPF cells (Figure 4d). The deposition of extracellular matrix proteins, like collagen and fibronectin in the lung, triggers respiratory failure(BUENO; CALYECA; ROJAS; MORA, 2020). Surprisingly, the treatment with pirfenidone enhanced *FN1* in IPF senescent lung fibroblast (Figure 4e).

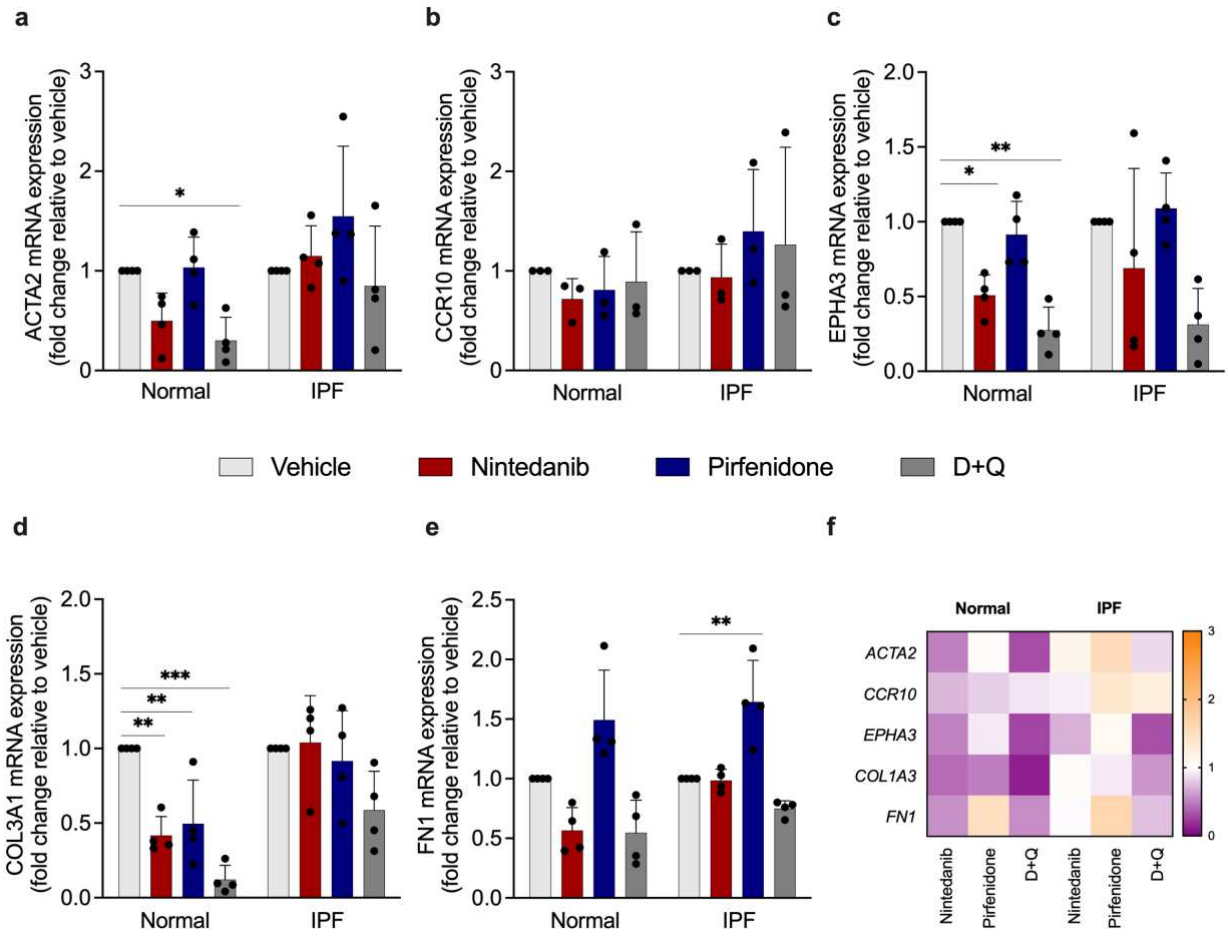

Figure 4. Effects of SOC drugs and D+Q on *ACTA2*, *CCR10*, *EPHA3, COL3A1*, and *FN1* mRNA expression in normal and IPF senescent lung fibroblasts. Heatmap of the expression of SASP and fibrosis-related genes in lung fibroblasts from normal and IPF

patients treated with nintedanib (300 nM), pirfenidone (2.5 mM), D+Q (20 μM/15 μM) for 24h. Each transcript was first normalized to the housekeeping gene RNA *18S*. Upregulation (orange) and downregulation (purple) of gene expression, compared with vehicle-treated cells. Data are presented as mean ± SD (n= 3 or 4 per group). P values were calculated using one-way ANOVA followed by Tukey's test. *P<0.05; **p<0.001; and ***p<0.0001 as indicated by the bars.

Pirfenidone increases COL1A1 expression in senescent IPF fibroblasts, and D+Q reduces collagen expression and release in senescent normal and IPF fibroblasts.

One of the fibrosis hallmarks is the excessive deposition of fibrotic extracellular matrix proteins, especially type I collagen(KLEAVELAND; VELIKOFF; YANG; AGARWAL *et al.*, 2014). To determine the effects of SOC drugs on the fibrosis-related marker type I collagen, we investigated the expression of *COL1A1* in senescent lung fibroblasts after the treatment with nintedanib, pirfenidone, or D+Q. RT-PCR and ELISA demonstrated that the expression levels of *COL1A1* were significantly increased in the IPF group after pirfenidone treatment (Figure 5a). On the other hand, D+Q treatment significantly decreased type I collagen secretion in normal and IPF lung fibroblasts (Figure 5b).

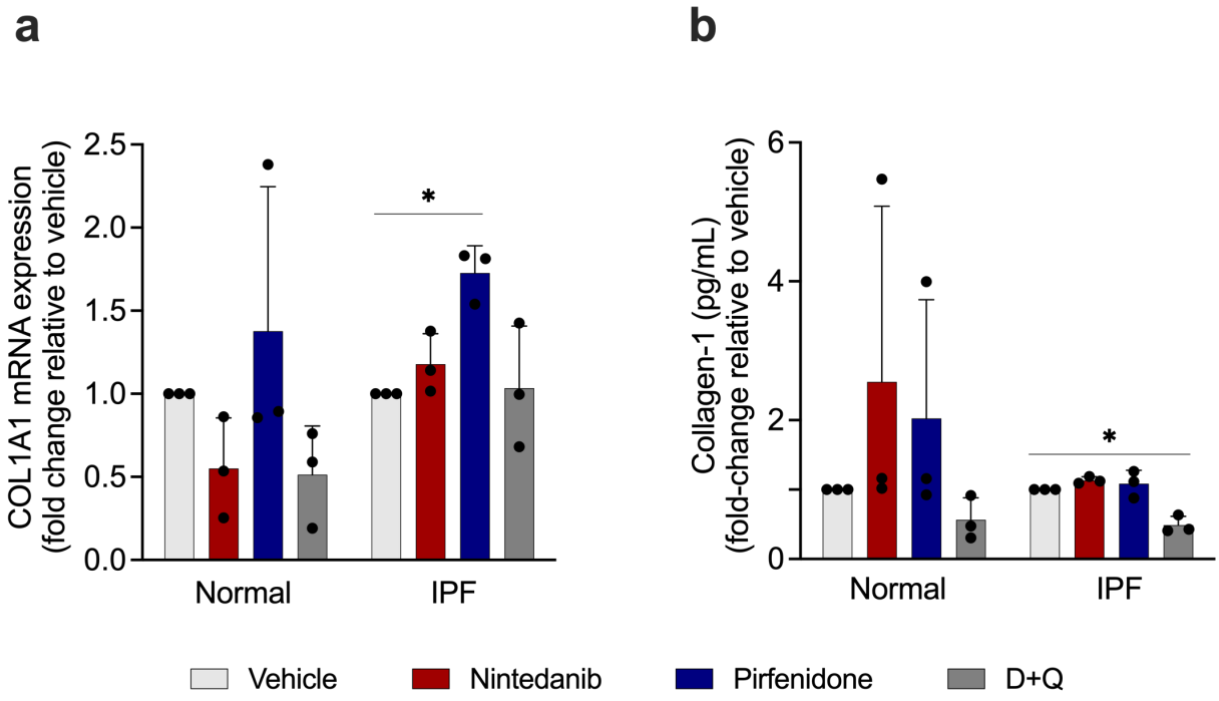

Figure 5. Influence of SOC drugs on *COL1A1* mRNA expression and type I collagen secretion. Effects of nintedanib (300 nM), pirfenidone (2.5 mM), D+Q (20 μM/15 μM) or vehicle (DMSO 0.05%), on *COL1A1* mRNA expression (A) and collagen-1 levels (B). *COL1A1* transcript was first normalized to the housekeeping gene RNA *18S*. Data represented as mean ± SD (n = 3 per group). P values were calculated using one-way ANOVA followed by Tukey's test. *p<0.05. **p<0.01 as indicated by the bars.

SOC drugs influence IL-6, IL-8, MCP-1, GDF-15, Wnt16 in senescent cells.

Next, we investigated whether SOC drugs affect senescent cells via modulation of their secretome. Senescent cells secrete interleukins, inflammatory cytokines, and growth factors that can affect neighboring cells(COPPE; DESPREZ; KRTOLICA; CAMPISI, 2010). Among SASP cytokines, the pleiotropic pro-inflammatory cytokine IL-6 is the most distinguished. We observed that SOC drugs and D+Q did not affect IL-6 in both cell types (Figure 6a). The treatment with D+Q reduced IL-8, a pro-inflammatory cytokine found in SASP, in senescent normal and IPF lung fibroblasts (Figure 6b).

Taking into consideration that senescent fibroblast can recruit leukocytes, and MCP-1 is a crucial component of SASP(JIN; LEE; HEO; LIM *et al.*, 2016), we evaluated the concentration of MCP-1 on normal and IPF senescent fibroblasts. The treatment with D+Q was able to reduce MCP-1 release in normal but not in IPF fibroblast, while SOC drugs showed no effect neither in normal nor IPF senescent fibroblast (Figure 6c). In addition, compelling studies showed GDF-15 emerging as part of the SASP repertoire(AL-MUDARES; REDDICK; REN; VENKATESH *et al.*, 2020). After the dosage of GDF-15, we observed a prominent increase after D+Q treatment only in normal senescent fibroblasts. However, no significant results were observed after the treatment with SOC drugs in normal or IPF senescent cells (Figure 6d). Moreover, we observed that after the treatment with SOC drugs and D+Q, there was no significant difference in Wnt16 release when compared with vehicle, showing that those drugs do not affect Wnt16 (Figure 6e).

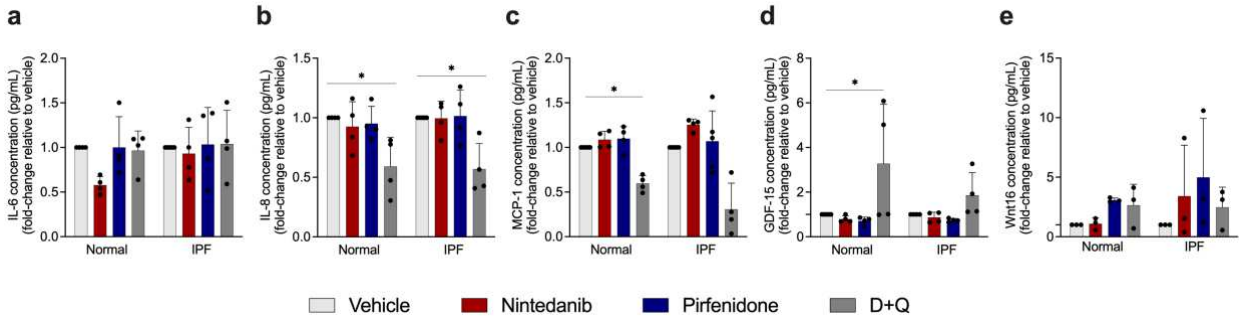

Figure 6. SOC do not have an impact on SASP release. Effects of treatment for 24 with nintedanib (300 nM), pirfenidone (2.5 mM), D+Q (20 μM/15 μM) or control (vehicle; DMSO 0.05%), on IL-6 (a), IL-8 (b), MCP-1 (c), GDF-15 (d), and Wnt16 (e) levels in the supernatant of normal and IPF senescent lung fibroblasts. Data are presented as mean ± SD (n= 3 or 4 per group). P values were calculated using one-way ANOVA followed by Tukey's test. p*<0.05 and **p<0.001 as indicated by the bars.

Pirfenidone triggers necroptosis in IPF lung fibroblasts

Subsequently, to address whether SOC drugs trigger other cell death types, we performed western blot to measure phosphorylated MLKL (p-MLKL) and total MLKL in senescent fibroblasts from normal and IPF patients. The activation of MLKL upon its phosphorylation initiates necroptosis, a form of programmed cell death in which rupture of cellular membranes leads to the release of intracellular components(YOON; KOVALENKO; BOGDANOV; WALLACH, 2017). Treatment with pirfenidone significantly increases the ratio p-MLKL/MLKL (Figure 7a-b), concluding that this drug leads to necroptosis. Although Nintedanib presented a mild increase of p-MLKL/MLKL, it was not significant when compared to the vehicle. We next evaluated the role of SOC drugs in necroptosis secreted factors, the release of cathepsin B, cathepsin D, and HMGB1. We observed that the treatment with nintedanib, pirfenidone and D+Q were able to reduce the levels of cathepsin B in IPF senescent lung fibroblast (Figure 7c), while no significant difference was observed in the normal group. Moreover, any of the treatments influenced cathepsin D and HMGB1 release (7d-e).

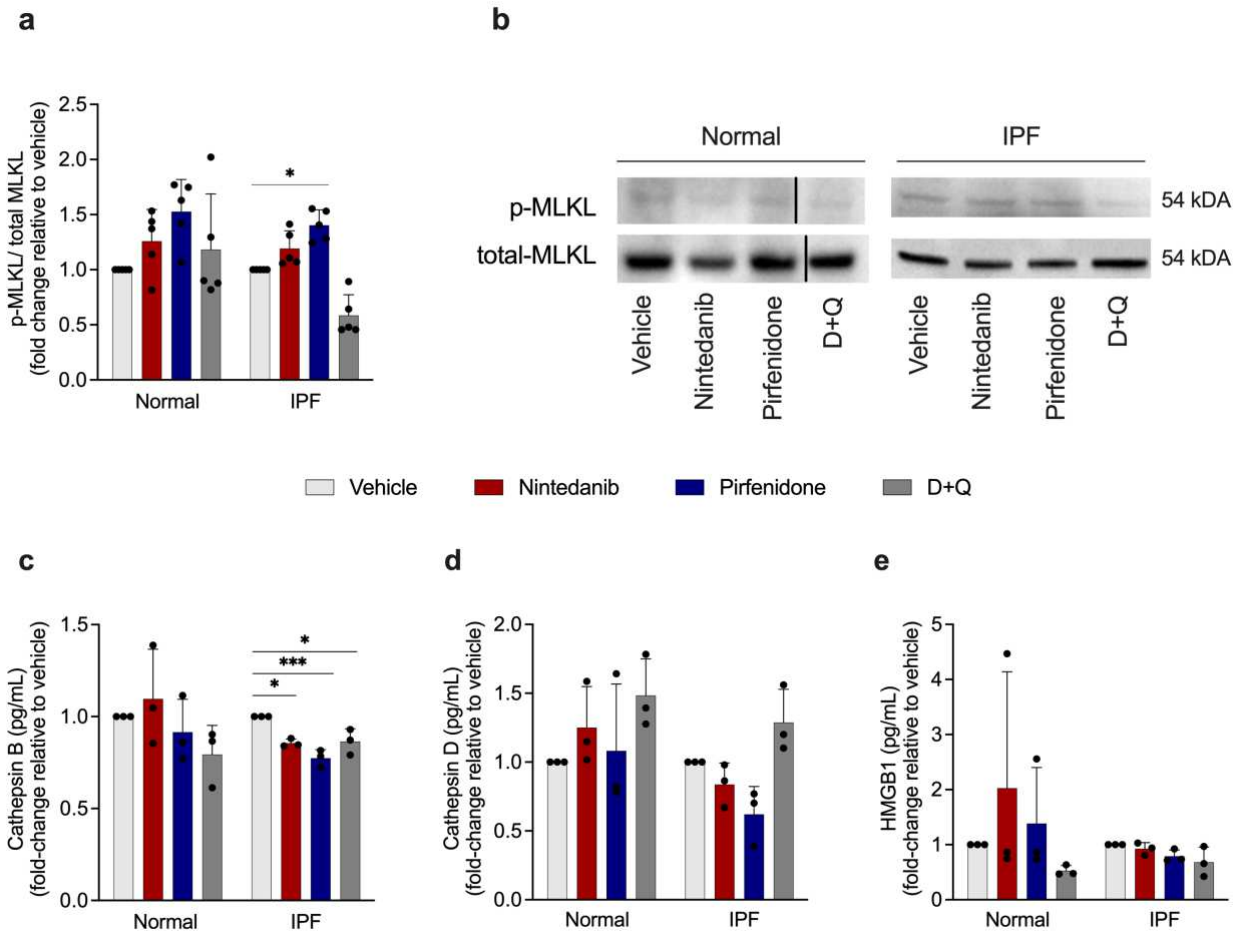

Figure 7. Pirfenidone enhances MLKL phosphorylation and reduces cathepsin B release. Western blot quantification of MLKL phosphorylated/MLKL total of normal and IPF senescent lung fibroblasts treated for 24h with nintedanib (300 nM), pirfenidone (2.5 mM), D+Q (20 μM/15 μM) or control (vehicle; DMSO 0.05%) (a). Representative western blot of p-MLKL/MLKL protein expression (b). Cathepsin B (c), cathepsin D (d) and HMGB1 (e) levels in senescent fibroblasts supernatant after treatment with nintedanib (300 nM), pirfenidone (2.5 mM), D+Q (20 μM/15 μM) or control (vehicle; DMSO 0.05%) for 24h. Data are presented as mean ± SD (n= 3 or 5 per group). P values were calculated using one-way ANOVA followed by Tukey's test. *p<0.05 **p<0.01 and ***p<0.001 as indicated by the bars.

