Alveolar Epithelial Cells Death in Idiopathic Pulmonary Fibrosis: Significance, Mechanisms and Contribution to Disease Progression

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Abstract

Idiopathic Pulmonary Fibrosis (IPF) is a rare but deadly interstitial lung disease mainly affecting people over 65 years of age in its sporadic form. As the world population is ageing, IPF prevalence is increasing, warranting better therapeutic options. Decades of intense research efforts resulted in the recent marketing for IPF treatment of the tyrosine kinase inhibitor Nintedanib and the anti-fibrotic drug Pirfenidone. Both drugs have been shown to improve patients’ life expectancy but fail to stop disease progression. According to the current hypothesis, repetitive injuries to the epithelial compartment play a critical role in IPF onset by precipitating their death. In the long run, overlapping cycles of repair and damages lead to the exhaustion of the alveolar epithelium regenerative capacities and the disruption of epithelial and mesenchymal cells communication. As a result, mesenchymal cells such as fibroblasts proliferate, differentiate into myofibroblasts, become resistant to apoptosis, leading to lung fibrosis development. This review will focus on the significance of alveolar epithelial cells death in IPF, and the mechanisms involved, particularly those linked to IPF risk factors and physiopathological cells interactions. We will also touch on the effect of efferocytosis on alveolar macrophages phenotype and its impact on tissue repair or fibrosis development. Finally, we will review the potential role of necrotic cell-derived damage-associated molecular patterns (DAMP) in lung fibrosis development.

Keywords: Idiopathic Pulmonary fibrosis; Alveolar Epithelial Cells; Apoptosis, Necroptosis, Efferocytosis; ER stress; Mitochondria dysfunction, damage-associated molecular patterns, Danger Receptors, Toll-Like receptors

Abbreviations

AEC: Alveolar Epithelial Cells; AECII: Alveolar Epithelial Cells Type II; AM: Alveolar Macrophages; ANG: Angiotensinogen; ANGX: Angiotensin X; ATFX: Activating Transcription Factor X; ATP: Adenosine TriPhosphate; ATX: Angiotensin Receptors X; BALF: Broncho-Alveolar Lavage Fluid; BAX: BCL2 Associated X; BID: BH3 Interacting domain Death Agonist; CDX: Cluster of Differentiation X; CHOP: C/EBP Homologous Protein; cNOS: constitutive Nitric Oxyde Synthase; COL1A1: Collagen 1 A1; COX-X: Cyclooxygenase-X; CTGF: Connective Tissue Growth Factor; DAMP: Damage-Associated Molecular Patterns; DDR: DNA Damage Response; DNA: Deoxyribonucleic Acid; ECM: Extracellular Matrix; EMT: Epithelial to Mesenchymal Transition; ER Stress: Endoplasmic Reticulum Stress; eROS: External Reactive Oxygen Species; FF: Fibroblastic Foci; HGF: Hepatocyte Growth Factor; HIF1-α: Hypoxia Inducible Factor 1-α; HMGB1: High Mobility Group Box 1; HSPX: Heat Shock Proteins X; IFNy: Interferony; IL-α: Interleukin-X; ILD: Interstitial Lung Diseases; iNOS: inducible Nitric Oxyde Synthase; IPF: Idiopathic Pulmonary Fibrosis; iPSC: inducible Pluripotent Stem Cells; iROS: Intracellular Reactive Oxygen Species; MnSOD: Manganese SuperOxide Dismutase; mtDNA: Mitochondrial Deoxyribonucleic acid; mtROS: Mitochondrial Reactive Oxygen Species; NF-B: nuclear factor kappa-light-chain-enhancer of activated B cells; OGG1: 8-OxoGuanine DNA Glycosylase 1; PAI-1: Plasminogen Activator Inhibitor-1; PAMP: Pathogens-Associated Molecular Patterns; PAR-1: Protease-Activated Receptor 1; PDGF-BB: Platelet-Derived Growth

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Introducing Idiopathic Pulmonary Fibrosis

Idiopathic Pulmonary Fibrosis (IPF) is a relentlessly progressive lung disease whose patients’ life expectancy range from two to four years in the absence of treatment [1-3]. The disease mainly affects people over 65 years old and is associated with decreased lung function (i.e., forced vital capacity, total lung capacity and carbon monoxide diffusing capacity) manifested by exertional dyspnoea [4]. Additional symptoms include dry cough, fatigue and hypoxemic manifestations such as clubbing and acrocyanosis [3].

