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Cellular Senescence in Idiopathic Pulmonary Fibrosis

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Abstract
Cellular senescence (CS) is increasingly implicated in the etiology of age-related diseases. While CS can facilitate physiological processes such as tissue repair and wound healing, senescent cells also contribute to pathophysiological processes involving macromolecular damage and metabolic dysregulation that characterize multiple morbid and prevalent diseases, including Alzheimer's disease, osteoarthritis, atherosclerotic vascular disease, diabetes mellitus, and idiopathic pulmonary fibrosis (IPF). Preclinical studies targeting senescent cells and the senescence-associated secretory phenotype (SASP) with "senotherapeutics" have demonstrated improvement in age-related morbidity associated with these disease states. Despite promising results from these preclinical trials, few human clinical trials have been conducted. A first-in-human, open-label, pilot study of the senolytic combination of dasatinib and quercetin (DQ) in patients with IPF showed improved physical function and mobility. In this review, we will discuss our current understanding of cellular senescence, its role in age-associated diseases, with a specific focus on IPF, and potential for senotherapeutics in the treatment of fibrotic lung diseases.

Keywords Cellular senescence · Senolytics · Senomorphics · Idiopathic pulmonary fibrosis

Cellular Senescence

Age-associated diseases (AADs) such as Alzheimer's disease, osteoarthritis, diabetes mellitus, and atherosclerosis will continue to become increasingly prevalent in our aging population. One of the increasingly recognized drivers for AADs is cellular senescence (CS), defined as stress-induced, non-reversible cell cycle arrest, which depletes regenerative capability and is associated with release of pro-inflammatory mediators. Indeed, markers of CS are associated with mortality and increase with normal aging or prematurely in pathologic states [1]. The complex molecular biology of senescence has been thoroughly reviewed elsewhere [2]. Briefly, senescent cells (SC) are characterized by growth arrest, expression of anti-proliferative molecules (e.g., p16INK4a), and activation of damage sensing signaling pathways (e.g., p38MAPK and NF-kB). The growth arrest of formerly replicative cells often results from a persistent DNA damage response (DDR) or stress signaling and is effected by sustained activation of the p16INK4a-RB and/or p53 pathways [3]. While senescent cells have exited the cell cycle, they remain metabolically active and produce a heterogeneous array of signaling molecules including proinflammatory cytokines, chemokines, growth factors, and proteases, termed the senescence-associated secretory phenotype (SASP) [4–6].

Conceptually, CS is a mechanism to counteract malignant transformation; over time, cells inevitably accumulate irreparable damage and, in response, may either undergo apoptosis or senescence to prevent the growth of damaged cells. These damaged but viable SC are required in processes including wound healing, embryogenesis, and tumor suppression by inducing immune clearance of potentially oncogenic cells [3, 7]. However, the generation and maintenance of senescent cells by activation of pro-survival pathways may outpace

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immune clearance, which creates self-expanding reservoirs of senescent cells and can ultimately lead to clinical disease.

The most commonly cited inducers of senescence are well established in cellular aging (e.g., DNA damage, proteotoxic stress, oxidative stress, mitochondrial dysfunction) [1, 8–10]. For example, telomeres are repetitive sequences of DNA that insulate the ends of chromosomes from damage. With repeated mitosis, telomeres are incompletely replicated, and thus shorten until reaching the theoretical Hayflick limit, where the loss of telomeric protective function results in p53- or p16-RB-mediated replicative senescence [11]. Shortened telomeres predispose cells to DNA damage. [12] Similarly, genotoxic agents (i.e., bleomycin) and ionizing radiation result in DNA damage. Regardless of the mechanism of activation, the DNA damage response (DDR) pathway is a major driver of cellular senescence [13]. Indeed, senescent cells have persistent evidence of DNA damage—segments with chromatin alterations reinforcing senescence (DNA-SCARs), which regulate cell cycle arrest and SASP [14].