Discussion

Senescence is a well-established feature in IPF(MEINERS; LEHMANN, 2020). Senescent cells dictate the generation of several age-related phenotypes, and their removal from aged tissues can prevent or delay organ dysfunction and lengthen healthspan(BAKER; WIJSHAKE; TCHKONIA; LEBRASSEUR *et al.*, 2011). Notwithstanding, senescent cells are remarkably resistant to apoptosis due to the upregulation of several proteins that contribute to anti-apoptotic pathways(HU; LI; ZI; LI *et al.*, 2022), the clearance of senescence cells has been shown to protect mice from lung fibrosis(LEHMANN; KORFEI; MUTZE; KLEE *et al.*, 2017). Under this senescent state, the cells secrete a myriad of cytokines and proteases and growth factors that play a pivotal impact on adjacent cells and the tissue microenvironment(KIRKLAND; TCHKONIA, 2017).

In the present study, we demonstrate that the treatment with IPF SOC drugs did not evoke apoptosis in senescent IPF fibroblasts, either spontaneously or via pro-apoptotic ligands such as FasL and TRAIL. This resistance to apoptosis is consistent with the findings of Moodley *et al*. who showed that myofibroblasts isolated from fibrotic lungs were more resistant to Fas ligand-induced apoptosis than control myofibroblasts when examined *in vitro*(MOODLEY; CATERINA; SCAFFIDI; MISSO *et al.*, 2004). The cocktail of D+Q has shown remarkable results in reducing pulmonary fibrosis in a bleomycin-induced fibrosis model, and this drug combination also drives cell death in radiation-induced senescent fibroblasts(SCHAFER; WHITE; IIJIMA; HAAK *et al.*, 2017). Surprisingly, our study also shows that D+Q fails to trigger cell death in senescent IPF lung fibroblasts, and these results might reflect the time point examined after treatment (i.e., 24h) or our method used for inducing cellular senescence in

primary human lung fibroblasts (i.e., replicative senescence versus stress-induced or oncogene driven senescence). Moreover, the accumulation of proteins from the Bcl-2 family contributes to the resistance to undergoing apoptosis(YOSEF; PILPEL; TOKARSKY-AMIEL; BIRAN *et al.*, 2016). Here, we discovered that, senescent IPF cells treated with nintedanib presented elevated levels of Bcl-2 when compared to normal senescent cells, despite previous studies showing nintedanib inhibits apoptotic proteins in lung-resident myofibroblasts(KASAM; REDDY; JEGGA; MADALA, 2019), although the effect was not evaluated in senescent cells, which are naturally resistant to apoptosis(SALMINEN; OJALA; KAARNIRANTA, 2011). Although Cho *et al* (2022) demonstrate that nintedanib triggers intrinsic apoptosis in human dermal senescent fibroblasts by inhibiting STAT3 pathway, we believe this discrepancy is due the higher concentration that was used their studies (2-20 μM)(CHO; HWANG; YANG; KIM *et al.*, 2022) in contrast to 300 nM concentration for 24h that we used in our studies, using senescent lung fibroblasts from patients and donors.

Mounting evidence indicates the progression of fibrosis involves ECM-driven mechanisms. Mesenchymal cells and their ECM products lead to the expansion of the alveolar wall, causing loss of the gas-exchange surface. The appearance of myofibroblast foci in IPF coincides with the production of several ECM components such as type I and III collagens, extra domain fibronectin, and fibrin, all of which contribute to progression in IPF(HERRERA; HENKE; BITTERMAN, 2018). The treatment with nintedanib diminished EPHA3 and COL3A1 expression in normal senescent fibroblast but no significant effect was found in IPF cells. We observed an increase in fibronectin 1 (*FN1*) and collagen 1 (*COL1A1*) gene expression after the treatment with pirfenidone, suggesting that although evidence shows its anti-fibrotic effect in proliferative IPF fibroblasts(COLLINS; RAGHU, 2019), our results suggest that

pirfenidone was unable to reduce those fibrosis markers. In agreement with our findings, Roach et al. (2021) and collaborators found increased deposition of collagen 1 and secretion of soluble collagen after treating fibroblasts with pirfenidone(ROACH; CASTELLS; DIXON; MASON *et al.*, 2021). Importantly, in the present study, we show that the cocktail D+Q reduced ACTA2, EPHA3 and COL3A1 expression in normal senescent fibroblasts, but this effect was not observed in IPF senescent fibroblasts. However, we found a decrease of soluble collagen 1 in senescent IPF fibroblasts.

In a bleomycin model, GDF-15 was previously observed to be the most upregulated protein and this cytokine appears to be a suitable biomarker of epithelial stress and severity of fibrotic lung disease(ZHANG; JIANG; NOURAIE; ROTH *et al.*, 2019). Moreover, Tanaka et al. used a proteomics analysis of lung samples and observed a positive association between GDF15 and age (AL-MUDARES; REDDICK; REN; VENKATESH *et al.*, 2020). In addition, GDF15 is reported to increase α-SMA expression in WI-38 lung fibroblasts, suggesting that elevated GDF15 in the fibrotic lung contributes to the fibrotic process(TAKENOUCHI; KITAKAZE; TSUBOI; OKAMOTO, 2020). Intriguingly, after D+Q treatment of senescent IPF fibroblasts, GDF-15 levels were significantly higher when compared to SOC drugs, suggesting that D+Q might be altering the synthetic activity of senescent IPF, which in turn might further contribute to the fibrotic process mediated by fibroblast progenitor or progeny.

Since pro-apoptotic caspase-3 release was not detected after SOC treatment in senescent IPF cells, we investigated whether other forms of cell death might be activated via these drugs. Necroptosis is an alternative form of cell death when caspase-dependent apoptosis is restricted or absent(HAN; ZHONG; ZHANG, 2011). It is present in many pathologies such as inflammatory diseases(TAO; SUN; WU; WANG *et al.*, 2020), ischemia-reperfusion injuries(MULLER; DEWITZ; SCHMITZ;

SCHRODER *et al.*, 2017), and degenerative diseases(GAUTHERON; VUCUR; SCHNEIDER; SEVERI *et al.*, 2016). It has been demonstrated that augmented levels of Receptor Interacting Serine/Threonine Kinase 3 (RIPK3) and phosphorylated MLKL in alveolar epithelial cells (AECs) in IPF lungs(LEE; YOSHIDA; KIM; LEE *et al.*, 2018) leading Lee et al. to postulate that cell-damaging agents injure AECs, leading RIPK-3–regulated necroptosis. In addition, damage-associated molecular patterns (DAMPS), including HMGB1 and IL-β, released from necroptotic AECs are responsible not only for inflammation but also for the development of fibrosis through enhanced myofibroblast differentiation during IPF pathogenesis. The necroptosis pathway can be induced by impaired apoptosis by ligand-dependent stimulation of cell death receptors, for instance, Fas(CHOI; PRICE; RYTER; CHOI, 2019). It is orchestrated by distinct proteins, namely RIPK1 and RIPK3 and the downstream protein MLKL, that once phosphorylated by RIPK3, lead to necroptosis by inducing the formation of oligomers, migration to the plasma membrane, and binding to phosphatidylinositol lipids to directly disrupt membrane integrity(RODRIGUEZ; WEINLICH; BROWN; GUY *et al.*, 2016). In the present study, we found some evidence that pirfenidone increases MLKL phosphorylation, the major hallmark of necroptosis, and the same effect was not observed after the treatment with nintedanib or D+Q. However, the mechanism whereby pirfenidone enhances this phosphorylation requires further investigation.

Previous work has shown that the inhibition of cathepsins provokes the induction of necroptosis in bone marrow-derived macrophages, suggesting that cathepsins act as anti-necroptotic factors(MCCOMB; SHUTINOSKI; THURSTON; CESSFORD *et al.*, 2014). This finding corroborates with our findings in the present study since we observed a decrease in cathepsin B after the treatment with nintedanib, pirfenidone, and, interestingly, D+Q. Similar results were obtained with cathepsin D.

Moreover, cathepsins are involved in the degradation of the anti-apoptotic protein Bcl-2, leading to apoptosis(DROGA-MAZOVEC; BOJIC; PETELIN; IVANOVA *et al.*, 2008). However, SOC drugs diminished cathepsin activity, which could potentially trigger cellular evasion of apoptosis and thereby increase the phosphorylation of MLKL ultimately leading to necroptosis.

We would like to acknowledge limitations in this study such as striking differences observed among the same group in IPF. We strongly believe that this phenomenon is due differences between the two fibroblasts subsets, brilliantly described by Levesque *et al.* (2021)(KARMAN; WANG; BODEA; CAO *et al.*, 2021).

Although SOC have been reported to be an important clinical strategy to treat IPF patients, over the years, the advance of senotherapeutics in preclinical model aligned with outstanding preliminary results of clinical trials, have proved the importance of targeting senescent cells in IPF, and clearly, SOC did not have a significant impact on senescent lung fibroblasts .

Together, these data demonstrate that SOC drugs fail to promote apoptosis or modulate the synthetic capacity of senescent IPF fibroblasts. Moreover, pirfenidone appeared to promote inflammatory cell death, however the mechanism which this drug mediates necroptosis requires future investigation. Indeed, further studies are necessary to determine whether SOC drugs prevent the appearance of senescent cells such as fibroblasts in IPF. Overall, this study sheds light on the need to develop more effective therapies for IPF, which target and selectively eliminate senescent cells in this progressive fibrotic lung disease.

Methods

Senescent fibroblast generation

Primary normal lung fibroblasts were derived from nonfibrotic lung samples without signs of disease from lung biopsies and primary IPF lung fibroblasts were derived from IPF patients from lung explants (Table 1). To obtain senescent fibroblasts, proliferating normal and IPF lung fibroblasts were repeatedly passaged in culture until they reached a senescent morphological phenotype (enlarged, flattened, and irregular shape) and SA-β-gal activity (HOHMANN; HABIEL; COELHO; VERRI *et al.*, 2019). Normal lung fibroblasts reached cellular senescence within 12-15 passages and IPF lung fibroblasts became senescent after 9-11 passages. Senescent fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM; Lonza, Basel, Switzerland) supplemented with 15% FBS (Atlas Biologicals, Inc, Fort Collins, CO), 1% penicillin/streptomycin (Mediatech, Manassas, VA), 1% glutamine (Mediatech) and 0.1% of primocin (Invivogen, San Diego, CA) at 37 °C, and 10% CO_2.

Table 1. IPF patient demographics

Sample name	Age	Gender	Diagnosis	Smoking history
CCA-18	67	Male	Normal	Smoked 1-2 cigarettes primarily socially at age 18.
CC01-19	47	Male	Normal	Smoked 0.5-1.5 packs of cigarettes for 35 years. Did not quit smoking.
CC07-19	18	Male	Normal	Nonsmoker
CC02-20	62	Male	Normal	Smoked 3 Cigarettes/day for 27 years. Quit at age 42
CC04-20	60	Male	Normal	Smoked less than 1 pack per week from 1978-1980. Quit in 1980.
CC06-20	57	Male	Normal	Cigarettes, ½ pack since age 16
CC01-21	54	Male	Normal	No history but lungs showed signs of smoking
IPF10-18	66	Male	IPF	Nonsmoker
IPF14-19	58	Male	IPF	Former smoker. Quit at 09/03/1994
IPF01-20	68	Male	IPF	Nonsmoker
IPF03-20	66	Female	IPF	Nonsmoker
IPF04-20	69	Male	IPF	Nonsmoker
IPF05-20	73	Male	IPF	Former smoker. Smoked 2.5 packs/day for 25 years. Quit 21.3 years ago
IPF08-20	64	Male	IPF	Never smoker
IPF07-21	74	Female	IPF	Nonsmoker

Co-staining of SA-β-gal and the DNA damage marker γ-H2Ax

Proliferating and senescence cells were plated in an 8-chamber slide (Thermo Scientific, Waltham, MA, USA) at 37°C overnight in a 5% incubator. SA-β-gal staining

was performed following manufacturer's protocol for living cells (Dojindo Cat #SG03-10). Next, cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature (RT). After three washes, cells were permeabilized with 0.1% Triton X-100/PBS for 30 min and blocked in 1% BSA/PBS for 1 hour. An anti- γ-H2A.x antibody (Cell Signaling Cat#2577L) diluted in 1% BSA/PBS was added to the cells and incubated overnight at 4°C. After 3 washes, an anti-rabbit secondary antibody (ThermoFisher Scientific, Alexa Fluor 594 Cat#A11012) was added to the cells and incubated for 2 hours at RT. Cells were washed with PBS 3 times and incubated with 2 µg/mL DAPI (ThermoFisher Scientific, Cat#62248) for 10 minutes. The same washing process was repeated, and cells were observed under a confocal microscope and analyzed using Zen 2.5 blue edition software and Image-Pro Premier E9.2 software.

SA- β-Gal staining

SA- β-Gal staining was performed following manufacturer's protocol (BioVision Inc., Milpitas, CA, US; Cat #K320-250). Cells were observed under light microscopy for the development of blue color.

Senescence associated β-galactosidase detection

To assess SA-β-galactosidase levels, a cellular senescence assay was performed (Dojindo, Kumamoto, Japan). After 24h of treatment with nintedanib, pirfenidone, or D+Q, cells were lysed with 50 µL of lysis buffer and incubated for 10 minutes. Then, 50 µL of SPiDER-βgal working solution was added to each well and incubated at 37 °C for 30 minutes. After that, 100 µL of stop solution was added to each well. Fluorescence values were assessed using a fluorescence excitation

wavelength of 500 nm and an emission of 540 nm with a fluorescent microplate reader (Biotek, Winooski, VT, USA).