As shown by histopathologic pieces of evidence, those symptoms result from an extensive remodelling and disruption of the lung architecture, which impairs the lungs’ gas exchange capacities [5,6]. The pattern of histopathologic lesions observed in IPF and other closely related lung diseases is termed Usual Interstitial Pneumonia (UIP). It includes 1) a patchy pattern of fibrosis mainly in the subpleural/paraseptal areas, 2) the presence of enlarged alveolar spaces filled with mucus and inflammatory cells lined by bronchiolar metaplastic epithelium called honeycombs, and 3) Fibroblastic Foci (FF), which are formed by actively proliferating fibroblasts/myofibroblasts, embedded in a pale myxoid matrix, and covered by hyperplastic pneumocytes [5,6].

As suggested by the term “Idiopathic”, IPF causes remain elusive. However, in the past decades, epidemiological studies identified among IPF patients behavioural/environmental risk factors such as cigarette smoking and dust inhalation in an occupational setting [7,8]. Moreover, some comorbidities, including gastroesophageal reflux, type 2 diabetes, and infections, were more frequent in IPF patients, suggesting their possible involvement in the disease onset [7,8]. Besides, genome-wide association studies of IPF patients also identified genetic risk factors, including mutations in genes relevant to lung epithelial cells functions (e.g., MUC5B, SFTPC, SFTPA2), telomere functioning (e.g., TERT, TERC, PARN, RTEL1) and innate immunity (e.g., TLR3, Tollip, IL-8) [9]. Thus, it seems that IPF results from a lifelong exposure of the lung to noxious agents in individuals whose genetics predispose to premature ageing, Endoplasmic Reticulum stress (ER stress), abnormal innate immune response or any combination of these [10].

IPF natural history: From chronic inflammation to fibrosis

In IPF, tissue damage mainly affects the alveoli, a strategic structure involved in gas exchanges and ensuring a mucosal barrier function [11]. The alveolar structure is often described as an alveolar-capillary membrane composed of immune and nonimmune cells [12]. The heart of the alveolar-capillary membrane is formed by the juxtaposition of a monolayer of alveolar epithelial cells (AEC) lying on a basement membrane with on the other side a monolayer of lung microvascular endothelial cells [12,13]. There are two types of AEC. Type 1 AEC (AECl) have a flat morphology and are specialised in gas exchanges and represent around 40% of all AEC while occupying 90% of the alveolar space. On the other end, AEC type 2 (AEClI) have a cuboidal shape and are involved in surfactant proteins secretion and serve as the stem cell compartment for the alveolar epithelium [14]. AEClI are located at alveolus’ corners and interact at their apical side with resident immune cells such as alveolar macrophages (AM) [15] but also at their basolateral side with interstitial cells in particular lipofibroblasts [13]. The basement membrane plays a key structural role in the alveolar-capillary membrane and is constituted of extracellular matrix components (ECM) such as laminin, collagen IV, perlecan and entactin [12].