Just as CS participates in multiple biological processes, intracellular signaling with reactive oxygen species (ROS) is a fundamental cellular function [15]. However, when ROS generation becomes excessive, oxidative stress (OS) results in a wide array of cellular damage. [16] Chronic OS plays a central role in the pathogenesis of CS through p53/$p21^{CIP1/WAF1}$ activation [17]. Mitochondrial dysfunction is an important generator of ROS and has been implicated in CS generation [18]. Moreover, a positive feedback loop involving mitochondrial dysfunction and OS can accelerate intracellular metabolic derangements, such as ATP depletion and calcium dysregulation [19].

The primary upstream cell cycle regulators that cause are $p21^{WAF1/Cip1}$ and $p16^{INK4A}$, which act through activation of the retinoblastoma (Rb) protein family to inhibit transactivation of E2F, resulting in cell cycle arrest. CS is also characterized by activation of canonical pro-survival pathways—EFNB1/3, PI3Kδ, BCL-x, and HSP-90 [20]. SASP protein production is largely regulated by mTOR activation [21]. In models of IPF, the SASP is pro-inflammatory and pro-fibrotic and includes cytokines such as TGF-β, IL-6, and MMP-12. [22, 23] While none of these is specific for CS, they all have a central role in pathogenesis.

SASPs vary widely among cell types but prominently feature pro-inflammatory cytokines that initiate immune clearance. However, over time, the imbalance of pro-survival pathways outpaces immune clearance, resulting in self-expanding reservoirs of senescent cells, which can ultimately lead to clinical disease Thus, chronic inflammation and CS are closely related processes [5]. IL-6 and IL-8 are robustly associated with SASPs and established inducers of the innate immune system, which plays a critical role in both malignant and senescent cell clearances [24] (Figure 1).

Senescence in Disease

As per the foregoing, CS plays physiological roles and is needed for tissue homeostasis; however, CS also represents a stress response triggered by insults associated with aging including genomic instability, telomere attrition, and other mechanisms [6]. The seminal study by Baker et al. first demonstrated a pathogenic role of CS by utilizing a transgenic mouse model to eliminate senescent cells by ablating $p16^{INK4A}$ [25]. An expanding body of preclinical and ex vivo studies has demonstrated a clear pathogenic role of CS in perpetuating organ dysfunction.

As previously mentioned, CS contributes to AADs and phenotypes including development of gray hair, loss of muscle mass, increases in adiposity, reduced neurogenesis, and increased tissue fibrosis [3]. The evidence linking senescence to these diverse pathologies includes accumulation of senescence markers in tissues with advancing age supporting an increased SC burden with advancing age. Indeed, the aging cellular phenotype prominently features altered extra-cellular matrix deposition, faltering stem cells, and senescent endothelial cells [26–28]. The far-reaching effects of a relatively small senescent reservoir are analogous to paraneoplastic syndromes. Moreover, if the development of SC occurs in particularly important fraction of cells in a given tissue, then the loss of function of those tissue cells could have an outsized effect on tissue integrity and function.

In contrast to disease in youth, AADs occur due to dysfunction of tissues changed by aging processes, which is expedited by CS. Increased senescence burden diminishes tissue resilience through cell cycle arrest as well as through SASP-induced stem cell and parenchymal cell dysfunction. As SC burden progressively increases, an additional wave of SC is generated, increasing SC burden further and further amplify AAD development and progression. This type of positive feedback process explains why disease vulnerability and incidence increase with age. For example, increased SC burden in adipose tissue drives diabetes mellitus, and aging endothelial cells drive atherosclerosis [3].

Although the most common histologic markers of SC burden are SA-β-galactosidase (SA-β-gal) and lipofuscin accumulation, assessing the SC burden in vivo is challenging because there are currently no robust clinical markers for CS [3]. A clinically useful marker of SC is clearly needed to guide future trials of senotherapeutics. Epigenetic investigations are promising [29]. Further characterization of gene expression via integration of genomic data and epigenetic markers of lung biopsies may provide a translatable assessment of CS burden [2]. In preclinical bleomycin models, growth and differentiation factor 15 (GDF15; TGF-β family member) is the most upregulated secreted protein [12]. Moreover, GDF15 levels are elevated in ILD patients prior to radiographic changes and are inversely associated with DLCO, FVC, and survival