Cell viability

Cell viability was evaluated using AlamarBlue Cell Viability Reagent (ThermoFisher Scientific). Senescent lung fibroblasts (3 x 10^4 cells/well) were treated with nintedanib (Ofev®, Boehringer Ingelheim, Germany; 300nM), pirfenidone (Esbriet®, Genentech, San Francisco; 2.5 mM) or dasatinib (Tocris, Bristol, UK; 20μM) + quercetin (Sigma-Aldrich, St. Louis, MO; 15 μM) for 24 hours followed by 3h with the cell death ligand Super Fas Ligand (100 ng/ml). AlamarBlue Cell Viability reagent was added to the cells and incubated for 4 hours at 37°C. Fluorescence values were assessed using a fluorescence excitation wavelength of 560 nm and an emission of 590 nm with a fluorescent microplate reader (Biotek). Results were expressed as fold-change compared to control cells.

Caspase-3 assay

The effect of SOC drugs on caspase-3 activity was observed by using Caspase-3/CPP32 Fluorometric Assay Kit (BioVision Inc). Senescent fibroblasts were treated with SOC drugs or D+Q for 24h, followed by 3h of Super FasL stimuli. Cells were lysed in 50 μl chilled cell lysis buffer on ice for 10 min before 50 μl of 2X reaction buffer (containing 10 mM Dithiothreitol) was added, followed by 50 μM DEVD-AFC substrate, incubated at 37 °C for 2 h. Fluorescence was measured at 505 nm with a fluorescent microplate reader (Biotek). Results were expressed as fold-change compared to control cells.

Lactate dehydrogenase (LDH) assay

To perform the assay, 50 µL of CyQUANT LDH Cytotoxicity Assay Kit reaction mixture was added to cell supernatants. After 30 minutes of incubation at RT, protected from light, the assay was stopped with a stop solution. Absorbance was measured at 490 nm and 680 nm using a microplate reader (Biotek, Winooski, VT, USA). Results were expressed as fold-change compared to control cells.

Soluble Collagen-1 direct ELISA

Senescent lung fibroblasts were plated into a 96-well plate and treated with nintedanib, pirfenidone, or D+Q for 24h. After 24 hours, lung fibroblast conditioned supernatants were harvested, and collagen-1 was assessed as previously described (HABIEL; ESPINDOLA; JONES; COELHO *et al.*, 2018). Results were expressed as fold-change compared to control cells.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cells were lysed in Trizol™ reagent (Thermo-Fisher Scientific), and RNA was extracted as recommended by the manufacturer. 3µg of RNA was reverse transcribed into cDNA using SuperScript™ II Reverse Transcriptase (Thermo-Fisher Scientific) as previously described (HOHMANN; HABIEL; ESPINDOLA; HUANG *et al.*, 2021). Gene expression analyses were performed using TaqMan master mix (Thermo-Fisher Scientific) probes for human *Smooth Muscle Actin Alpha 2 (ACTA2), C-C Motif Chemokine Receptor (CCR)10, CDKN1A, CDKN2A, Collagen (COL)1A, COL3A1, (EPH Receptor A3) EPHA3, Fibronectin (FN)1, GDF15, and WNT16 (*all *Thermo-Fisher Scientific*). Quantitative PCR analysis was performed using Viia7 Thermocycler (Thermo-Fisher Scientific). Results were normalized to *RNA18S5* expression and

presented as fold-change values compared to control cells by using DataAssist software version 3.01 (Thermo-Fisher Scientific).

ELISA

IL-6, IL-8, monocyte chemoattractant protein (MCP)-1, GDF-15 were determined in senescent fibroblast supernatant, and WNT16 levels were determined in senescent fibroblast lysates using a standardized sandwich ELISA technique (R&D Systems, Minneapolis, MN, USA), according to manufacturer's protocol. Results were expressed as fold-change compared to control cells.

Western blotting

Cells were lysed using RIPA lysis buffer (Thermo-Fisher Scientific) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo-Fisher Scientific). Protein concentrations were measured by using a Detergent Compatible protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the same amount of protein was loaded into a 4–15% NuPAGE Bis-Tris Protein gel. Gels were transferred using an iBlot Dry blotting system onto nitrocellulose membranes (Thermo-Fisher Scientific), and the transferred samples were blocked for 1 hour at RT in 5% non-fat-dry-milk in tris-buffered saline (TBS). Primary antibodies used included: Phospho-MLKL (Cat# 916895, Cell Signaling, Danvers, MA), Mixed Lineage Kinase Domain Like Pseudokinase (MLKL) (Cat #14993S, Cell Signaling), and B cell lymphoma (Bcl)-2 (Cat#Ab182858, Abcam, Cambridge, UK). Images of chemiluminescent bands were acquired using a Bio-Rad Gel documentation system (Bio-Rad Laboratories, Inc.). Membranes were washed in TBS-T, blotted with anti-tubulin antibody (Abcam CAT#Ab6046), and developed similarly. Image Lab Software

version 6.0.1 (Bio-Rad Laboratories, Inc.) was used to perform densitometric analysis. Results were expressed as fold-change compared to control cells.

Statistics

Statistical analyses were performed using GraphPad Prism 9.1.2 (GraphPad Software, San Diego, CA, USA). Data were presented as standard deviation (SD) and evaluated for significance by one-way Analysis of Variance (ANOVA) followed by Tukey's test. A P value less than 0.05 was considered statistically significant.

Study approval

This Institutional Review Board at Cedars-Sinai Medical Center approved all experiments with primary human tissue, and informed consent was obtained before inclusion in the studies described herein. All methods were performed in accordance with relevant guideline and regulations.

References

1 Martinez, F. J. *et al.* Idiopathic pulmonary fibrosis. *Nat Rev Dis Primers* **3**, 17074, doi:10.1038/nrdp.2017.74 (2017).

2 Raghu, G., Chen, S. Y., Hou, Q., Yeh, W. S. & Collard, H. R. Incidence, and prevalence of idiopathic pulmonary fibrosis in US adults 18-64 years old. *Eur Respir J* **48**, 179-186, doi:10.1183/13993003.01653-2015 (2016).

3 King, T. E., Jr., Pardo, A. & Selman, M. Idiopathic pulmonary fibrosis. *Lancet* **378**, 1949-1961, doi:10.1016/S0140-6736(11)60052-4 (2011).

4 Maher, T. M. *et al.* Rationale, design, and objectives of two phase III, randomised, placebo-controlled studies of GLPG1690, a novel autotaxin inhibitor, in idiopathic pulmonary fibrosis (ISABELA 1 and 2). *BMJ Open Respir Res* **6**, e000422, doi:10.1136/bmjresp-2019-000422 (2019).

5 Zaman, T. & Lee, J. S. Risk factors for the development of idiopathic pulmonary fibrosis: A review. *Curr Pulmonol Rep* **7**, 118-125, doi:10.1007/s13665-018-0210-7 (2018).

6 Salama, R., Sadaie, M., Hoare, M. & Narita, M. Cellular senescence, and its effector programs. *Genes Dev* **28**, 99-114, doi:10.1101/gad.235184.113 (2014).

7 Soto-Gamez, A., Quax, W. J. & Demaria, M. Regulation of Survival Networks in Senescent Cells: From Mechanisms to Interventions. *J Mol Biol* **431**, 2629-2643, doi:10.1016/j.jmb.2019.05.036 (2019).

8 Baker, D. J. *et al.* Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan. *Nature* **530**, 184-189, doi:10.1038/nature16932 (2016).

9 Kendall, R. T. & Feghali-Bostwick, C. A. Fibroblasts in fibrosis: novel roles and mediators. *Front Pharmacol* **5**, 123, doi:10.3389/fphar.2014.00123 (2014).

10 Parimon, T., Hohmann, M. S. & Yao, C. Cellular Senescence: Pathogenic Mechanisms in Lung Fibrosis. *Int J Mol Sci* **22**, doi:10.3390/ijms22126214 (2021).

11 Karimi-Shah, B. A. & Chowdhury, B. A. Forced vital capacity in idiopathic pulmonary fibrosis--FDA review of pirfenidone and nintedanib. *N Engl J Med* **372**, 1189-1191, doi:10.1056/NEJMp1500526 (2015).

12 Raghu, G. & Selman, M. Nintedanib and pirfenidone. New antifibrotic treatments indicated for idiopathic pulmonary fibrosis offer hopes and raises questions. *Am J Respir Crit Care Med* **191**, 252-254, doi:10.1164/rccm.201411-2044ED (2015).

13 Hostettler, K. E. *et al.* Anti-fibrotic effects of nintedanib in lung fibroblasts derived from patients with idiopathic pulmonary fibrosis. *Respir Res* **15**, 157, doi:10.1186/s12931-014-0157-3 (2014).

14 Kato, K. *et al.* Leveraging ageing models of pulmonary fibrosis: the efficacy of nintedanib in ageing. *Eur Respir J* **58**, doi:10.1183/13993003.00759-2021 (2021).

15 Kellogg, D. L., Kellogg, D. L., Jr., Musi, N. & Nambiar, A. M. Cellular Senescence in Idiopathic Pulmonary Fibrosis. *Curr Mol Biol Rep* **7**, 31-40, doi:10.1007/s40610-021-00145-4 (2021).

16 Schafer, M. J. *et al.* Cellular senescence mediates fibrotic pulmonary disease. *Nat Commun* **8**, 14532, doi:10.1038/ncomms14532 (2017).

17 Hohmann, M. S., Habiel, D. M., Coelho, A. L., Verri, W. A., Jr. & Hogaboam, C. M. Quercetin Enhances Ligand-induced Apoptosis in Senescent Idiopathic Pulmonary Fibrosis Fibroblasts and Reduces Lung Fibrosis In Vivo. *Am J Respir Cell Mol Biol* **60**, 28-40, doi:10.1165/rcmb.2017-0289OC (2019).

18 de Mera-Rodriguez, J. A. *et al.* Is Senescence-Associated beta-Galactosidase a Reliable in vivo Marker of Cellular Senescence During Embryonic Development? *Front Cell Dev Biol* **9**, 623175, doi:10.3389/fcell.2021.623175 (2021).

19 Tominaga, K. The emerging role of senescent cells in tissue homeostasis and pathophysiology. *Pathobiol Aging Age Relat Dis* **5**, 27743, doi:10.3402/pba.v5.27743 (2015).

20 Zhang, Y. *et al.* GDF15 is an epithelial-derived biomarker of idiopathic pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* **317**, L510-L521, doi:10.1152/ajplung.00062.2019 (2019).

21 Binet, R. *et al.* WNT16B is a new marker of cellular senescence that regulates p53 activity and the phosphoinositide 3-kinase/AKT pathway. *Cancer Res* **69**, 9183-9191, doi:10.1158/0008-5472.CAN-09-1016 (2009).

22 Bueno, M., Calyeca, J., Rojas, M. & Mora, A. L. Mitochondria dysfunction and metabolic reprogramming as drivers of idiopathic pulmonary fibrosis. *Redox Biol* **33**, 101509, doi:10.1016/j.redox.2020.101509 (2020).

23 Kleaveland, K. R. *et al.* Fibrocytes are not an essential source of type I collagen during lung fibrosis. *J Immunol* **193**, 5229-5239, doi:10.4049/jimmunol.1400753 (2014).

24 Coppe, J. P., Desprez, P. Y., Krtolica, A. & Campisi, J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol* **5**, 99-118, doi:10.1146/annurev-pathol-121808-102144 (2010).

25 Jin, H. J. *et al.* Senescence-Associated MCP-1 Secretion Is Dependent on a Decline in BMI1 in Human Mesenchymal Stromal Cells. *Antioxid Redox Signal* **24**, 471-485, doi:10.1089/ars.2015.6359 (2016).

26 Al-Mudares, F. *et al.* Role of Growth Differentiation Factor 15 in Lung Disease and Senescence: Potential Role Across the Lifespan. *Front Med (Lausanne)* **7**, 594137, doi:10.3389/fmed.2020.594137 (2020).

27 Yoon, S., Kovalenko, A., Bogdanov, K. & Wallach, D. MLKL, the Protein that Mediates Necroptosis, Also Regulates Endosomal Trafficking and Extracellular Vesicle Generation. *Immunity* **47**, 51-65 e57, doi:10.1016/j.immuni.2017.06.001 (2017).

28 Meiners, S. & Lehmann, M. Senescent Cells in IPF: Locked in Repair? *Front Med (Lausanne)* **7**, 606330, doi:10.3389/fmed.2020.606330 (2020).

29 Baker, D. J. *et al.* Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* **479**, 232-236, doi:10.1038/nature10600 (2011).

30 Hu, L. *et al.* Why Senescent Cells Are Resistant to Apoptosis: An Insight for Senolytic Development. *Front Cell Dev Biol* **10**, 822816, doi:10.3389/fcell.2022.822816 (2022).

31 Lehmann, M. *et al.* Senolytic drugs target alveolar epithelial cell function and attenuate experimental lung fibrosis ex vivo. *Eur Respir J* **50**, doi:10.1183/13993003.02367-2016 (2017).

32 Kirkland, J. L. & Tchkonia, T. Cellular Senescence: A Translational Perspective. *EBioMedicine* **21**, 21-28, doi:10.1016/j.ebiom.2017.04.013 (2017).

33 Moodley, Y. P. *et al.* Comparison of the morphological and biochemical changes in normal human lung fibroblasts and fibroblasts derived from lungs of patients with idiopathic pulmonary fibrosis during FasL-induced apoptosis. *J Pathol* **202**, 486-495, doi:10.1002/path.1531 (2004).

34 Yosef, R. *et al.* Directed elimination of senescent cells by inhibition of BCL-W and BCL-XL. *Nat Commun* **7**, 11190, doi:10.1038/ncomms11190 (2016).

35 Kasam, R. K., Reddy, G. B., Jegga, A. G. & Madala, S. K. Dysregulation of Mesenchymal Cell Survival Pathways in Severe Fibrotic Lung Disease: The Effect of Nintedanib Therapy. *Front Pharmacol* **10**, 532, doi:10.3389/fphar.2019.00532 (2019).

36 Salminen, A., Ojala, J. & Kaarniranta, K. Apoptosis and aging: increased resistance to apoptosis enhances the aging process. *Cell Mol Life Sci* **68**, 1021-1031, doi:10.1007/s00018-010-0597-y (2011).

37 Cho, H. J. *et al.* Nintedanib induces senolytic effect via STAT3 inhibition. *Cell Death Dis* **13**, 760, doi:10.1038/s41419-022-05207-8 (2022).

38 Herrera, J., Henke, C. A. & Bitterman, P. B. Extracellular matrix as a driver of progressive fibrosis. *J Clin Invest* **128**, 45-53, doi:10.1172/JCI93557 (2018).

39 Collins, B. F. & Raghu, G. Antifibrotic therapy for fibrotic lung disease beyond idiopathic pulmonary fibrosis. *Eur Respir Rev* **28**, doi:10.1183/16000617.0022-2019 (2019).