Figure 1: Cellular Events Involved in Idiopathic Pulmonary Fibrosis Onset and Development

Damages to the lung epithelium by noxious agents such as cigarette smoke particles, viruses, dust and micro-aspiration of stomach fluids lead to their death, secretion of inflammatory mediators and activation of the coagulation cascade resulting in the recruitment of inflammatory cells such as neutrophils. By secreting pro-inflammatory and apoptotic factors such as cytokines, ROS and proteases, inflammatory cells inflict further damage to the alveolar-capillary membrane. AECII proliferates to repair the damaged epithelium, while fibroblasts are recruited in the damaged basement membrane to repair it. Years of chronic exposure to noxious agents and continuous inflammatory cells recruitment further damage the basement membrane impairing epithelial cells regulation of mesenchymal cells proliferation. As a result, fibroblasts proliferate abnormally, differentiate into myofibroblasts, and become resistant to apoptosis, contributing to excessive ECM deposition and fibrosis. Besides, AECII and endothelial cells, by undergoing respectively epithelial and endothelial to mesenchymal transition, further support mesenchymal cells expansion and fibroblastic foci (FF) formation. Finally, a pathological ECM deposition whose profibrotic signalling promotes an autonomous fibrotic development is observed in later IPF development stages.

As suggested by epidemiological studies, micro-injuries to the alveolar epithelium by noxious agents (e.g., cigarette smoke, dust, viruses) are likely initiating events in IPF onset (See Figure 1). The resulting inflammatory response (i.e., cytokines and chemokines secretion) leads to AM and lung microvascular endothelial cells activation followed by the recruitment of inflammatory cells such as neutrophils, monocytes and lymphocytes [12,16]. While this initial response is physiologic, its recurrence fosters chronic inflammation and fibrosis of the lung. Thus, chronic inflammation causes further tissue damages (e.g., Epithelial and Endothelial cells death, basement membrane degradation), which, in a vicious cycle, exacerbate the inflammatory response [12,16]. In the long run, this leads to an exhaustion of the alveolar epithelium regenerative potential, a disruption of the alveolar epithelial lining and a loss of the basement membrane [11,12]. Some researchers believe the basement membrane loss and the subsequent collapse of adjacent alveolus represent a point of no return at which fibrosis become irreversible [12].

Following the destruction of the alveolar-capillary membrane, epithelial/mesenchyme interactions regulating the balance between regeneration and scaring are impaired, leading to the excessive deposition of ECM components and scar tissue formation [11]. At this stage, molecular pathways favouring scars formation, such as the TGF-β pathway, become overwhelmingly prevalent, favouring fibroblasts proliferation, differentiation in myofibroblasts, Epithelial/Endothelial to mesenchyme transition and further destruction of the alveolar structure by Fibroblastic Foci [11]. Lastly, the pathologic ECM found in advanced stages of IPF has been shown to promote fibroblasts differentiation into myofibroblasts establishing a positive feedback loop that supports the relentless progression of fibrosis in IPF [17].

Epithelial cells death in IPF: significance, mechanisms and contribution to lung fibrosis

Although there is no certainty regarding the identity of the noxious agents triggering IPF, the damages they inflict to lung cells can be observed on tissue sections. Depending on the toxicity of the noxious agents they are exposed to, the intensity and duration of this ex-
posure, as well as their internal capacities (e.g., ATP availability), to cope with the damages suffered, cells will either recover or die [18]. Initially, the scientific community distinguished two types of cell death; accidental cell death (i.e., necrosis) and programmed cell death (i.e., apoptosis) [19]. However, recent studies revealed that accidental cell death caused by acute injuries (e.g., heat) also present regulatory mechanisms [19]. This is the case of pyronecrosis, ferroptosis and necroptosis, to name a few [19].

Necrosis typically happens when the damages sustained by cells largely exceeded their coping capacities. It results in the release in their environment of intracellular molecules (e.g., mtDNA, HSP, HGMB1), which acts as damage-associated molecular patterns (DAMP) [19]. The recognition of these DAMP by surroundings cells’ danger receptors (e.g., TLR) triggers their expression of inflammatory cytokines and chemokines, and the subsequent recruitment in the damaged tissue of immune (e.g., neutrophils, monocytes) and mesenchymal cells (e.g., fibrocytes) [19,20].