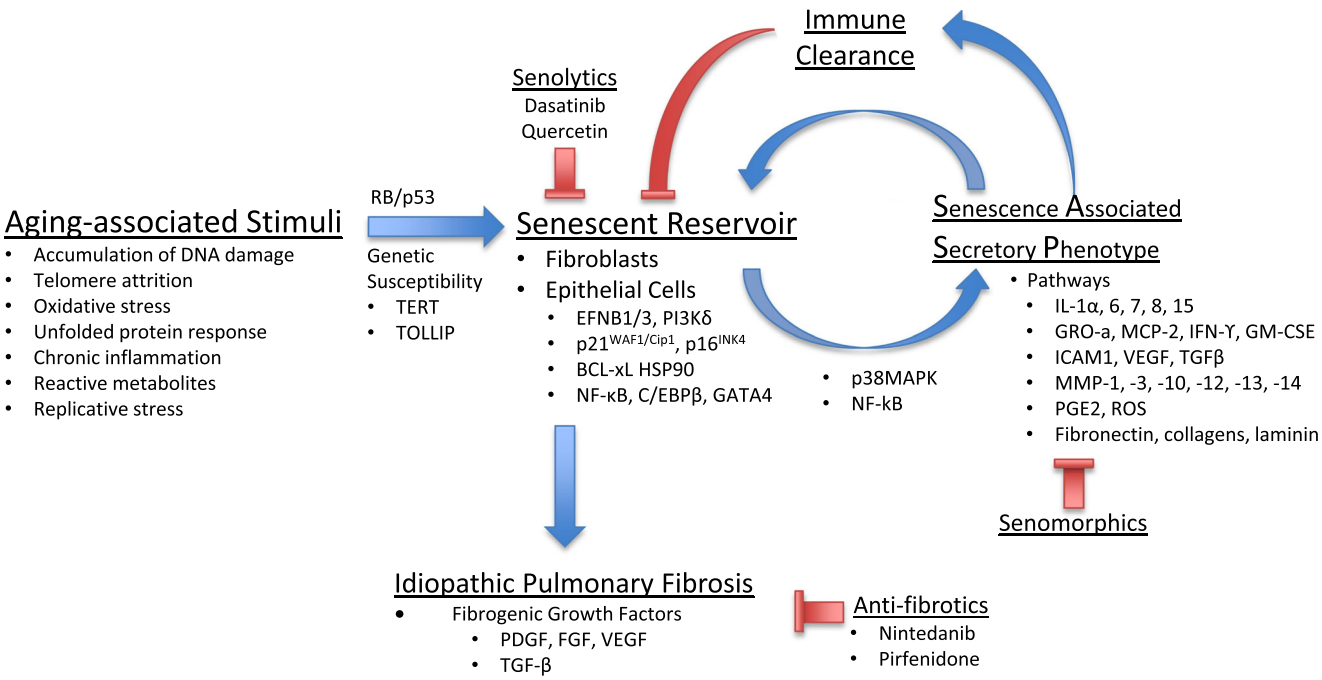

Figure 1 Cellular senescence and idiopathic pulmonary fibrosis: pathways and therapeutic approaches

[30, 31]. Together, the foregoing suggests GDF15 or other aging biomarkers may allow for risk stratification of progressive ILDs [32, 33].

Senomorphics and Senolytics

After extensive laboratory investigations, significant progress in preclinical studies is strongly supportive of potential clinical benefits for therapeutic treatment of SC (senotherapeutics). The diversity of SASPs has necessitated the use of bioinformatic experiments to characterize the underlying molecular networks and discern possible drug targets. There are established pro-survival regulators that are central in the cellular networks of senescent preadipocytes (the most abundant SC type), including ephrins, PI3K, BCL-2, and HSP-90 [20]. These have formed the basis for numerous senotherapeutics (Table 1).