40 Roach, K. M. *et al.* Evaluation of Pirfenidone and Nintedanib in a Human Lung Model of Fibrogenesis. *Front Pharmacol* **12**, 679388, doi:10.3389/fphar.2021.679388 (2021).
41 Takenouchi, Y., Kitakaze, K., Tsuboi, K. & Okamoto, Y. Growth differentiation factor 15 facilitates lung fibrosis by activating macrophages and fibroblasts. *Exp Cell Res* **391**, 112010, doi:10.1016/j.yexcr.2020.112010 (2020).
42 Han, J., Zhong, C. Q. & Zhang, D. W. Programmed necrosis: backup to and competitor with apoptosis in the immune system. *Nat Immunol* **12**, 1143-1149, doi:10.1038/ni.2159 (2011).
43 Tao, P. *et al.* A dominant autoinflammatory disease caused by non-cleavable variants of RIPK1. *Nature* **577**, 109-114, doi:10.1038/s41586-019-1830-y (2020).
44 Muller, T. *et al.* Necroptosis and ferroptosis are alternative cell death pathways that operate in acute kidney failure. *Cell Mol Life Sci* **74**, 3631-3645, doi:10.1007/s00018-017-2547-4 (2017).
45 Gautheron, J. *et al.* The necroptosis-inducing kinase RIPK3 dampens adipose tissue inflammation and glucose intolerance. *Nat Commun* **7**, 11869, doi:10.1038/ncomms11869 (2016).
46 Lee, J. M. *et al.* Involvement of Alveolar Epithelial Cell Necroptosis in Idiopathic Pulmonary Fibrosis Pathogenesis. *Am J Respir Cell Mol Biol* **59**, 215-224, doi:10.1165/rcmb.2017-0034OC (2018).
47 Choi, M. E., Price, D. R., Ryter, S. W. & Choi, A. M. K. Necroptosis: a crucial pathogenic mediator of human disease. *JCI Insight* **4**, doi:10.1172/jci.insight.128834 (2019).
48 Rodriguez, D. A. *et al.* Characterization of RIPK3-mediated phosphorylation of the activation loop of MLKL during necroptosis. *Cell Death Differ* **23**, 76-88, doi:10.1038/cdd.2015.70 (2016).
49 McComb, S. *et al.* Cathepsins limit macrophage necroptosis through cleavage of Rip1 kinase. *J Immunol* **192**, 5671-5678, doi:10.4049/jimmunol.1303380 (2014).
50 Droga-Mazovec, G. *et al.* Cysteine cathepsins trigger caspase-dependent cell death through cleavage of bid and antiapoptotic Bcl-2 homologues. *J Biol Chem* **283**, 19140-19150, doi:10.1074/jbc.M802513200 (2008).
51 Karman, J., Wang, J., Bodea, C., Cao, S. & Levesque, M. C. Lung gene expression and single cell analyses reveal two subsets of idiopathic pulmonary fibrosis (IPF) patients associated with different pathogenic mechanisms. *PLoS One* **16**, e0248889, doi:10.1371/journal.pone.0248889 (2021).
52 Habiel, D. M. *et al.* CCR10+ epithelial cells from idiopathic pulmonary fibrosis lungs drive remodeling. *JCI Insight* **3**, doi:10.1172/jci.insight.122211 (2018).
53 Hohmann, M. S. *et al.* Antibody-mediated depletion of CCR10+EphA3+ cells ameliorates fibrosis in IPF. *JCI Insight* **6**, doi:10.1172/jci.insight.141061 (2021).

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Authors contributions

SBG and MSH conceived, designed, performed experiments, and analyzed results. SBG wrote and edited the manuscript. WAVJ and CMH conceived and designed the experiments and wrote and edited the manuscript. SBG and MSH performed experiments and edited the manuscript. ALC assisted with experiments.

Data availability statement

The datasets used and analyzed during this study are available from the corresponding author upon reasonable request.

Competing interests: The authors have declared that no competing interest exists.

4. ARTICLE II FOR PUBLICATION

The present study was performed in Hogaboam laboratory at Cedars-Sinai Medical Center, Los Angeles, EUA in collaboration with Laboratório de Dor, Inflamação, Neuropatia e Câncer, from Universidade Estadual de Londrina, Londrina, Brazil. The results are described in the article entitled "*Effects of specialized pro-resolving lipid mediator Resolvin D2 on proliferative and senescent human lung fibroblasts,*" to be submitted to *Nature Scientific Reports*.

Effects of specialized pro-resolving lipid mediator Resolvin D2 on proliferative and senescent human lung fibroblasts

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Abstract

Cellular senescence is considered a key mechanism in idiopathic pulmonary fibrosis. Specialized pro-resolving lipid mediators are effective at improving infection clearance and hold strong therapeutic potential in the management of COVID-19 as they can regulate macrophage infiltration and cytokine production but also promote a pro-resolving macrophage phenotype. It is presently unclear whether Resolvin D2 (RvD2) presents antifibrotic or senolytic properties. In this study, we attempted to illuminate the effects of Resolvin D2 on proliferative and senescent lung fibroblasts from normal and IPF patients. After RvD2 addition, with or without FasL, cell viability, caspase-3 release, and LDH leakage were measured. Colorimetric/fluorimetric assays, phase-contrast imaging, RT-qPCR, and western blotting were used to evaluate the effect of RvD2 on senescent normal and IPF fibroblasts. The treatment with RvD2 did not provoke cell death in senescence lung fibroblast. In addition, RvD2 did not influence fibroblast invasion in proliferative lung fibroblasts. We found that RvD2 was not able to influence SASP released factors. However, it significantly decreases transcript levels of *BIRC5*, *CCR10*, *COL1A1*, *COL3A1*, *FN1*, GDF15, and *WNT16* in senescent IPF fibroblasts. Our results suggest that RvD2 can reduce the expression of fibrosis and senescence markers. However, further studies are necessary to fully elucidate the effects of RvD2 in IPF.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a destructive, chronic, and age-related disease characterized by excessive deposition of extracellular matrix leading to irreversible loss of lung function and structure and progressive dyspnea (RAGHU; COLLARD; EGAN; MARTINEZ *et al.*, 2011). It is the most common type of interstitial pneumonia, occurs in elderly adults over the age of 50, with a median survival time between 3 and 5 years after diagnosis (KING; PARDO; SELMAN, 2011).

The treatment available consists of two drugs approved by US Food and Drug Administration, nintedanib and pirfenidone. Although both drugs suppress disease progression by reducing lung function decline, they are associated with tolerability issues and several side effects such as nausea, fatigue, and diarrhea (SHENDEROV; COLLINS; POWELL; HORTON, 2021). Therefore, there is a need to seek other therapeutic approaches for IPF

Resolvins are specialized pro-resolving lipid mediators (SPMs), derived from the ω-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), classified as D-series and E-series respectively (BASIL; LEVY, 2016). RvD2 exerts its potent actions on phagocytes, cell type- and organ-specific actions promoting tissue protection, repair, and regeneration (CHIANG; SERHAN, 2020). RvD2 binds to its selective receptor G protein-coupled receptor 18 (GPR18), which plays a role in the resolution of inflammation by modulating the expression of neutrophils, monocyte-derived macrophages, and lymphocytes (KYTIKOVA; NOVGORODTSEVA; DENISENKO; ANTONYUK *et al.*, 2019).

Several studies have demonstrated that RvD2 attenuates early brain injury in a rat model (ZHANG; ZUO; ZHANG, 2021), prevents experimental colitis (BENTO, ALLISSON FREIRE; CLAUDINO, RAFAELA FRANCO; DUTRA, RAFAEL

CYPRIANO; MARCON, RODRIGO *et al.*, 2011), reduces fibromyalgia-induced pain (KLEIN; SPEROTTO; MACIEL; LEITE *et al.*, 2014), decreases renal injury in mice, and prevents periodontitis (MIZRAJI; HEYMAN; VAN DYKE; WILENSKY, 2018).

In bleomycin-induced pulmonary fibrosis in mice, 17(R)-resolvin D1 attenuated the inflammation by stimulating the resolution of neutrophilic inflammation and downregulating IL-1β mRNA expression (YATOMI; HISADA; ISHIZUKA; KOGA *et al.*, 2015). Moreover, macrophages treated with RvD1 or RvD2 presented decreased levels of cytokine release and enhanced phagocytic activity, providing *in vitro* evidence that SPMs regulate the resolution of COVID-19 (RECCHIUTI; PATRUNO; MATTOSCIO; ISOPI *et al.*, 2021).

However, there are no studies that show the role of RvD2 in lung fibroblasts or idiopathic pulmonary fibrosis. Given the effects of RvD2, we sought to investigate whether RvD2 could employ a senolytic or anti-fibrotic effects in normal or IPF senescent lung fibroblast and evaluate the effect of this drug on fibroblast invasion.

Results

Resolvin D2 does not alter cell viability, LDH leakage, or caspase-3 release

To evaluate whether Resolvin D2 modulates apoptosis in senescent normal and IPF lung fibroblasts, cells were treated with RvD2 (1nm) or vehicle for 24h, followed by Super Fas ligand (100 ng/mL) for 3h. We observed that RvD2 did not show an effect on cell viability or lactate dehydrogenase (LDH) leakage. However, it was observed an increase in caspase-3 release in normal fibroblasts after the treatment with RvD2 when combined with the cell death ligand, FasL (Figure 1C).

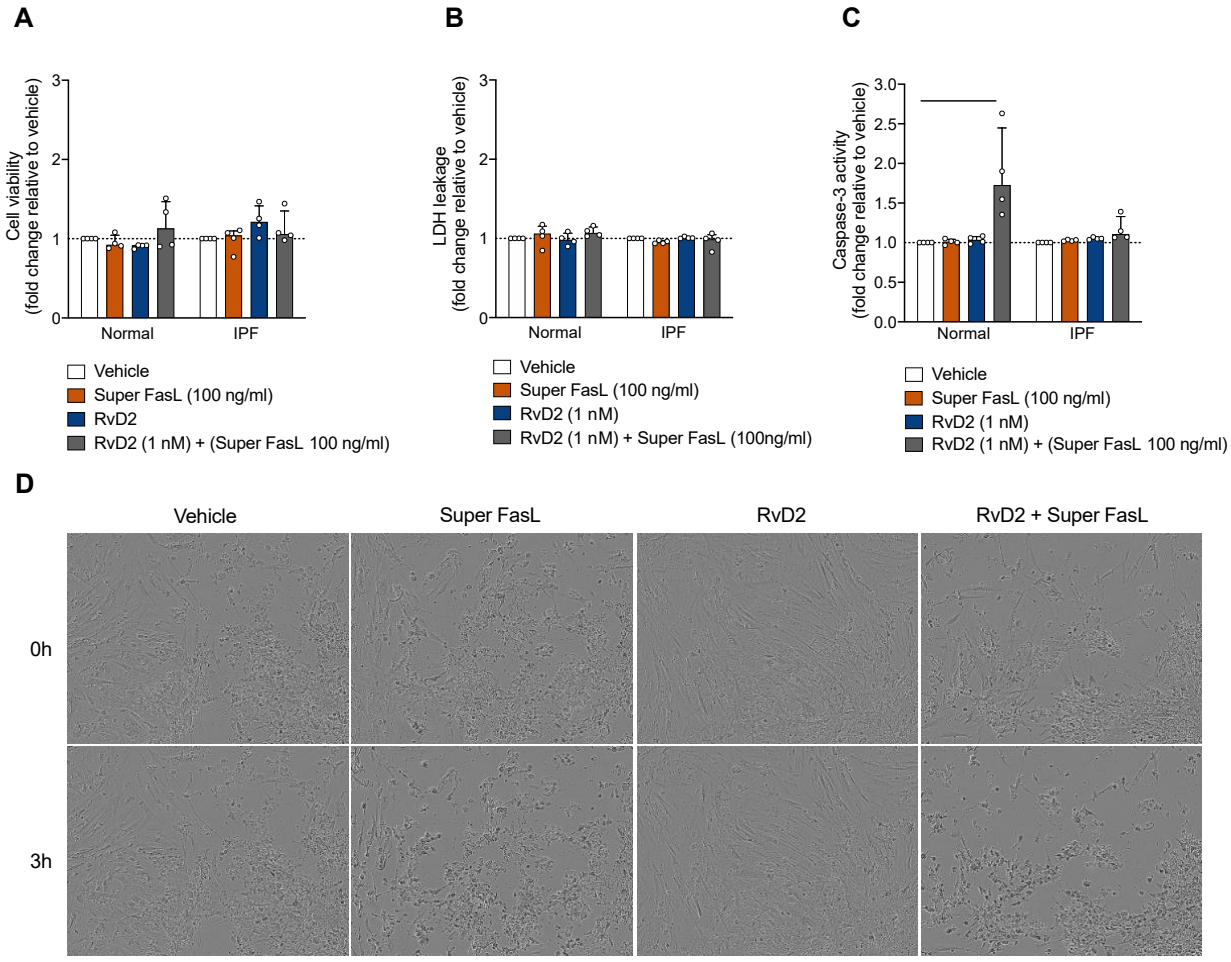

Figure 1. Cell viability, lactate dehydrogenase (LDH) release, caspase-3 activity, and phase-contrast images of senescent lung fibroblasts from normal and IPF patients after 24-hour treatment with RvD2 (1nM) or vehicle, followed by 3h of Super FasL (100ng/mL). Cell viability was expressed as optical density (OD) values at 450 nm, LDH release as the difference in the absorbance values at 490 and 680 nm, and caspase-3 activity as relative AFC. Data are presented as median with an interquartile range (n = 3 or 4 per group). *p<0.05, **p<0.01 as indicated by the bars.

Resolvin D2 does not influence normal or IPF lung fibroblast invasion

To assess the modulation of lung fibroblasts invasion by RvD2, normal and IPF proliferative fibroblasts were plated into 96-well plates, scratched using a

WoundMaker, and treated with vehicle or RvD2 (1nM), and monitored using an Incucyte Zoom live-cell imager for 72 hours. When treated with RvD2, control (Figure 2A-D) and IPF (Figure 2E-F) fibroblasts showed the same pattern of invasive wound healing when compared to the vehicle, demonstrating that RvD2 does not influence fibroblast invasion.

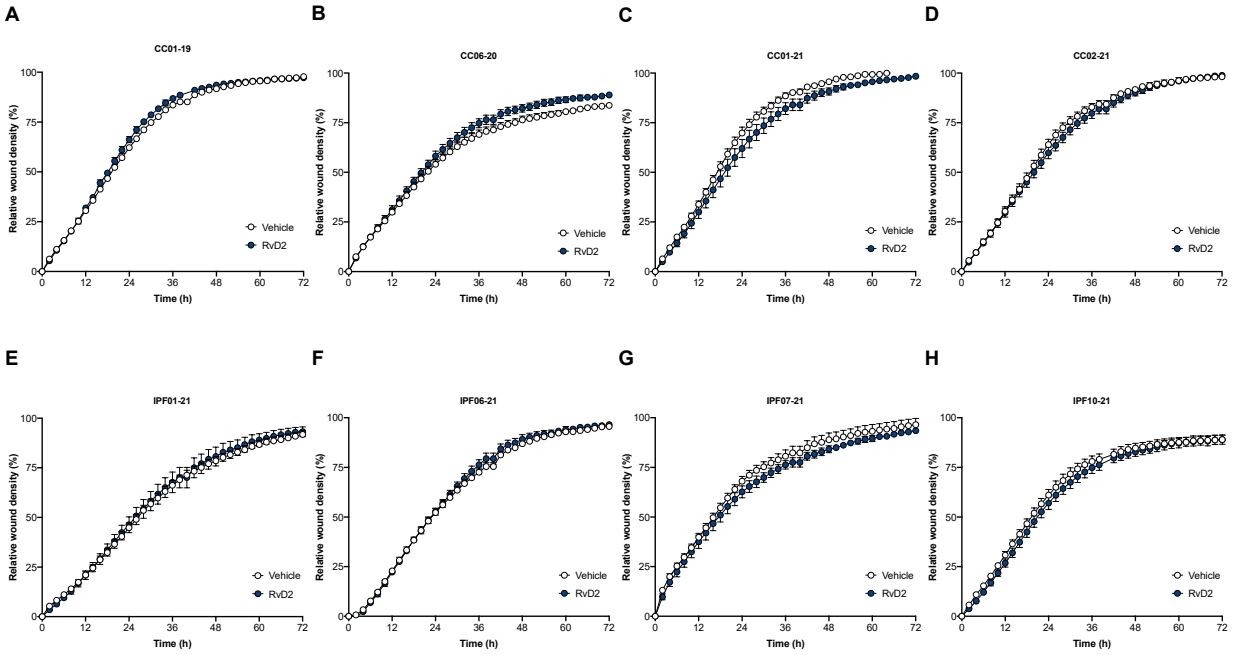

Figure 2. Normal and IPF proliferative lung fibroblasts were plated into 96-well plates, scratched using a WoundMaker, treated with vehicle or RvD2 (1nM), and monitored using an Incucyte Zoom live-cell imager for 72 hours. Depicted is the average wound closure (relative to the initial wound) over 72 hours of normal (n = 4) or IPF (n = 4) fibroblasts. Data shown are the mean and error ± SEM.