Necroptosis is the most studied and, consequently, the better characterised regulated form of necrosis. It is a TNF-induced form of necrosis, regulated by a multiprotein complex formed by Receptor Interacting Protein Kinase-1 and -3 (RIPK1 and 3) and its substrate, mixed lineage kinase-like (MLKL), which can be inhibited by a class of RIPK1 inhibitors called necrostatins [19].

Apoptosis, on the other end, is a much more regulated death process, often happening in physiological responses such as normal wound healing [21], which does not result in significant DAMP releases and is therefore regarded as less immunogenic than necrosis [19]. Whereas necrosis is characterised by organelles swelling and limited chromatin condensation, apoptosis involves cells shrinking, chromatin condensation, and apoptotic bodies formation. All these processes facilitate apoptotic cells phagocytosis in a process called efferocytosis [19].

Apoptosis can be triggered by an extrinsic or an intrinsic pathway. The extrinsic pathway is triggered by the activation at the cells surface of death receptors by immune cells or inflammatory mediators such as the TNF-related apoptosis-inducing ligand receptor (TRAIL) and FAS [22]. On the other hand, the intrinsic pathway is triggered in response to stimuli, such as oxidative stress, DNA damage and ER stress [22]. Both pathways result in caspases activation, which plays a central role in apoptosis regulation [22]. Whereas the stimulation of death receptors directly activates pro-caspases (e.g., pro-caspase-8 and -10), caspases activation by the intrinsic pathway requires a process called mitochondrial outer membrane permeabilisation (MOMP) [23]. The MOMP results in the release of mitochondrial cytochrome c into the cytoplasm, where it activates a protein complex named apoptosome, which is responsible for caspases activation and subsequently apoptosis [23].

Significance of epithelial cells death in IPF pathogenesis

As said earlier, damages to the alveolar epithelium are regarded by the research community as the initial event in IPF pathogenesis. This section will review findings regarding epithelial cells death in IPF, the mechanisms involved, and its impact on lung fibrosis development.

As early as 1988, Meyers., et al, using light and electron microscopy, described in a case report the presence of AEC necrosis associated with the alveolar collapse in lung samples presenting a UIP pattern characteristic of IPF [24]. Although in this study, these changes were mainly observed nearby FF [24,25], another study observed an increased AECII apoptosis in histologically normal alveoli of IPF lungs, suggesting AEC apoptosis is an early event of the disease onset [26]. Ten years later, Uhal., et al, using in situ end labelling of fragmented DNA and α-SMA staining (i.e., myofibroblasts marker) on paraffin sections, reported the presence in IPF lungs of necrotic and apoptotic AEC, particularly in cells immediately adjacent to FF containing α-SMA+ myofibroblasts [27]. Both this study and a later one by Barbas-Filho., et al, using the same technics, identified the dying cells as AECII based on their typical cuboidal shape [26]. The staining of pro and anti-apoptotic markers in IPF samples later confirmed an increased DNA fragmentation (i.e., TUNEL staining) and expression of pro-apoptotic markers such as p53, BAX and caspase-3 in hyperplastic AECII, with a concomitant decrease in their expression of the

anti-apoptotic protein B-cell lymphoma 2 (BCL-2) [28]. In the same way, Nakashima, et al., showed in AEC from IPF samples increased expression and phosphorylation of p53 along with increased mouse double minute 2 (MDM2) and p21 expression. Interestingly, most of those markers except for the senescence-associated marker p21 were expressed at higher levels in IPF than in non-specific interstitial pneumonitis, another interstitial lung disease responsive to anti-inflammatory treatments with a better prognosis than IPF [29]. The significance of AEC death contribution to IPF pathogenesis is further supported by experiments showing that administration of mediators with anti-apoptotic/pro-proliferative effects on AEC can abrogate lung fibrosis development. Thus, hepatocyte growth factor (HGF) gene transfer [30,31] or administration of recombinant HGF [32,33] prevented bleomycin-induced epithelial cells apoptosis and lung fibrosis development. Moreover, intratracheal administration of human induced pluripotent stem cells (iPSC)-derived AECII to rats 15 days after lung fibrosis induction by bleomycin instillation reduced TGF-β1 and α-SMA expression as well as pulmonary fibrosis [34].