Two approaches to senotherapy have been proposed: senescent-selective apoptosis (senolytic) and SASP suppression (senomorphic). Senolytic drugs inhibit anti-apoptotic pathways and thus restore selective clearance of SC [20, 63]. Senolytics alleviate multiple chronic disease and physical dysfunction in mouse models of a wide arrange of diseases but are difficult to develop due to off-target effects on physiologic survival pathways [3]. Thus, numerous in vitro studies have been required to screen for possible off-target effects. From a clinical perspective, a benefit for senolytics is the efficacy of intermittent treatment, which reduces senescent reservoirs and mitigates adverse effects that occur with continued treatment. Senomorphic medications, such as the mTOR antagonist rapamycin, abrogate the SASP and reduce the proliferation of senescent reservoirs. Such agents are likely to be used as continuous therapy to allow for physiologic CS but attenuate pathogenic CS. Senomorphic agents are undergoing clinical trials as age-modulating agents and for some AADs including Alzheimer's disease [64]. Combination therapy consisting of senolytic induction treatment with subsequent senomorphic maintenance treatment has been proposed by our group.

The first two and best-characterized senolytics are dasatinib and quercetin (DQ). They have proven in vitro synergism and preclinical health span improvement [34, 35, 65]. Quercetin is a plant-based polyphenol flavonoid that has antioxidant properties and has been shown to induce autophagy through proteasome activation in vitro. In fibroblasts from mouse models, quercetin restored Fas-L- and TNF-mediated apoptosis [66]. Dasatinib is an orally available tyrosine kinase inhibitor (TKI), originally developed to target SRC and ABL kinases [67], and is a second-line treatment for chronic myeloid leukemia (CML) [68].

Idiopathic Pulmonary Fibrosis: Pathophysiology

Idiopathic pulmonary fibrosis (IPF) is a classic AAD and devastating interstitial lung disease (ILD) characterized by restricted ventilation and compromised gas exchange leading to progressive dyspnea, impaired quality of life, and ultimately death [69–71]. The pathophysiology of IPF

Table 1 An overview of selected senotherapeutics

	Mechanism of action	In vitro targets	Key preclinical results	Clinical trials
Dasatinib + quercetin	Tyrosine kinase Inhibitor Inhibitor of multiple anti-apoptotic targets	HUVECs Myofibroblasts Pancreatic B cells Pre-eclampsia SC Preadipocytes Mesenchymal stem cells	Osteoporosis [20, 34] Physical dysfunction [20, 35] Lifepsan [35] Atherosclerosis [36] Pulmonary fibrosis [22] Obesity-induced anxiety [37] Hepatic steatosis [38]	CML and ALL (FDA approved 2006) Improved functional capacity in IPF patients (phase 1B 2019) Improved senescent cell burden in DN patients (phase 1 2019)
Flavonoids (Fisetin)	Antioxidant	HUVECs [39]	Extended lifespan [40] Improved oxidative stress [41] Neuroprotection [42] Beneficial in DM [43] Airway inflammation [44]	
Rapamycin	mTOR inhibitor	Nrf2 activation	Extended health span [45] Osteoarthritis [46]	Renal transplant anti-rejection (FDA approved 1999) No significant change in SASP (phase 1) Improved vaccine response in older patients
UBX0101	p53/MDM2 inhibitor		Osteoarthritis [47]	Osteoarthritis—no functional improvement at 12 weeks (phase I)
Navitoclax Venetoclax	BCL inhibitor	HUVECs, human lung fibroblasts, mouse fibroblasts (MEFs) [48]	Genotoxic senescence [49] Pulmonary fibrosis [50] Increased bone loss [51] Etoposide or doxorubicin senescence [52]	
	JAK/STAT inhibitor	Preadipocytes and HUVECs	Reduced inflammation, improved metabolic function and physical function	Myelofibrosis, polycythemia vera (FDA approved)
	HSP90 inhibitor		Geldanamycin—physical function [53] Significant diarrhea, hepatotoxicity, retinal damage	17-AAG—phase I/II chemotherapy [54–56] 17-DMAG—worsening age morbidity [53] Ganetespib—phase I/III chemotherapy [57–59] Onalespib—phase I chemotherapy [60] Luminespib—phase I chemotherapy [61] BIIB021—phase I chemotherapy [62]