RvD2 does not influence interleukin (IL)-6, IL-8, monocyte chemoattractant protein (*MCP-1),* growth differentiation factor 15 (*GDF15), and WNT16 levels in senescent cells*

Senescent cells secrete a myriad of interleukins, cytokines, and growth factors constituting the SASP which exerts paracrine effects (COPPE; DESPREZ; KRTOLICA; CAMPISI, 2010). Among SASP factors, we investigated the effect of RvD2 on IL-6, IL-8, MCP-1, GDF-1,5, and WNT16. However, we observed that RvD2 has no significant difference in those SASP components in senescent fibroblasts when compared to vehicle (Figure 3A-E).

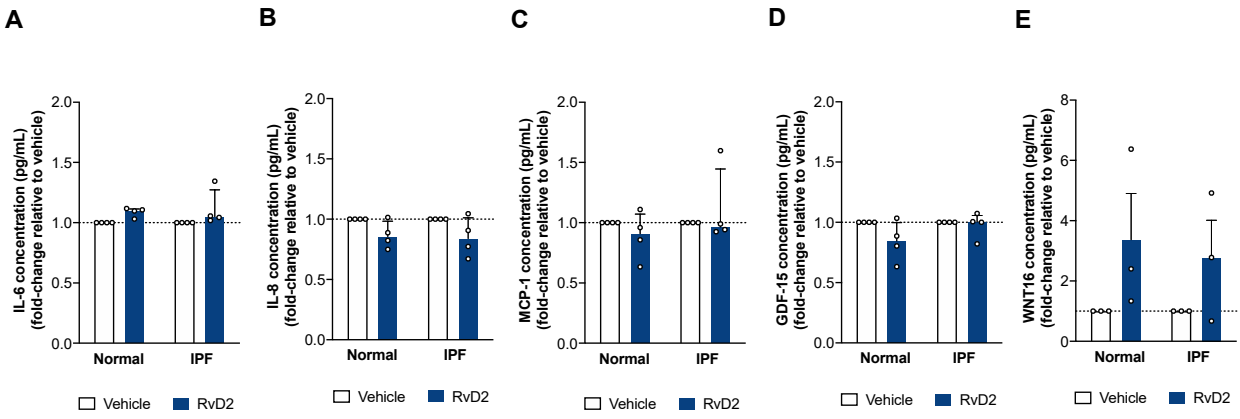

Figure 3. RvD2 does not have an impact on the SASP release. Effects of RvD2 (1nM) or control on IL-6 (A), IL-8 (B), MCP-1 (C), GDF-15 (D) and WNT16 (E) levels. Data are presented as median with an interquartile range (n= 3 or 4 per group). *P<0.05 as indicated by the bars.

Effects of RvD2 on senescent lung fibroblasts

Next, we aimed to investigate the effect of RvD2 on senescent lung fibroblasts. BIRC5 gene encodes the protein survivin, which inhibits both intrinsic and extrinsic pathways of apoptosis (GARG; SURI; GUPTA; TALWAR *et al.*, 2016). RvD2 inhibited *Baculoviral IAP Repeat Containing 5* (*BIRC5)* mRNA expression in IPF senescent fibroblasts but not in normal fibroblasts. *C-C motif chemokine receptor (CCR10)* expression is increased in rapidly progressive IPF (HOHMANN; HABIEL;

ESPINDOLA; HUANG *et al.*, 2021), we observed that cells treatment with RvD2 presented a reduction of CCR10 mRNA expression only in IPF senescent cells. EPH Receptor A3 (Epha3) is a recognized marker for mesenchymal cells (HOHMANN; HABIEL; ESPINDOLA; HUANG *et al.*, 2021). Its mRNA expression was increased only in normal lung fibroblast after the treatment with RvD2. Deposition of the extracellular matrix is crucial to the pathogenesis of IPF (HYNES, 2009). Resolvin D2 was able to reduce the mRNA expression of genes encoding collagen-1 (*COL1A1*), collagen-3 (*COL3A1*), and fibronectin-1 (FN1) in IPF fibroblasts. Moreover, GDF15, IL-6, ILand -33, and WNT16 presented reduced mRNA expression after RvD2 treatment in normal and IPF fibroblasts. The expression of *GPR18* mRNA was increased after the treatment with RvD2 in normal senescent fibroblast. *Alpha-smooth muscle actin cyclin-dependent kinase inhibitor (CDK)1A1, CDK2A2, decoy receptor 3 (DCR3), IL-1β, MCP-1, matrix metallopeptidase (MMP-9), NADPH oxidase 4 (NOX-4), plasminogen activator inhibitor 1 (PAI-1) and ST2* mRNA expression were altered by RvD2 treatment neither in normal nor IPF senescent lung fibroblasts.

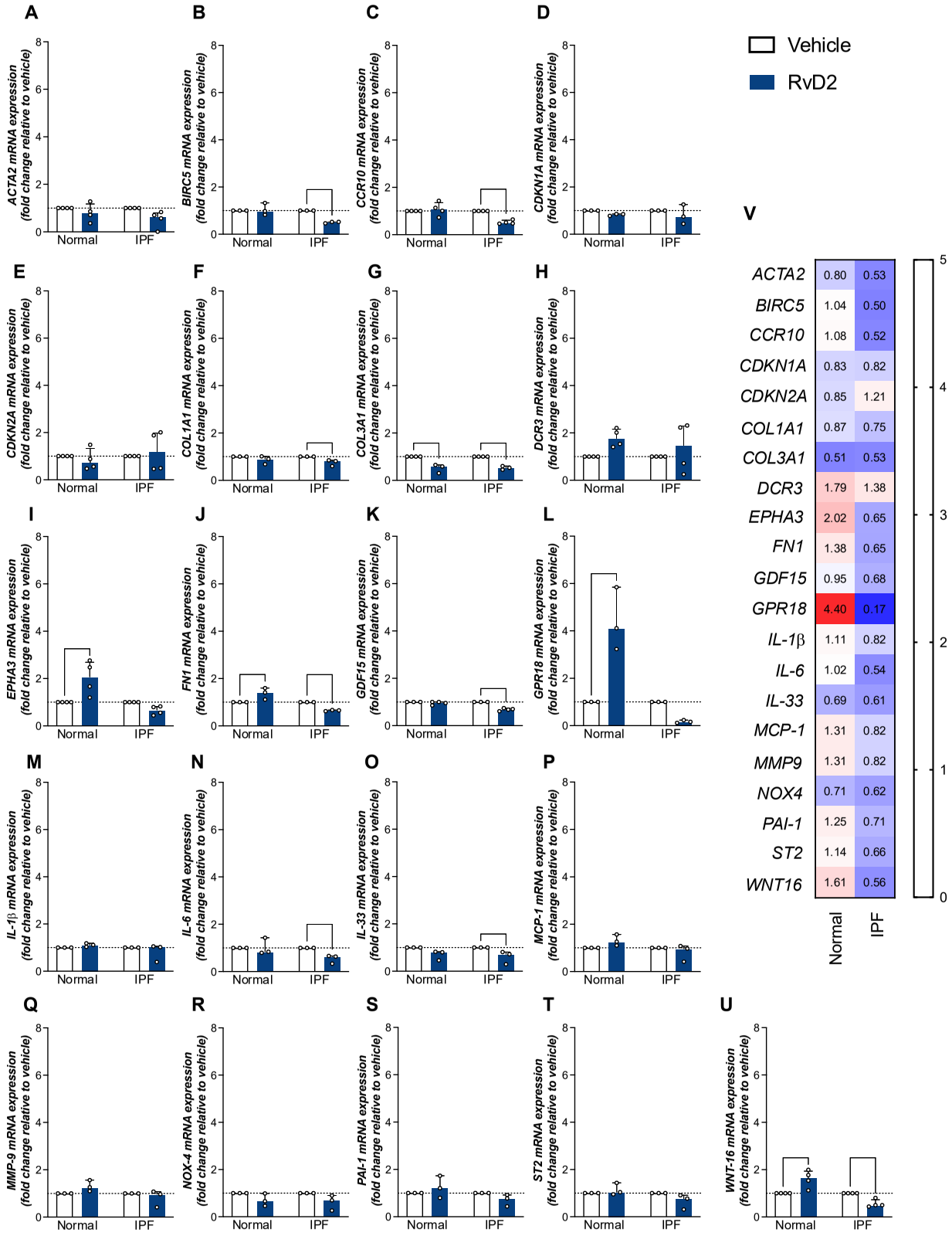

Figure 4. Effects of RvD2 drugs on *ACTA2, BIRC5, CCR10, CDKN1A, CDKN2A, COL1A1, COL3A1, DCR3, EPHA3, FN1, GDF15, GPR18, IL-1β, IL-6, IL-33, MCP-1, MMP9, NOX-4, PAI-1, ST2* and *WNT16* mRNA expression in normal and IPF

senescent lung fibroblasts (Figure 4A-U). Heatmap of the expression of SASP, inflammation-, antioxidant-, apoptosis- and fibrosis-related genes in lung fibroblasts from normal and IPF patients treated with RvD2 (1nM) or vehicle for 24h (Figure 4V). All transcripts were first normalized to the housekeeping gene 18S. Red shows the upregulation of gene expression, and blue represents the downregulation of gene expression compared with vehicle-treated cells. Data are presented as median with an interquartile range (n= 3 or 4 per group). *P<0.05; **P<0.01; ***P<0.001 and ***P<0.0001 as indicated by the bars.

RvD2 does not alter GPR18 protein levels

Next, we evaluated the influence of RvD2 on its receptor GPR18 protein level. We did not see an increase in GPR18 protein levels after the treatment with RvD2 in normal senescent fibroblasts (Figure 5A), and the same effect was observed in senescent IPF fibroblasts (Figure 5B).

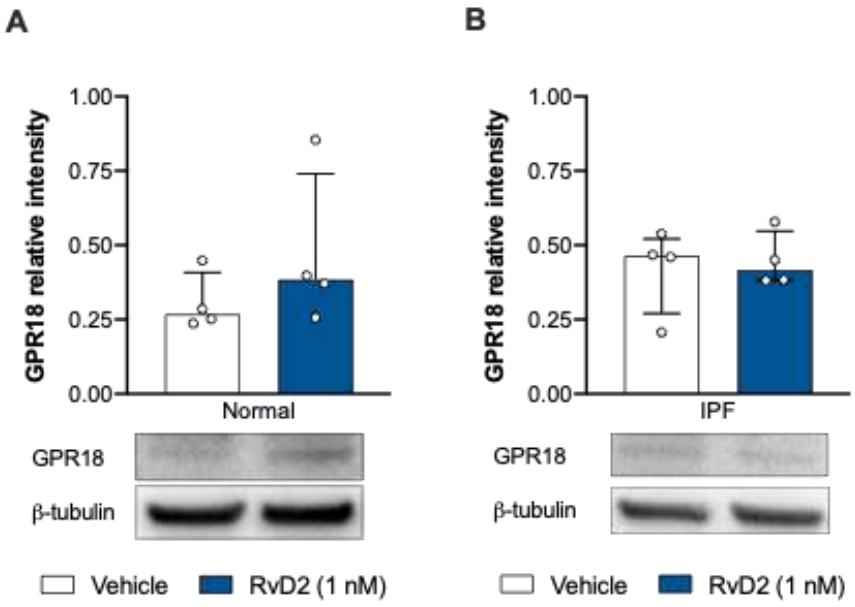

Figure 5. Effects of RvD2 on GPR18 (A) Representative western blot of GPR18 protein expression on normal senescent lung fibroblasts. (B) Representative western blot of GPR18 protein expression on IPF senescent lung fibroblasts. Data are presented as median with interquartile range (n = 5 per group), one-way ANOVA followed by Tukey's test. *p<0.05 **p<0.01 and p<0.001 as indicated by the bars.

Discussion

IPF is a chronic, fibroproliferative lung disease of unknown etiology and limited therapeutic options (FRASER; HOYLES, 2016). Cellular senescence has been associated with the etiology of IPF. One of the hypotheses of how cell senescence contributes to the progression of IPF is that it protects myofibroblasts, the major producer of extracellular matrix proteins, from undergoing apoptosis (LIU; LIU, 2020). Thus, it is of great importance to explore the mechanisms and develop better therapeutic strategies for IPF that target the removal of senescence cells.

In the present study, we showed that RvD2 does not trigger apoptosis in senescent lung fibroblasts. Survivin suppresses cell death by suppressing the activities of caspase-3 and caspase-7 to resist apoptosis induced by specific stimuli (LI; HU; LI, 2018). Following that, we observed that RvD2 inhibits *BIRC5* mRNA expression in IPF senescent fibroblasts, which justifies the reason why it was not observed an effect on caspase-3 release. The same inhibition was not observed in normal senescent cells, which were more susceptible to apoptosis as it was observed by the increased release of caspase-3 in normal senescent fibroblasts. In addition, we did not detect alterations in the LDH release or cell viability after RvD2 treatment, confirming that this drug does not drive apoptosis of senescent cells.

To further explore its therapeutic potential, the effect of RvD2 was evaluated this time in proliferative lung fibroblasts. We observed there was no effect on invasion neither in normal nor in IPF fibroblasts. These results suggest that RvD2 does not influence invasive wound healing.

One of IPF hallmarks is the deposition of ECM disrupting the lung architecture resulting in a decrease in lung volume and weakened gas exchange (LEDERER; MARTINEZ, 2018). Among cell types contributing to the pathogenesis of IPF, fibroblast produces extensive amounts of ECM proteins, such as type I and III collagen and fibronectin (CLARKE; CARRUTHERS; MUSTELIN; MURRAY, 2013). Our data showed that RvD2 was capable of decreasing the expression of collagen-1, collagen-3, and fibronectin-1 by senescent fibroblasts, which play a pivotal role in the disease pathogenesis. By reducing the ECM components, the treatment with RvD2 could improve gas exchange in the lung, resulting in an improvement in the patient's quality of life.

Subsequently, we observed diminished CCR10 mRNA expression after the treatment with RvD2. Habiel et al. proposed that higher expression of CCR10 in IPF lungs could promote lung remodeling of IPF (HABIEL; ESPINDOLA; JONES; COELHO *et al.*, 2018). Moreover, it has been demonstrated that the depletion of $CCR10^{+}EphA3^{+}$ cells ameliorates fibrosis in IPF (HOHMANN; HABIEL; ESPINDOLA; HUANG *et al.*, 2021). However, we did not detect the decrease of EphA3 when senescent cells were treated with RvD2. In addition, RvD2 increased the expression of its receptor, GPR18, in normal senescent fibroblasts but not in IPF senescent cells. Considering our observation that GPR18 mRNA expression was decreased in IPF senescent fibroblast, we hypothesize that this receptor may be internalized in IPF cells. Internalization of G-protein-coupled receptors (GPCRs) generally occurs in response to agonist activation

of the receptors, leading to a redistribution of receptors away from the plasma membrane toward endosomes (KOENIG, 2004). However, the consequence of this possible internalization still needs to be further explored.

In addition, we observed a reduction of GDF15 mRNA expression after RvD2 treatment. GDF15 seems to be elevated only when telomeres reach their functional threshold length, and it is known as a novel biomarker for IPF severity, expressed predominantly by epithelial cells (ZHANG; JIANG; NOURAIE; ROTH *et al.*, 2019). Lambrecht et al. have shown that GDF15 levels were increased in the bleomycin-exposed lungs and a reduction of expression of IL-6 in the lung fibroblasts when they exposed GDF15 deficient mice to bleomycin (LAMBRECHT; SMITH; DE WILDE; COUDENYS *et al.*, 2014). We observed a reduction of IL-6 and a decrease in IL-33 expression. Studies have shown IL-33 as an important profibrogenic cytokine that stimulates the initiation and progression of pulmonary fibrosis by recruiting IL-6-dependent alternative activated macrophages, leading to pulmonary fibrosis (FANNY; NASCIMENTO; BARON; SCHRICKE *et al.*, 2018).