Conversely, intratracheal administration of apoptotic cells in rats induces lung cells secondary apoptosis, inflammation and pulmonary fibrosis [35]. On the other hand, administration of caspases inhibitors attenuated bleomycin-induced pneumopathy in mice [36,37].

Recently, necroptosis appeared to be in addition to apoptosis, a significant mechanism of AEC death in IPF. Indeed, Lee, et al., recently reported an increased expression of the necroptosis inducer RIPK3 in AEC from IPF and experimental lung fibrosis samples [38]. In experimental lung fibrosis, both RIPK3 deficiency and intranasal administration of necrostatin (i.e., necroptosis inhibitor) protected mice from lung fibrosis development [38]. Consistently, a second research team has recently shown the protective role of necrostatin administration in experimental lung fibrosis [39]. Moreover, using primary mouse cells, the authors highlighted in vitro the role of necroptosis in AEC death induced by bleomycin and hydrogen peroxide [38]. Interestingly, the authors found that while apoptotic cells (i.e. cleaved caspase-3+ cells) and necrotic cells (i.e., RIPK-3+ cells) were very scarce in normal lungs, they represented respectively 18 and 9% of cells in IPF samples, with an additional 5% of double-positive cells [38]. Whether these proportions evolve with disease activity is unknown; however, a shift towards increasing necroptosis could exacerbate inflammatory cells recruitment and lead to further AEC damage. Interestingly, some data showed a higher proportion of immune cells (i.e., CD45+ cells) in the lung of rapidly progressing IPF patients compared with stable ones [40].

Finally, the extent of AEC loss in IPF lungs is difficult to quantify because of various technical reasons (e.g., AECI fragility, Incomplete tissue digestion; Lack of stable surface markers; Tissue accessibility...). However, some data suggest it is massive; indeed, Xu, et al., using the AECII marker HTII-280, found that AECII represented more than 90% of normal distal lung epithelial cells (i.e., CD326+ cells) recovered following enzymatic dissociation against around 5% in distal IPF lung [41]. This observation was independently confirmed by Liang, et al., who also observed, a reduced renewal capacity (i.e., in vitro colony-forming capacity) of IPF-derived AEC compare to AEC derived from normal lungs [42]. However, these results should be taken with caution since the authors did not report the expression of other AEC markers (e.g., SFTPC) by CD326+/HTII-280- cells. Therefore, it is not clear whether the loss of HTII-280 reflects a loss of AECII identity or a simple phenotypic shift involving the downregulation of HTII-280 expression.

Mechanisms of epithelial cells death in IPF

This section will explore the literature addressing the mechanisms involved in AEC apoptosis by focusing on the link between known IPF risk factors and AEC dysfunctions affecting the endoplasmic reticulum and mitochondria. We will also explore the cellular interactions and mediators supporting AEC death in IPF.

The oxidant/antioxidant imbalance in IPF

In their efforts to uncover the molecular mechanisms supporting AEC death in IPF, scientists rapidly identified oxidative stress as a potential contributor. Oxidative stress is defined as an imbalance between the production of oxidants (e.g., Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS)) and antioxidants (e.g., Glutathione, Vitamins, Superoxide dismutase, N-acetylcysteine), resulting in excessive oxidation and damages to cellular molecules such as DNA, proteins and lipids [43].
In IPF, early investigations revealed an increased level of oxidised methionine in patients' Bronchoalveolar Lavage Fluid (BALF) [44], while the cells recovered from these samples (e.g., neutrophils, AM) were found to be releasing abnormally high levels of ROS such as superoxide [45,46]. Similarly, Psathakis., et al found an increase in hydrogen peroxide and 8-isoprostane concentration inside IPF patients’ expired breath condensate [47], while others found an increase in lipid peroxidation within their BALF and plasma [48,49].