is largely extrapolated from preclinical models of pulmonary fibrosis and ex vivo cell culture of lung epithelial cells. There are several hypothesized mechanisms for IPF, including chronic micro-aspiration, viral infection, and environmental exposures [72]. The most common mouse model for IPF employs intra-tracheal bleomycin to induce fibrosis and has elucidated numerous molecular mechanisms [73, 74]. There is mounting evidence that cellular senescence significantly contributes to chronic matrix remodeling and fibrosis and may be central to IPF pathophysiology [13, 22, 75, 76].

Inflammation has long been postulated to be the inciting factor in IPF patients [77]. Multiple studies have implicated inflammatory derangements. There is evidence of early alveolar macrophage activation and increased production of IL-1, IL-6, IL-8, MCP-1, and TNF-α [78, 79]. This inflammatory milieu transitions to a chronic fibrotic phase that is mediated by a SASP primarily of TGF-β, PDGF, and GM-CSF [22]. Despite these preliminary findings, anti-inflammatory drugs such as corticosteroids have not been effective in modifying disease progression but, in fact, harmful [80]. This is likely because chronic inflammation has multiple positive feedback loops that reinforce immune activation and induce senescence, which are accelerated based on patient risk factors.

There has been extensive investigation into the genetic risk factors. Familial interstitial pneumonia (FIP) is an inherited form of idiopathic interstitial pneumonia (IIP), and studies of patients with FIP have provided some insight regarding the role of genetic risk factors of pulmonary fibrosis. Surfactant-related proteins were identified by genome-wide association studies (GWAS) and account for ~30% of spontaneous IPF cases [81–83]. Other GWAS of IPF identified mutations of telomerase-associated genes in up to 25% of non-familial IPF cases [13]. Short telomeres are histologically associated with CS markers in IPF patients [29]. An important observation in IPF is shortening of telomeres in about 10% of IPF patients, which has implications for clinical outcomes [84–86]. However, defects in telomerase pathways are not specific for IPF and have also been associated with other ILDs and emphysema [87]. An association of Toll interacting protein (TOLLIP) variants with IPF susceptibility has also been described and associated with mortality [88]. TOLLIP negatively regulates Toll-like receptor 3 (TLR3) activity, which is a key step in immune activation. Taken together, these findings support a role for inflammatory activation during the pathogenesis of IPF but have not translated to effective risk stratification of IPF patients without a family history [89]. Genetic risk stratification has not been widely adopted, but the implicated genes are compatible with cellular senescence.

IPF Clinical Presentation, Diagnosis, and Senescent Cell Burden

IPF presents with nonspecific symptoms of dyspnea and nonproductive cough. A thorough history and high-resolution computed tomography (HRCT) of the chest are critical for confirming the diagnosis of IPF. The radiologic hallmark of IPF is a usual interstitial pneumonia (UIP) pattern, which includes subpleural and basilar predominant reticular opacities, honeycombing, and traction bronchiectasis [90]. Histopathologic UIP pattern obtained by surgical lung biopsies includes patchy, paraseptal destructive fibrosis and fibroblastic foci without granulomas or inflammatory infiltrates [90]. Bronchoalveolar lavage (BAL) cellular analysis is usually not helpful in confirming IPF diagnosis but may be supportive of an alternative diagnosis (e.g., significant BAL lymphocytosis may suggest hypersensitivity pneumonitis) [91, 92]. Transbronchial lung biopsies (TBLB) are usually non-diagnostic due to inadequate tissue sample size. However, next-generation RNA sequencing of TBLB specimens has potential to be a less invasive approach to confirm the presence of UIP histopathology. In two recent trials, genomic classification (GC) of lung biopsies improved the ability to differentiate IPF from other ILDs [93, 94]. Thus, GC may be useful to identify patients with significant senescence burden and benefit from senotherapeutics [95]. Transbronchial cryobiopsy (TBCB) is a promising diagnostic procedure that is less invasive than a surgical lung biopsy, but is only available at a few expert ILD centers and is not yet established as standard of care. Twenty genetic variants have been associated with IPF by genome-wide association studies [30]. While genotyping is not a routine diagnostic approach, the implicated genes may provide avenues for further investigation (e.g., DEPTOR, which inhibits mTOR signaling) and develop into a risk assessment tool for possible IPF patients.