The treatment with RvD2 showed a striking reduction of WNT16 mRNA expression in senescent IPF fibroblasts and an increase in its expression in normal senescent fibroblasts. Wnt16 has been described to facilitate cell senescence and increase SASP mRNA expression in ankylosing spondylitis osteoprogenitor cells after H_2O_2-induced stimulation, which was reverted after Wnt16 knockdown cultures (JO; WEON; NAM; JANG *et al.*, 2021). Later, Binet *et al*. proposed Wnt16b as a novel marker of cellular senescence, postulating its activation is dependent on p53 activation through AKT pathway (BINET; YTHIER; ROBLES; COLLADO *et al.*, 2009). Considering that cellular senescence is pivotal to IPF pathophysiology (KELLOGG; KELLOGG; MUSI; NAMBIAR, 2021), and Wnt16 rises as a new IPF marker, the

reduction of its expression after RvD2 constitutes an important finding, suggesting RvD2 could exert senomorphic activities. Notwithstanding, the role of Wnt16 in senescent fibroblasts has not been explored until this moment.

Overall, we demonstrated for the first time that RvD2 could act by reducing pro-fibrotic and senomorphic mechanisms. Our findings highlight the importance of the SPM RvD2 as a potential therapeutic approach for IPF.

Methods

Study approval

All the experiments with primary human tissue were approved by the Institutional Review Board at Cedars-Sinai Medical Center (PRO 00035396 AND PRO 00034067). Informed consent was obtained prior to inclusion in the studies described.

Senescent fibroblast generation

Normal and IPF lung explants were obtained from consenting patients. Proliferative and senescent lung fibroblasts were cultured in DMEM supplemented with 15% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% glutamine at 37 ºC, and 10% CO_2. Primary normal lung fibroblasts were derived from nonfibrotic lung samples without signs of disease. To obtain senescent fibroblasts, proliferative normal and IPF lung fibroblasts were repeatedly passaged in culture until they reached a senescent morphological phenotype (enlarged, flattened, and irregular shape) and senescence-associated β-galactosidase activity (HOHMANN; HABIEL; COELHO; VERRI *et al.*, 2019).

Cell viability

Cell viability was evaluated using the AlamarBlue Cell Viability Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Senescent lung fibroblasts (3 x 10^4 cells/well) were treated with RvD2 (1nm) for 24h, followed by 3h of Super FasL (100ng/mL) stimuli in a 96-well plate. After 27h, 1:10 volume of cell viability reagent was added to the cells and incubated for 4 hours at 37°C. Fluorescence values were assessed using a fluorescence excitation wavelength of 560 nm and an emission of 590 nm.

Caspase-3 assay

To evaluate the effect of RvD2 on caspase-3 activity, a Caspase-3/CPP32 Fluorometric Assay Kit (BioVision Inc., Milpitas, CA, USA) was used. Senescent fibroblasts in a 96-well plate were treated with RvD2 (1nm) for 24h, followed by 3h of Super FasL stimuli. After 27h of treatment, cells were lysed in 50 µl chilled cell lysis buffer on ice for 10 min. Then, 50 µl of 2X reaction buffer (containing 10 mM DTT) was added, followed by 50 µM DEVD-AFC substrate, incubated at 37 °C for 2 h. Results were read at 400 nm excitation filter and 505 nm emission filter with a fluorescent microplate reader (Biotek). Results were expressed as fold-change compared to control cells.

Lactate dehydrogenase (LDH) assay

To perform the assay, 50 µL of cell culture media was transferred to a new plate, and 50µL of CyQUANT LDH Cytotoxicity Assay Kit reaction mixture was added. After 30 minutes of incubation at room temperature, protected from light, the assay was stopped by the addition of stop solution, and then absorbance was measured at 490 nm and 680 nm using a microplate reader.

Senescence associated β-galactosidase detection

After 24h of treatment with RvD2 (1nm), cells were lysed with 50 µL of lysis buffer in a 96-well plate and incubated for 10 minutes. Then, 50 µL of SPiDER- βgal working solution was added to each well and incubated at 37 °C for 30 minutes. The assay was stopped by the addition of stop solution, and then fluorescence signals were measured at excitation 540nm and emission 580nm.

qPCR-RT

Cells were lysed in Trizol™ reagent (Thermo Fisher Scientific, Waltham, MA, USA), and RNA was extracted as recommended by the manufacturer. 3µg of RNA was reverse transcribed into cDNA using SuperScript™ II Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) as previously described (HOHMANN; HABIEL; ESPINDOLA; HUANG *et al.*, 2021). Complementary DNA (cDNA) was loaded into a TaqMan plate (Thermo Fisher Scientific, Waltham, MA, USA), and gene expression analyses were performed using TaqMan master mix (Thermo-Fisher Scientific) probes for human alpha-smooth muscle actin (*ACTA2), C-C motif chemokine receptor 10 (CCR10), cyclin-dependent kinase inhibitor (CDK)N1A, CDKN2A, collagen (COL)1A1, COL3A1, EPH Receptor A3 (EPHA3), (fibronectin) FN1, GDF15, GPR18* and *WNT16* (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative PCR analysis was performed using Viia7 Thermocycler (Thermo Fisher Scientific, Waltham, MA, USA). Results were exported and normalized to *RNA18S5* expression. Fold change values were calculated using DataAssist software version 3.01 (Thermo Fisher Scientific, Waltham, MA, USA).

ELISA

IL-6, IL-8, MCP-1, and GDF15 were determined in senescent fibroblast supernatant, and WNT16 levels were determined in senescent fibroblast lysates using a standardized sandwich ELISA technique (R&D Systems, Minneapolis, MN, USA).

Fibroblast invasion analysis

To perform fibroblast scratch wound invasion, 96 well ImageLockTM plates (Essen BioScience, Ann Arbor, MI, USA) were coated with 50 µg/mL of basement membrane extract (BME) (Bio-techne, Minneapolis, MN, USA) for 1 h at room temperature. After coating, the BME solution was removed, and normal and IPF proliferating fibroblasts were added at a concentration of 3 x 10^4/well. Cells were then incubated overnight at 37^{o}C and 10% CO_2. After incubation, cells were then scratched using the WoundMakerTM (Essen BioScience, Ann Arbor, MI, USA), washed with PBS, and then treated with RvD2 in a solution containing 4 mg/mL BME diluted with a complete medium. Cells were then placed into the IncuCyte ZOOM live-cell imager (Essen BioScience, Ann Arbor, MI, USA) for imaging once every 2 hours at 100x magnification for 72h at 37^{o}C and 10% CO_2. Wound closure was measured using the IncuCyte ZOOM Software (Essen BioScience, Ann Arbor, MI, USA) as relative wound density.

Western blotting

Cells were lysed using RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Protein concentrations were measured by using a DC protein assay (Bio-Rad Laboratories Inc., Hercules, CA, USA). and the same amount of protein was loaded into a 4–15% NuPAGE Bis-Tris Protein gel. Gels were transferred using an iBlot Dry blotting system onto nitrocellulose membranes (Thermo

Fisher Scientific, Waltham, MA, USA), and the transferred samples were blocked for 1 hour at room temperature in 5% non-fat-dry-milk in tris-buffered saline (TBS). The primary antibody Anti-GPCR GPR18 antibody (ab76258, Abcam, Cambridge, UK) was used to detect GPR18 levels. Images of chemiluminescent bands were acquired using a Bio-Rad Gel documentation system (Bio-Rad Laboratories, Inc.). Membranes were washed in TBS-T, blotted with anti-tubulin antibody (ab6046, Abcam, Cambridge, UK), and developed. Image Lab Software version 6.0.1 (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used to perform densitometric analysis.

Data analysis

Statistical analyses were performed using GraphPad Prism 9.0.1 (GraphPad Software, San Diego, CA, USA). Data were expressed as mean ± SEM and evaluated for significance by one-way ANOVA followed by Tukey's test. A value of $P < 0.05$ was considered statistically significant.

Authors contributions

SBG, MSH, and MSE conceived, designed, performed experiments, and analyzed results. SBG, MSH, and MSE wrote and edited the manuscript. WAVJ and CMH conceived and designed the experiments and wrote and edited the manuscript. SBG, MSH, and MSE performed experiments and edited the manuscript. ALC assisted with experiments.

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Conflict of interest

There is no conflict of interest to be declared.

References

1 Raghu, G. *et al.* An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. *Am J Respir Crit Care Med* **183**, 788-824, doi:10.1164/rccm.2009-040GL (2011).

2 King, T. E., Jr., Pardo, A. & Selman, M. Idiopathic pulmonary fibrosis. *Lancet* **378**, 1949-1961, doi:10.1016/S0140-6736(11)60052-4 (2011).

3 Shenderov, K., Collins, S. L., Powell, J. D. & Horton, M. R. Immune dysregulation as a driver of idiopathic pulmonary fibrosis. *J Clin Invest* **131**, doi:10.1172/JCI143226 (2021).

4 Basil, M. C. & Levy, B. D. Specialized pro-resolving mediators: endogenous regulators of infection and inflammation. *Nat Rev Immunol* **16**, 51-67, doi:10.1038/nri.2015.4 (2016).

5 Chiang, N. & Serhan, C. N. Specialized pro-resolving mediator network: an update on production and actions. *Essays Biochem* **64**, 443-462, doi:10.1042/EBC20200018 (2020).

6 Zhang, T., Zuo, G. & Zhang, H. GPR18 Agonist Resolvin D2 Reduces Early Brain Injury in a Rat Model of Subarachnoid Hemorrhage by Multiple Protective Mechanisms. *Cell Mol Neurobiol*, doi:10.1007/s10571-021-01114-2 (2021).

7 Bento, A. F., Claudino, R. F., Dutra, R. C., Marcon, R. & Calixto, J. B. Omega-3 fatty acid-derived mediators 17 (R)-hydroxy docosahexaenoic acid, aspirin-triggered resolvin D1, and resolvin D2 prevent experimental colitis in mice. *The Journal of Immunology* **187**, 1957-1969 (2011).

8 Klein, C. P. *et al.* Effects of D-series resolvins on behavioral and neurochemical changes in a fibromyalgia-like model in mice. *Neuropharmacology* **86**, 57-66 (2014).

9 Mizraji, G., Heyman, O., Van Dyke, T. E. & Wilensky, A. Resolvin D2 Restrains Th1 Immunity and Prevents Alveolar Bone Loss in Murine Periodontitis. *Front Immunol* **9**, 785, doi:10.3389/fimmu.2018.00785 (2018).

10 Yatomi, M. *et al.* 17(R)-resolvin D1 ameliorates bleomycin-induced pulmonary fibrosis in mice. *Physiol Rep* **3**, doi:10.14814/phy2.12628 (2015).

11 Recchiuti, A. *et al.* Resolvin D1, and D2 reduce SARS-CoV-2-induced inflammatory responses in cystic fibrosis macrophages. *FASEB J* **35**, e21441, doi:10.1096/fj.202001952R (2021).

12 Kytikova, O., Novgorodtseva, T., Denisenko, Y., Antonyuk, M. & Gvozdenko, T. Pro-Resolving Lipid Mediators in the Pathophysiology of Asthma. *Medicina (Kaunas)* **55**, doi:10.3390/medicina55060284 (2019).

13 Coppe, J. P., Desprez, P. Y., Krtolica, A. & Campisi, J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol* **5**, 99-118, doi:10.1146/annurev-pathol-121808-102144 (2010).

14 Garg, H., Suri, P., Gupta, J. C., Talwar, G. P. & Dubey, S. Survivin: a unique target for tumor therapy. *Cancer Cell Int* **16**, 49, doi:10.1186/s12935-016-0326-1 (2016).

15 Hohmann, M. S. *et al.* Antibody-mediated depletion of CCR10+EphA3+ cells ameliorates fibrosis in IPF. *JCI Insight* **6**, doi:10.1172/jci.insight.141061 (2021).

16 Hynes, R. O. The extracellular matrix: not just pretty fibrils. *Science* **326**, 1216-1219, doi:10.1126/science.1176009 (2009).

17 Fraser, E. & Hoyles, R. K. Therapeutic advances in idiopathic pulmonary fibrosis. *Clin Med (Lond)* **16**, 42-51, doi:10.7861/clinmedicine.16-1-42 (2016).

18 Liu, R. M. & Liu, G. Cell senescence and fibrotic lung diseases. *Exp Gerontol* **132**, 110836, doi:10.1016/j.exger.2020.110836 (2020).

19 Li, D., Hu, C. & Li, H. Survivin as a novel target protein for reducing the proliferation of cancer cells. *Biomed Rep* **8**, 399-406, doi:10.3892/br.2018.1077 (2018).

20 Lederer, D. J. & Martinez, F. J. Idiopathic pulmonary fibrosis. *New England Journal of Medicine* **378**, 1811-1823 (2018).

21 Clarke, D. L., Carruthers, A. M., Mustelin, T. & Murray, L. A. Matrix regulation of idiopathic pulmonary fibrosis: the role of enzymes. *Fibrogenesis Tissue Repair* **6**, 20, doi:10.1186/1755-1536-6-20 (2013).

22 Habiel, D. M. *et al.* CCR10+ epithelial cells from idiopathic pulmonary fibrosis lungs drive remodeling. *JCI Insight* **3**, doi:10.1172/jci.insight.122211 (2018).

23 Koenig, J. A. Assessment of receptor internalization and recycling. *Methods Mol Biol* **259**, 249-273, doi:10.1385/1-59259-754-8:249 (2004).

24 Zhang, Y. *et al.* GDF15 is an epithelial-derived biomarker of idiopathic pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* **317**, L510-L521, doi:10.1152/ajplung.00062.2019 (2019).

25 Lambrecht, S. *et al.* Growth differentiation factor 15, a marker of lung involvement in systemic sclerosis, is involved in fibrosis development but is not indispensable for fibrosis development. *Arthritis Rheumatol* **66**, 418-427, doi:10.1002/art.38241 (2014).

26 Fanny, M. *et al.* The IL-33 Receptor ST2 Regulates Pulmonary Inflammation and Fibrosis to Bleomycin. *Front Immunol* **9**, 1476, doi:10.3389/fimmu.2018.01476 (2018).

27 Jo, S. *et al.* WNT16 elevation induced cell senescence of osteoblasts in ankylosing spondylitis. *Arthritis Res Ther* **23**, 301, doi:10.1186/s13075-021-02670-0 (2021).

28 Binet, R. *et al.* WNT16B is a new marker of cellular senescence that regulates p53 activity and the phosphoinositide 3-kinase/AKT pathway. *Cancer Res* **69**, 9183-9191, doi:10.1158/0008-5472.CAN-09-1016 (2009).

29 Kellogg, D. L., Kellogg, D. L., Jr., Musi, N. & Nambiar, A. M. Cellular Senescence in Idiopathic Pulmonary Fibrosis. *Curr Mol Biol Rep* **7**, 31-40, doi:10.1007/s40610-021-00145-4 (2021).

30 Hohmann, M. S., Habiel, D. M., Coelho, A. L., Verri, W. A., Jr. & Hogaboam, C. M. Quercetin Enhances Ligand-induced Apoptosis in Senescent Idiopathic Pulmonary Fibrosis Fibroblasts and Reduces Lung Fibrosis In Vivo. *Am J Respir Cell Mol Biol* **60**, 28-40, doi:10.1165/rcmb.2017-0289OC (2019).

5 CONCLUSIONS

In the presence and absence of the extrinsic pro-apoptotic ligands, SOC drugs failed to trigger apoptosis in senescent fibroblasts, possibly due to enhanced Bcl-2 levels and the activation of the necroptosis pathway. SOC drugs elevated fibrotic and senescence markers in IPF lung fibroblasts. These data demonstrated the inefficacy of SOC in targeting senescent cells. Further investigation is required to fully elucidate the therapeutic implications of SOC drugs on other senescent cell types in IPF.

In addition, we demonstrated for the first time that RvD2 could act by reducing pro-fibrotic and senomorphic mechanisms. Our findings highlight the importance of the SPM RvD2 as a potential therapeutic approach for IPF.

REFERENCES

AL-MUDARES, F.; REDDICK, S.; REN, J.; VENKATESH, A. *et al.* Role of Growth Differentiation Factor 15 in Lung Disease and Senescence: Potential Role Across the Lifespan. **Front Med (Lausanne)**, 7, p. 594137, 2020.