Consistent with the neutrophilia observed in IPF, affected patients’ epithelial lining fluid also presented higher concentrations of myeloperoxidase, a protein released by activated neutrophils and transforming hydrogen peroxide into the highly toxic hypoalide anion [45].

In addition to ROS, there is in IPF pieces of evidence of molecular damages induced by RNS. Indeed, IPF is associated with increased NO and peroxynitrite formation, which correlates with a shift in nitrite oxide synthases expression in IPF lungs (i.e., cNOS downregulation and iNOS upregulation) [50]. Consistently, Saleh., et al., found in IPF lungs an increased level of Nitrotyrosine, a marker of protein nitration by peroxynitrite [50].

Conversely, several investigators found in IPF samples decreased levels of antioxidants. Thus, Rahman., et al., observed a marked reduction in antioxidant capacities (i.e., Trolox equivalent) within the plasma and BALF of IPF patients [49], which correlates with decreased glutathione concentration in the epithelial lining fluid [51], sputum and plasma of IPF patients [52]. Altogether, these observations reveal a strong imbalance between oxidants and antioxidants production in IPF lungs that have locals and systemic impacts. Lavrentiadou., et al., showed that GSH treatment at physiologic concentration inhibited H$_2$O$_2$-induced ceramide production and apoptosis in the AEC line A549 [53]. Similarly, catalase inhibition in these cells increased their production of H2O2 and ceramides, leading to their apoptosis. Interestingly, this process could be blocked by the administration of the antioxidant N-acetylcysteine [53].

Interestingly, many risk factors potentially associated with IPF onsets, such as cigarette smoking or exposure to dust (i.e., Silica), can directly induce oxidative stress [54]. Moreover, experimental evidence suggests that bleomycin-induced lung fibrosis involves ROS-dependent apoptosis of AEC [55]. These initial oxidative stress sources, by damaging nucleic acids, proteins and lipids, impair subcellular organelles’ functioning, including the nucleus, the endoplasmic reticulum and the mitochondria [43]. To manage those dysfunctions, cells activate several cellular processes, such as the DNA damage responses (DDR), the unfolded protein response (UPR) and autophagy. Failure to repair these damages can lead to excessive mitochondrial ROS production, further molecular damages, higher activation of inflammatory pathways and ultimately cells death [43,56].

### Nuclear DNA (nDNA) damages

The genetic information contained in our nuclear DNA is fundamental to our cell functioning, and the maintenance of its integrity is critical to avoid genes loss of function, cell transformation and ultimately, death. The integrity of DNA is threatened by many agents, including ultraviolet, ionising radiation, and ROS [57]. DNA damaging agents can induce chemical modifications of nucleotides, DNA strand breaks (i.e., Single or Double) and interstrand cross-linking [57]. DNA damage such as double-strand breaks induces a DDR involving the early activation of the protein kinase ATM and subsequent activation of the genome guardian p53 as well as that of cell cycle inhibitors (e.g., p16, p21) and pro-inflammatory transcription factors such as NF-κB. Depending on the extent of DNA damage and the cell’s ability to repair them, the DDR can lead to cell senescence or apoptosis [57].

In IPF, the role of oxidative stress in AEC death is supported by the observation in these cells of significant ROS-specific DNA damage such as 8-hydroxy-deoxyguanosine (8-OHdG) [58]. Moreover, Kuwano., et al., also reported increased DNA double-strand breaks in bronchial and alveolar epithelial cells of IPF patients, which were associated with an increased expression of p53 and p21 [59]. Besides, apoptotic AEC from IPF samples also present changes in MAP Kinases activation with increased activation of the stress-activated and pro-inflammatory MAP kinases JNK and p38 Map Kinase [60]. Conversely, these cells present a decreased activation of the pro-proliferative and pro-survival MAP Kinase, ERK1/2 [60]. Finally, the significant role played by DNA damage in IPF pathogenesis is supported by the
observation that DNA damaging agent such as bleomycin and ionising radiation are efficient promoters of lung fibrosis [61,62].