The key parameters for assessing IPF severity and prognosis are age, gender, forced vital capacity (FVC), and diffusing capacity of lung for carbon monoxide (DLCO), which have been incorporated into a scoring system to predict short-term mortality [96]. A six-minute walk test (6MWT) is an objective measurement of exercise tolerance and symptom severity. Regular monitoring of pulmonary function is important to identify disease progression and acute exacerbations (AE-IPF), which are associated with acute to subacute clinical deterioration and new bilateral GGO superimposed on UIP background on HRCT. AE-IPF becomes more common with advanced disease and associated with poor prognosis. Unless a reversible trigger is present, there does not exist a proven safe and effective treatment. The unrelentingly predictable course of IPF reinforces the importance of early palliative care discussions.

Evaluating SC burden may mitigate the difficulties with diagnosis and uncertainties in prognosis. In a small study of

IPF patient lung biopsies, SA-β-gal, a specific SC marker, was increased compared to patients with COPD or hypersensitivity pneumonitis [29, 97]. We hypothesized that in patients with different fibrotic lung diseases, a lung biopsy and staining for SA-β-gal may help identify subtypes that may be more responsive to senolytic treatments. Specificity may be increased with other preclinical markers such as p16 and p21, but these have not yet been investigated in IPF patients. Uncertainties that require further research include the most appropriate SC biomarker, precise measurement of SC burden, ideal SC assay compartment (e.g., BAL, lung biopsy, blood, urine, skin, etc.), and ultimately impact of clinically important patient outcomes.

Current Therapy of IPF

The majority of IPF therapy is supportive and includes supplemental oxygen if needed, pulmonary rehabilitation, and smoking cessation. Early referral for transplant evaluation is critical, as bilateral lung transplant is the only known curative treatment for IPF.

Numerous clinical trials have shown no benefit or harm in IPF. Currently, pirfenidone and nintedanib are the only FDA-approved medications for treatment of IPF. Both treatments slow the progression of fibrosis in some patients with IPF but do not halt or reverse progressive fibrosis. Early identification of IPF is important for initiation of anti-fibrotic treatment as patients with advanced IPF (FVC <50% or DLCO<35%) may demonstrate less benefit than those with mild or moderate disease [98].

Pirfenidone negatively regulates lung fibroblasts through inhibition of transforming growth factor beta (TGF-β) and reduces extracellular matrix production. A meta-analysis of multiple randomized controlled trials evaluating pirfenidone demonstrated a benefit in progression-free survival, 6MWT, and subjective symptoms [99]. Nintedanib inhibits activation of fibrogenic growth factors through blocking receptor-associated tyrosine kinases (PDGF, FGF, and VEGF) [100]. In the INPULSIS-1 and INPULSIS-2 trials, nintedanib reduced the decline in FVC and increased time to first exacerbation [101]. In a meta-analysis, nintedanib also reduced the risk of AE-IPF [102]. Nintedanib has also shown benefits with advanced disease after the initial trials only included mild-to-moderate disease [98, 103, 104]. Dose-dependent diarrhea is a common adverse effect and can result in discontinuation of treatment.

Senotherapeutics for IPF

Senomorphics have not been studied in human clinical trials yet. However, senolytics have been successfully used in a single open-label pilot study followed by a yet unpublished small randomized controlled trial as detailed below. DQ showed significant improvement in senescent burden, physical function, and pulmonary function in bleomycin-induced fibrosis mouse models [22], as well as ex vivo senolysis of alveolar epithelial cells and lung fibroblasts [105].