BADDINI-MARTINEZ, J.; PEREIRA, C. A. How many patients with idiopathic pulmonary fibrosis are there in Brazil? **J Bras Pneumol**, 41, n. 6, p. 560-561, Nov-Dec 2015.

BAKER, D. J.; CHILDS, B. G.; DURIK, M.; WIJERS, M. E. *et al.* Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan. **Nature**, 530, n. 7589, p. 184-189, Feb 11 2016.

BAKER, D. J.; WIJSHAKE, T.; TCHKONIA, T.; LEBRASSEUR, N. K. *et al.* Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. **Nature**, 479, n. 7372, p. 232-236, Nov 2 2011.

BASIL, M. C.; LEVY, B. D. Specialized pro-resolving mediators: endogenous regulators of infection and inflammation. **Nat Rev Immunol**, 16, n. 1, p. 51-67, Jan 2016.

BENTO, A. F.; CLAUDINO, R. F.; DUTRA, R. C.; MARCON, R. *et al.* Omega-3 fatty acid-derived mediators 17 (R)-hydroxy docosahexaenoic acid, aspirin-triggered resolvin D1 and resolvin D2 prevent experimental colitis in mice. **The Journal of Immunology**, 187, n. 4, p. 1957-1969, 2011.

BENTO, A. F.; CLAUDINO, R. F.; DUTRA, R. C.; MARCON, R. *et al.* Omega-3 fatty acid-derived mediators 17(R)-hydroxy docosahexaenoic acid, aspirin-triggered resolvin D1 and resolvin D2 prevent experimental colitis in mice. **J Immunol**, 187, n. 4, p. 1957-1969, Aug 15 2011.

BINET, R.; YTHIER, D.; ROBLES, A. I.; COLLADO, M. *et al.* WNT16B is a new marker of cellular senescence that regulates p53 activity and the phosphoinositide 3-kinase/AKT pathway. **Cancer Res**, 69, n. 24, p. 9183-9191, Dec 15 2009.

BOOTS, A. W.; VEITH, C.; ALBRECHT, C.; BARTHOLOME, R. *et al.* The dietary antioxidant quercetin reduces hallmarks of bleomycin-induced lung fibrogenesis in mice. **BMC Pulm Med**, 20, n. 1, p. 112, Apr 29 2020.

BORODKINA, A. V.; DERYABIN, P. I.; GIUKOVA, A. A.; NIKOLSKY, N. N. "Social Life" of Senescent Cells: What Is SASP and Why Study It? **Acta Naturae**, 10, n. 1, p. 4-14, Jan-Mar 2018.

BUENO, M.; CALYECA, J.; ROJAS, M.; MORA, A. L. Mitochondria dysfunction and metabolic reprogramming as drivers of idiopathic pulmonary fibrosis. **Redox Biol**, 33, p. 101509, Jun 2020.

CHANDA, D.; OTOUPALOVA, E.; SMITH, S. R.; VOLCKAERT, T. *et al.* Developmental pathways in the pathogenesis of lung fibrosis. **Mol Aspects Med**, 65, p. 56-69, Feb 2019.

CHAUDHARY, N. I.; ROTH, G. J.; HILBERG, F.; MULLER-QUERNHEIM, J. *et al.* Inhibition of PDGF, VEGF and FGF signalling attenuates fibrosis. **Eur Respir J**, 29, n. 5, p. 976-985, May 2007.

CHIANG, N.; DALLI, J.; COLAS, R. A.; SERHAN, C. N. Identification of resolvin D2 receptor mediating resolution of infections and organ protection. **J Exp Med**, 212, n. 8, p. 1203-1217, Jul 27 2015.

CHIANG, N.; SERHAN, C. N. Specialized pro-resolving mediator network: an update on production and actions. **Essays Biochem**, 64, n. 3, p. 443-462, Sep 23 2020.

CHO, H. J.; HWANG, J. A.; YANG, E. J.; KIM, E. C. *et al.* Nintedanib induces senolytic effect via STAT3 inhibition. **Cell Death Dis**, 13, n. 9, p. 760, Sep 2 2022.

CHOI, M. E.; PRICE, D. R.; RYTER, S. W.; CHOI, A. M. K. Necroptosis: a crucial pathogenic mediator of human disease. **JCI Insight**, 4, n. 15, Aug 8 2019.

CLARKE, D. L.; CARRUTHERS, A. M.; MUSTELIN, T.; MURRAY, L. A. Matrix regulation of idiopathic pulmonary fibrosis: the role of enzymes. **Fibrogenesis Tissue Repair**, 6, n. 1, p. 20, Nov 26 2013.

COLLINS, B. F.; RAGHU, G. Antifibrotic therapy for fibrotic lung disease beyond idiopathic pulmonary fibrosis. **Eur Respir Rev**, 28, n. 153, Sep 30 2019.

COPPE, J. P.; DESPREZ, P. Y.; KRTOLICA, A.; CAMPISI, J. The senescence-associated secretory phenotype: the dark side of tumor suppression. **Annu Rev Pathol**, 5, p. 99-118, 2010.

DAVIS, J. M.; MURPHY, E. A.; CARMICHAEL, M. D. Effects of the dietary flavonoid quercetin upon performance and health. **Curr Sports Med Rep**, 8, n. 4, p. 206-213, Jul-Aug 2009.

DE MERA-RODRIGUEZ, J. A.; ALVAREZ-HERNAN, G.; GANAN, Y.; MARTIN-PARTIDO, G. *et al.* Is Senescence-Associated beta-Galactosidase a Reliable in vivo Marker of Cellular Senescence During Embryonic Development? **Front Cell Dev Biol**, 9, p. 623175, 2021.

DODIG, S.; CEPELAK, I.; PAVIC, I. Hallmarks of senescence and aging. **Biochem Med (Zagreb)**, 29, n. 3, p. 030501, Oct 15 2019.

DROGA-MAZOVEC, G.; BOJIC, L.; PETELIN, A.; IVANOVA, S. *et al.* Cysteine cathepsins trigger caspase-dependent cell death through cleavage of bid and antiapoptotic Bcl-2 homologues. **J Biol Chem**, 283, n. 27, p. 19140-19150, Jul 4 2008.

FAGET, D. V.; REN, Q.; STEWART, S. A. Unmasking senescence: context-dependent effects of SASP in cancer. **Nat Rev Cancer**, 19, n. 8, p. 439-453, Aug 2019.

FANNY, M.; NASCIMENTO, M.; BARON, L.; SCHRICKE, C. *et al.* The IL-33 Receptor ST2 Regulates Pulmonary Inflammation and Fibrosis to Bleomycin. **Front Immunol**, 9, p. 1476, 2018.

FLAHERTY, K. R.; WELLS, A. U.; COTTIN, V.; DEVARAJ, A. *et al.* Nintedanib in Progressive Fibrosing Interstitial Lung Diseases. **N Engl J Med**, 381, n. 18, p. 1718-1727, Oct 31 2019.

FRASER, E.; HOYLES, R. K. Therapeutic advances in idiopathic pulmonary fibrosis. **Clin Med (Lond)**, 16, n. 1, p. 42-51, Feb 2016.

GARG, H.; SURI, P.; GUPTA, J. C.; TALWAR, G. P. *et al.* Survivin: a unique target for tumor therapy. **Cancer Cell Int**, 16, p. 49, 2016.

GAUTHERON, J.; VUCUR, M.; SCHNEIDER, A. T.; SEVERI, I. *et al.* The necroptosis-inducing kinase RIPK3 dampens adipose tissue inflammation and glucose intolerance. **Nat Commun**, 7, p. 11869, Jun 21 2016.

GLASSBERG, M. K. Overview of idiopathic pulmonary fibrosis, evidence-based guidelines, and recent developments in the treatment landscape. **Am J Manag Care**, 25, n. 11 Suppl, p. S195-S203, Jul 2019.

HABIEL, D. M.; ESPINDOLA, M. S.; JONES, I. C.; COELHO, A. L. *et al.* CCR10+ epithelial cells from idiopathic pulmonary fibrosis lungs drive remodeling. **JCI Insight**, 3, n. 16, Aug 23 2018.

HAN, J.; ZHONG, C. Q.; ZHANG, D. W. Programmed necrosis: backup to and competitor with apoptosis in the immune system. **Nat Immunol**, 12, n. 12, p. 1143-1149, Nov 16 2011.

HAYFLICK, L.; MOORHEAD, P. S. The serial cultivation of human diploid cell strains. **Exp Cell Res**, 25, p. 585-621, Dec 1961.

HERRERA, J.; HENKE, C. A.; BITTERMAN, P. B. Extracellular matrix as a driver of progressive fibrosis. **J Clin Invest**, 128, n. 1, p. 45-53, Jan 2 2018.

HOHMANN, M. S.; HABIEL, D. M.; COELHO, A. L.; VERRI, W. A., Jr. *et al.* Quercetin Enhances Ligand-induced Apoptosis in Senescent Idiopathic Pulmonary Fibrosis Fibroblasts and Reduces Lung Fibrosis In Vivo. **Am J Respir Cell Mol Biol**, 60, n. 1, p. 28-40, Jan 2019.

HOHMANN, M. S.; HABIEL, D. M.; ESPINDOLA, M. S.; HUANG, G. *et al.* Antibody-mediated depletion of CCR10+EphA3+ cells ameliorates fibrosis in IPF. **JCI Insight**, 6, n. 11, Jun 8 2021.

HOSTETTLER, K. E.; ZHONG, J.; PAPAKONSTANTINOU, E.; KARAKIULAKIS, G. *et al.* Anti-fibrotic effects of nintedanib in lung fibroblasts derived from patients with idiopathic pulmonary fibrosis. **Respir Res**, 15, p. 157, Dec 12 2014.

HU, L.; LI, H.; ZI, M.; LI, W. *et al.* Why Senescent Cells Are Resistant to Apoptosis: An Insight for Senolytic Development. **Front Cell Dev Biol**, 10, p. 822816, 2022.

HUTCHINSON, J.; FOGARTY, A.; HUBBARD, R.; MCKEEVER, T. Global incidence and mortality of idiopathic pulmonary fibrosis: a systematic review. **Eur Respir J**, 46, n. 3, p. 795-806, Sep 2015.

HYNES, R. O. The extracellular matrix: not just pretty fibrils. **Science**, 326, n. 5957, p. 1216-1219, Nov 27 2009.

JENSEN, K.; NIZAMUTDINOV, D.; GUERRIER, M.; AFROZE, S. *et al.* General mechanisms of nicotine-induced fibrogenesis. **FASEB J**, 26, n. 12, p. 4778-4787, Dec 2012.

JIN, H. J.; LEE, H. J.; HEO, J.; LIM, J. *et al.* Senescence-Associated MCP-1 Secretion Is Dependent on a Decline in BMI1 in Human Mesenchymal Stromal Cells. **Antioxid Redox Signal**, 24, n. 9, p. 471-485, Mar 20 2016.

JO, S.; WEON, S.; NAM, B.; JANG, M. A. *et al.* WNT16 elevation induced cell senescence of osteoblasts in ankylosing spondylitis. **Arthritis Res Ther**, 23, n. 1, p. 301, Dec 8 2021.

JORGENSEN, E.; STINSON, A.; SHAN, L.; YANG, J. *et al.* Cigarette smoke induces endoplasmic reticulum stress and the unfolded protein response in normal and malignant human lung cells. **BMC Cancer**, 8, p. 229, Aug 11 2008.

JUSTICE, J. N.; NAMBIAR, A. M.; TCHKONIA, T.; LEBRASSEUR, N. K. *et al.* Senolytics in idiopathic pulmonary fibrosis: Results from a first-in-human, open-label, pilot study. **EBioMedicine**, 40, p. 554-563, Feb 2019.

KANEMATSU, T.; KITAICHI, M.; NISHIMURA, K.; NAGAI, S. *et al.* Clubbing of the fingers and smooth-muscle proliferation in fibrotic changes in the lung in patients with idiopathic pulmonary fibrosis. **Chest**, 105, n. 2, p. 339-342, Feb 1994.

KANTARJIAN, H.; JABBOUR, E.; GRIMLEY, J.; KIRKPATRICK, P. Dasatinib. **Nat Rev Drug Discov**, 5, n. 9, p. 717-718, Sep 2006.

KARIMI-SHAH, B. A.; CHOWDHURY, B. A. Forced vital capacity in idiopathic pulmonary fibrosis--FDA review of pirfenidone and nintedanib. **N Engl J Med**, 372, n. 13, p. 1189-1191, Mar 26 2015.

KARMAN, J.; WANG, J.; BODEA, C.; CAO, S. *et al.* Lung gene expression and single cell analyses reveal two subsets of idiopathic pulmonary fibrosis (IPF) patients associated with different pathogenic mechanisms. **PLoS One**, 16, n. 3, p. e0248889, 2021.

KASAM, R. K.; REDDY, G. B.; JEGGA, A. G.; MADALA, S. K. Dysregulation of Mesenchymal Cell Survival Pathways in Severe Fibrotic Lung Disease: The Effect of Nintedanib Therapy. **Front Pharmacol**, 10, p. 532, 2019.

KATO, K.; SHIN, Y. J.; PALUMBO, S.; PAPAGEORGIOU, I. *et al.* Leveraging ageing models of pulmonary fibrosis: the efficacy of nintedanib in ageing. **Eur Respir J**, 58, n. 5, Nov 2021.

KAUR, A.; MATHAI, S. K.; SCHWARTZ, D. A. Genetics in Idiopathic Pulmonary Fibrosis Pathogenesis, Prognosis, and Treatment. **Front Med (Lausanne)**, 4, p. 154, 2017.

KELLOGG, D. L.; KELLOGG, D. L., Jr.; MUSI, N.; NAMBIAR, A. M. Cellular Senescence in Idiopathic Pulmonary Fibrosis. **Curr Mol Biol Rep**, 7, n. 3, p. 31-40, 2021.

KENDALL, R. T.; FEGHALI-BOSTWICK, C. A. Fibroblasts in fibrosis: novel roles and mediators. **Front Pharmacol**, 5, p. 123, 2014.

KING, T. E., Jr.; PARDO, A.; SELMAN, M. Idiopathic pulmonary fibrosis. **Lancet**, 378, n. 9807, p. 1949-1961, Dec 3 2011.

KIRKLAND, J. L.; TCHKONIA, T. Cellular Senescence: A Translational Perspective. **EBioMedicine**, 21, p. 21-28, Jul 2017.

KIRKLAND, J. L.; TCHKONIA, T. Senolytic drugs: from discovery to translation. **J Intern Med**, 288, n. 5, p. 518-536, Nov 2020.

KISHABA, T. Evaluation and management of Idiopathic Pulmonary Fibrosis. **Respir Investig**, 57, n. 4, p. 300-311, Jul 2019.

KISTLER, K. D.; NALYSNYK, L.; ROTELLA, P.; ESSER, D. Lung transplantation in idiopathic pulmonary fibrosis: a systematic review of the literature. **BMC Pulm Med**, 14, p. 139, Aug 16 2014.

KLEAVELAND, K. R.; VELIKOFF, M.; YANG, J.; AGARWAL, M. *et al.* Fibrocytes are not an essential source of type I collagen during lung fibrosis. **J Immunol**, 193, n. 10, p. 5229-5239, Nov 15 2014.

KLEIN, C. P.; SPEROTTO, N. D.; MACIEL, I. S.; LEITE, C. E. *et al.* Effects of D-series resolvins on behavioral and neurochemical changes in a fibromyalgia-like model in mice. **Neuropharmacology**, 86, p. 57-66, 2014.

KOENIG, J. A. Assessment of receptor internalization and recycling. **Methods Mol Biol**, 259, p. 249-273, 2004.

KOLB, M.; BONELLA, F.; WOLLIN, L. Therapeutic targets in idiopathic pulmonary fibrosis. **Respir Med**, 131, p. 49-57, Oct 2017.

KYTIKOVA, O.; NOVGORODTSEVA, T.; DENISENKO, Y.; ANTONYUK, M. *et al.* Pro-Resolving Lipid Mediators in the Pathophysiology of Asthma. **Medicina (Kaunas)**, 55, n. 6, Jun 18 2019.