Based on these preclinical data on the emerging pathogenic role of CS in IPF, we undertook a first-in-human, two-stage, prospective, clinical trial of intermittent administration of DQ (D, 100 mg/day; Q, 1250 mg/day, 3 days/week over 3 weeks) in older adults with stable IPF [106]. First, an open-label study (OL) was performed at two clinical sites followed by a single-site double-blind randomized placebo-controlled trial (RCT; ongoing). The primary endpoints demonstrated excellent therapeutic feasibility (e.g., participant retention, planned assessment completion rate, DQ adherence). Secondary endpoints were safety, functional health status, and changes in SASP. Although DQ was associated with greater mild-to-moderate adverse events, it was generally well tolerated. Interestingly, IPF patients treated with intermittent DQ showed significantly improved physical function and mobility by 6MWT, 4-minute gait speed, timed chair stands, and short physical performance battery (SPPB). Functional and reported health measures were unchanged. Although DQ effects on circulating SASP factors were inconclusive, improved physical function correlated with reduced SASP-related factors (23/48 markers, $r \geq 0.50$). Analysis of our small completed RCT of DQ in IPF is pending.

Although not done in IPF, Martyanov et al. tested dasatinib alone (100mg daily for 6 months) in 31 patients with scleroderma-associated ILD [107]. In scleroderma patients, dasatinib treatment (without quercetin) manifested no significant clinical efficacy. While scleroderma ILD has similarities with IPF, it also has many divergent features reflecting differences in mechanistic pathways, immune dysregulation, and fibroblast responses. As such, further prospective clinical trials of the safety and efficacy of senotherapeutics (such as senolytics DQ) are greatly needed in fibrotic lung diseases, such as scleroderma-associated ILD and IPF.

Future Directions

Senotherapeutics, particularly DQ, are potentially important therapeutic interventions for IPF and perhaps other fibrotic ILDs that feature an initial inflammatory, followed by fibroproliferative, phase such as viral pneumonia and acute respiratory distress syndrome (ARDS). Could senotherapeutics be used for pulmonary fibrosis in patients with post-acute sequelae of COVID-19 (PASC)? Elderly patients who require ICU care and mechanical ventilation appear to be at the highest risk of developing PASC ILD, including pulmonary fibrosis [108]. Considering the vast prevalence of

COVID-19 worldwide, even a small proportion of PASC lung fibrosis would have a tremendous deleterious impact on our health-care system. While the prevalence of PASC pulmonary fibrosis will become apparent with time, early data from such patients suggest that 25–65% of recovered patients develop fibrotic lung abnormalities at 3 months on HRCT [108, 109]. Currently, no proven options are available for their treatment though anti-fibrotic agents are in clinical trials, albeit none with DQ.

As another example, there are other pulmonary diseases that feature interstitial fibrosis following an initial inflammatory phase. Fibrotic/chronic hypersensitivity pneumonitis (HP) is initiated by an antigen that results in a robust T_H1 predominant response, which may be visualized by GGOs on HRCT or non-necrotizing granulomas on lung biopsy. There are many clinical and pathogenetic parallels between fibroproliferative HP and IPF, including an association with advanced age, shortened telomeres, and lack of significant improvement with anti-inflammatory treatments [110–113]. Interestingly, telomere shortening has been identified and is prognostically significant in many ILDs. However, given the disparate results with scleroderma-associated ILD, use of senolytics in fibrotic HP should undergo a proof of concept trial first [114, 115]. Indeed, there are many unanswered questions in the general fields of senescence and senotherapeutics in fibrotic pulmonary diseases.

Abbreviations *AE-IPF,* Acute exacerbation of IPF; *COPD,* Chronic obstructive pulmonary disease; *DLCO,* Diffusing capacity of lung for carbon monoxide; *FVC,* Forced vital capacity; *HRCT,* High-resolution computed tomography; *ILD,* Interstitial lung disease; *IPF,* Idiopathic pulmonary fibrosis; *UIP,* Usual interstitial pneumonia; *NSIP,* Nonspecific interstitial pneumonia; *TBCB,* Transbronchial cryobiopsy; *TBLB,* Transbronchial lung biopsy; *SPPB,* Short physical performance battery; *6MWT,* Six-minute walk test

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