LAMBRECHT, S.; SMITH, V.; DE WILDE, K.; COUDENYS, J. *et al.* Growth differentiation factor 15, a marker of lung involvement in systemic sclerosis, is involved in fibrosis development but is not indispensable for fibrosis development. **Arthritis Rheumatol**, 66, n. 2, p. 418-427, Feb 2014.

LEDERER, D. J.; MARTINEZ, F. J. Idiopathic pulmonary fibrosis. **New England Journal of Medicine**, 378, n. 19, p. 1811-1823, 2018.

LEE, J. M.; YOSHIDA, M.; KIM, M. S.; LEE, J. H. *et al.* Involvement of Alveolar Epithelial Cell Necroptosis in Idiopathic Pulmonary Fibrosis Pathogenesis. **Am J Respir Cell Mol Biol**, 59, n. 2, p. 215-224, Aug 2018.

LEHMANN, M.; KORFEI, M.; MUTZE, K.; KLEE, S. *et al.* Senolytic drugs target alveolar epithelial cell function and attenuate experimental lung fibrosis ex vivo. **Eur Respir J**, 50, n. 2, Aug 2017.

LEY, B.; COLLARD, H. R. Epidemiology of idiopathic pulmonary fibrosis. **Clin Epidemiol**, 5, p. 483-492, Nov 25 2013.

LI, D.; HU, C.; LI, H. Survivin as a novel target protein for reducing the proliferation of cancer cells. **Biomed Rep**, 8, n. 5, p. 399-406, May 2018.

LI, Y.; YAO, J.; HAN, C.; YANG, J. *et al.* Quercetin, Inflammation and Immunity. **Nutrients**, 8, n. 3, p. 167, Mar 15 2016.

LIN, Y.; XU, Z. Fibroblast Senescence in Idiopathic Pulmonary Fibrosis. **Front Cell Dev Biol**, 8, p. 593283, 2020.

LIU, R. M.; LIU, G. Cell senescence and fibrotic lung diseases. **Exp Gerontol**, 132, p. 110836, Apr 2020.

LOPEZ-OTIN, C.; BLASCO, M. A.; PARTRIDGE, L.; SERRANO, M. *et al.* The hallmarks of aging. **Cell**, 153, n. 6, p. 1194-1217, Jun 6 2013.

MAHER, T. M.; BENDSTRUP, E.; DRON, L.; LANGLEY, J. *et al.* Global incidence and prevalence of idiopathic pulmonary fibrosis. **Respir Res**, 22, n. 1, p. 197, Jul 7 2021.

MAHER, T. M.; KREUTER, M.; LEDERER, D. J.; BROWN, K. K. *et al.* Rationale, design and objectives of two phase III, randomised, placebo-controlled studies of GLPG1690, a novel autotaxin inhibitor, in idiopathic pulmonary fibrosis (ISABELA 1 and 2). **BMJ Open Respir Res**, 6, n. 1, p. e000422, 2019.

MARCOTTE, R.; LACELLE, C.; WANG, E. Senescent fibroblasts resist apoptosis by downregulating caspase-3. **Mech Ageing Dev**, 125, n. 10-11, p. 777-783, Oct-Nov 2004.

MARTINEZ, F. J.; COLLARD, H. R.; PARDO, A.; RAGHU, G. *et al.* Idiopathic pulmonary fibrosis. **Nat Rev Dis Primers**, 3, p. 17074, Oct 20 2017.

MARTINEZ, F. J.; SAFRIN, S.; WEYCKER, D.; STARKO, K. M. *et al.* The clinical course of patients with idiopathic pulmonary fibrosis. **Ann Intern Med**, 142, n. 12 Pt 1, p. 963-967, Jun 21 2005.

MCCOMB, S.; SHUTINOSKI, B.; THURSTON, S.; CESSFORD, E. *et al.* Cathepsins limit macrophage necroptosis through cleavage of Rip1 kinase. **J Immunol**, 192, n. 12, p. 5671-5678, Jun 15 2014.

MEINERS, S.; LEHMANN, M. Senescent Cells in IPF: Locked in Repair? **Front Med (Lausanne)**, 7, p. 606330, 2020.

MIZRAJI, G.; HEYMAN, O.; VAN DYKE, T. E.; WILENSKY, A. Resolvin D2 Restrains Th1 Immunity and Prevents Alveolar Bone Loss in Murine Periodontitis. **Front Immunol**, 9, p. 785, 2018.

MOODLEY, Y. P.; CATERINA, P.; SCAFFIDI, A. K.; MISSO, N. L. *et al.* Comparison of the morphological and biochemical changes in normal human lung fibroblasts and fibroblasts derived from lungs of patients with idiopathic pulmonary fibrosis during FasL-induced apoptosis. **J Pathol**, 202, n. 4, p. 486-495, Apr 2004.

MULLER, T.; DEWITZ, C.; SCHMITZ, J.; SCHRODER, A. S. *et al.* Necroptosis and ferroptosis are alternative cell death pathways that operate in acute kidney failure. **Cell Mol Life Sci**, 74, n. 19, p. 3631-3645, Oct 2017.

NAKAMURA, Y.; SUDA, T. Idiopathic Pulmonary Fibrosis: Diagnosis and Clinical Manifestations. **Clin Med Insights Circ Respir Pulm Med**, 9, n. Suppl 1, p. 163-171, 2015.

PADILLA, M. Idiopathic pulmonary fibrosis: the role of pathobiology in making a definitive diagnosis. **Am J Manag Care**, 21, n. 14 Suppl, p. s276-283, Oct 2015.

PAEZ-RIBES, M.; GONZALEZ-GUALDA, E.; DOHERTY, G. J.; MUNOZ-ESPIN, D. Targeting senescent cells in translational medicine. **EMBO Mol Med**, 11, n. 12, p. e10234, Dec 2019.

PARDO, A.; SELMAN, M. The Interplay of the Genetic Architecture, Aging, and Environmental Factors in the Pathogenesis of Idiopathic Pulmonary Fibrosis. **Am J Respir Cell Mol Biol**, 64, n. 2, p. 163-172, Feb 2021.

PARIMON, T.; HOHMANN, M. S.; YAO, C. Cellular Senescence: Pathogenic Mechanisms in Lung Fibrosis. **Int J Mol Sci**, 22, n. 12, Jun 9 2021.

PARK, Y.; AHN, C.; KIM, T. H. Occupational and environmental risk factors of idiopathic pulmonary fibrosis: a systematic review and meta-analyses. **Sci Rep**, 11, n. 1, p. 4318, Mar 2 2021.

PRATA, L.; OVSYANNIKOVA, I. G.; TCHKONIA, T.; KIRKLAND, J. L. Senescent cell clearance by the immune system: Emerging therapeutic opportunities. **Semin Immunol**, 40, p. 101275, Dec 2018.

PSATHAKIS, K.; MERMIGKIS, D.; PAPATHEODOROU, G.; LOUKIDES, S. *et al.* Exhaled markers of oxidative stress in idiopathic pulmonary fibrosis. **Eur J Clin Invest**, 36, n. 5, p. 362-367, May 2006.

RAGHU, G.; CHEN, S. Y.; HOU, Q.; YEH, W. S. *et al.* Incidence and prevalence of idiopathic pulmonary fibrosis in US adults 18-64 years old. **Eur Respir J**, 48, n. 1, p. 179-186, Jul 2016.

RAGHU, G.; COLLARD, H. R.; EGAN, J. J.; MARTINEZ, F. J. *et al.* An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. **Am J Respir Crit Care Med**, 183, n. 6, p. 788-824, Mar 15 2011.

RAGHU, G.; SELMAN, M. Nintedanib and pirfenidone. New antifibrotic treatments indicated for idiopathic pulmonary fibrosis offer hopes and raises questions. **Am J Respir Crit Care Med**, 191, n. 3, p. 252-254, Feb 1 2015.

RECCHIUTI, A.; PATRUNO, S.; MATTOSCIO, D.; ISOPI, E. *et al.* Resolvin D1 and D2 reduce SARS-CoV-2-induced inflammatory responses in cystic fibrosis macrophages. **FASEB J**, 35, n. 4, p. e21441, Apr 2021.

ROACH, K. M.; CASTELLS, E.; DIXON, K.; MASON, S. *et al.* Evaluation of Pirfenidone and Nintedanib in a Human Lung Model of Fibrogenesis. **Front Pharmacol**, 12, p. 679388, 2021.

RODRIGUEZ, D. A.; WEINLICH, R.; BROWN, S.; GUY, C. *et al.* Characterization of RIPK3-mediated phosphorylation of the activation loop of MLKL during necroptosis. **Cell Death Differ**, 23, n. 1, p. 76-88, Jan 2016.

SACK, C.; RAGHU, G. Idiopathic pulmonary fibrosis: unmasking cryptogenic environmental factors. **Eur Respir J**, 53, n. 2, Feb 2019.

SAGIV, A.; BIRAN, A.; YON, M.; SIMON, J. *et al.* Granule exocytosis mediates immune surveillance of senescent cells. **Oncogene**, 32, n. 15, p. 1971-1977, Apr 11 2013.

SALAMA, R.; SADAIE, M.; HOARE, M.; NARITA, M. Cellular senescence and its effector programs. **Genes Dev**, 28, n. 2, p. 99-114, Jan 15 2014.

SALMINEN, A.; OJALA, J.; KAARNIRANTA, K. Apoptosis and aging: increased resistance to apoptosis enhances the aging process. **Cell Mol Life Sci**, 68, n. 6, p. 1021-1031, Mar 2011.

SANDERS, Y. Y.; LIU, H.; ZHANG, X.; HECKER, L. *et al.* Histone modifications in senescence-associated resistance to apoptosis by oxidative stress. **Redox Biol**, 1, p. 8-16, 2013.

SCHAFER, M. J.; WHITE, T. A.; IIJIMA, K.; HAAK, A. J. *et al.* Cellular senescence mediates fibrotic pulmonary disease. **Nat Commun**, 8, p. 14532, Feb 23 2017.

SEIFIRAD, S. Pirfenidone: A novel hypothetical treatment for COVID-19. **Med Hypotheses**, 144, p. 110005, Nov 2020.

SERHAN, C. N.; CHIANG, N.; VAN DYKE, T. E. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. **Nat Rev Immunol**, 8, n. 5, p. 349-361, May 2008.

SERHAN, C. N.; PETASIS, N. A. Resolvins and protectins in inflammation resolution. **Chem Rev**, 111, n. 10, p. 5922-5943, Oct 12 2011.

SGALLA, G.; IOVENE, B.; CALVELLO, M.; ORI, M. *et al.* Idiopathic pulmonary fibrosis: pathogenesis and management. **Respir Res**, 19, n. 1, p. 32, Feb 22 2018.

SHENDEROV, K.; COLLINS, S. L.; POWELL, J. D.; HORTON, M. R. Immune dysregulation as a driver of idiopathic pulmonary fibrosis. **J Clin Invest**, 131, n. 2, Jan 19 2021.

SOTO-GAMEZ, A.; QUAX, W. J.; DEMARIA, M. Regulation of Survival Networks in Senescent Cells: From Mechanisms to Interventions. **J Mol Biol**, 431, n. 15, p. 2629-2643, Jul 12 2019.

TAKEDA, Y.; TSUJINO, K.; KIJIMA, T.; KUMANOGOH, A. Efficacy and safety of pirfenidone for idiopathic pulmonary fibrosis. **Patient Prefer Adherence**, 8, p. 361-370, 2014.

TAKENOUCHI, Y.; KITAKAZE, K.; TSUBOI, K.; OKAMOTO, Y. Growth differentiation factor 15 facilitates lung fibrosis by activating macrophages and fibroblasts. **Exp Cell Res**, 391, n. 2, p. 112010, Jun 15 2020.

TANIGUCHI, H.; EBINA, M.; KONDOH, Y.; OGURA, T. *et al.* Pirfenidone in idiopathic pulmonary fibrosis. **Eur Respir J**, 35, n. 4, p. 821-829, Apr 2010.

TAO, P.; SUN, J.; WU, Z.; WANG, S. *et al.* A dominant autoinflammatory disease caused by non-cleavable variants of RIPK1. **Nature**, 577, n. 7788, p. 109-114, Jan 2020.

TEPPER, C. G.; SELDIN, M. F.; MUDRYJ, M. Fas-mediated apoptosis of proliferating, transiently growth-arrested, and senescent normal human fibroblasts. **Exp Cell Res**, 260, n. 1, p. 9-19, Oct 10 2000.

TOMINAGA, K. The emerging role of senescent cells in tissue homeostasis and pathophysiology. **Pathobiol Aging Age Relat Dis**, 5, p. 27743, 2015.

VAN MANEN, M. J. G.; VERMEER, L. C.; MOOR, C. C.; VRIJENHOEFF, R. *et al.* Clubbing in patients with fibrotic interstitial lung diseases. **Respir Med**, 132, p. 226-231, Nov 2017.

WANG, E. Senescent human fibroblasts resist programmed cell death, and failure to suppress bcl2 is involved. **Cancer Res**, 55, n. 11, p. 2284-2292, Jun 1 1995.

WOLLIN, L.; DISTLER, J. H. W.; REDENTE, E. F.; RICHES, D. W. H. *et al.* Potential of nintedanib in treatment of progressive fibrosing interstitial lung diseases. **Eur Respir J**, 54, n. 3, Sep 2019.

WOLLIN, L.; WEX, E.; PAUTSCH, A.; SCHNAPP, G. *et al.* Mode of action of nintedanib in the treatment of idiopathic pulmonary fibrosis. **Eur Respir J**, 45, n. 5, p. 1434-1445, May 2015.

WOLTERS, P. J.; COLLARD, H. R.; JONES, K. D. Pathogenesis of idiopathic pulmonary fibrosis. **Annu Rev Pathol**, 9, p. 157-179, 2014.

WUYTS, W. A.; AGOSTINI, C.; ANTONIOU, K. M.; BOUROS, D. *et al.* The pathogenesis of pulmonary fibrosis: a moving target. **Eur Respir J**, 41, n. 5, p. 1207-1218, May 2013.

XU, M.; PIRTSKHALAVA, T.; FARR, J. N.; WEIGAND, B. M. *et al.* Senolytics improve physical function and increase lifespan in old age. **Nat Med**, 24, n. 8, p. 1246-1256, Aug 2018.

YANG, J.; LIU, X.; BHALLA, K.; KIM, C. N. *et al.* Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. **Science**, 275, n. 5303, p. 1129-1132, Feb 21 1997.

YATOMI, M.; HISADA, T.; ISHIZUKA, T.; KOGA, Y. *et al.* 17(R)-resolvin D1 ameliorates bleomycin-induced pulmonary fibrosis in mice. **Physiol Rep**, 3, n. 12, Dec 2015.

YOON, S.; KOVALENKO, A.; BOGDANOV, K.; WALLACH, D. MLKL, the Protein that Mediates Necroptosis, Also Regulates Endosomal Trafficking and Extracellular Vesicle Generation. **Immunity**, 47, n. 1, p. 51-65 e57, Jul 18 2017.

YOSEF, R.; PILPEL, N.; TOKARSKY-AMIEL, R.; BIRAN, A. *et al.* Directed elimination of senescent cells by inhibition of BCL-W and BCL-XL. **Nat Commun**, 7, p. 11190, Apr 6 2016.

ZAMAN, T.; LEE, J. S. Risk factors for the development of idiopathic pulmonary fibrosis: A review. **Curr Pulmonol Rep**, 7, n. 4, p. 118-125, Dec 2018.

ZHANG, T.; ZUO, G.; ZHANG, H. GPR18 Agonist Resolvin D2 Reduces Early Brain Injury in a Rat Model of Subarachnoid Hemorrhage by Multiple Protective Mechanisms. **Cell Mol Neurobiol**, Jun 5 2021.

ZHANG, Y.; JIANG, M.; NOURAIE, M.; ROTH, M. G. *et al.* GDF15 is an epithelial-derived biomarker of idiopathic pulmonary fibrosis. **Am J Physiol Lung Cell Mol Physiol**, 317, n. 4, p. L510-L521, Oct 1 2019.