Mitochondrial dysfunction

Mitochondria are maternally inherited intracellular organelles often considered the cell’s powerhouse as they produce life’s molecular fuel, also known as adenosine triphosphate (ATP) [63]. As by-products of their respiration, mitochondria also produce ROS (mtROS) such as superoxide anion (\(O_2^-\)), hydrogen superoxide (\(H_2O_2\)) and hydroxyl radical (\(OH^-\)). At physiologic concentrations, mtROS activates antioxidant pathways in a process called mitohormesis [64].

The number of mitochondria per cell is variable between tissues, and for a given tissue between individuals depending on parameters such as age and physical activity, for instance [63]. Interestingly, mitochondria are the only sub-cellular organelle to have their own DNA, which is composed of 16569 nucleotides coding 13 polypeptides of the electron transport chain, 22 transfer RNA and two ribosomal RNA [63]. Most of the mitochondrial proteins (~1200) are coded by nuclear DNA, produced in the cytoplasm and imported into the mitochondria [63]. In addition to ATP production, mitochondria play a significant role in cell fate by regulating cellular processes such as apoptosis, cell cycling, or differentiation [63].

In IPF, several studies reported in AECII an accumulation of dysmorphic mitochondria with a swollen appearance and disorganised cristae [65,66]. The accumulation of damaged mitochondria in IPF has been attributed to defective mitophagy due to a decreased expression of PTEN-induced putative kinase 1 (PINK1). According to the authors, the decreased expression of PINK1 in AECII from IPF lungs results from the activation of its transcriptional repressor ATF3 in response to ER stress [65,67]. Consistently, PINK1 deficiency aggravated lung fibrosis induced by bleomycin, whereas mice with a conditional deletion of ATF3 in AEC were protected [66,67]. Although both studies identified PINK1 deficiency as deleterious in experimental lung fibrosis, Patel, et al., reported an increased PINK1 expression in IPF-derived AECII and suggested based on in vitro experiments that TGF-β1 stimulation could be responsible for this increase [66]. Interestingly, AEC derived from PINK1 deficient mice produce more ROS and are more sensitive to TGF-β1-induced apoptosis [66].

However, in IPF, excessive AEC exposure to ROS might be the primary cause of mitochondrial damage. Indeed, high ROS concentrations might lead to damages to mitochondrial DNA, lipids and proteins, and subsequent uncoupling of the electron transport chain involved in mitochondrial respiration and ATP production [64]. Consistently, AEC from IPF lungs presents a reduced activity of mitochondria’s electron transport chain complex I and IV [65], leading to increased mtROS production. Interestingly, in vitro experiments showed that TGF-β1 stimulation induces a reduction in ETC complex IV activity and increased mtROS production in AEC [68]. These data provide a rationale for the increased in ROS-induced DNA lesions such as 8-hydroxyguanine (8-OXO-G) and 8-hydroxy-2’-deoxyguanosine (8-OH-dG) observed in mtDNA from IPF patients [69]. The critical role of mtDNA damage in AECII apoptosis and lung fibrosis development is supported by their increases during experimental lung fibrosis (i.e., asbestos and bleomycin) in mice deficient for the mtDNA repair protein 8-oxoguanine DNA glycosylase 1 (OGG1) [70]. In addition to its DNA repair function, in vitro experiments suggest that OGG1 protect mtDNA from ROS induced damage by chaperoning the oxidative stress biosensor aconitase-2 [71]. Moreover, recent data showed that mtOGG1 overexpression mitigates mtDNA damage and AECII apoptosis associated with PINK1 deficiency [72]. Additional data revealed that mitochondrial proteins with antioxidant (i.e., manganese superoxide dismutase) and DNA-repair (i.e., OGG1) function were inactive in AEC from IPF patients [73]. In vitro, treatment of AEC with asbestos or \(H_2O_2\) diminished the expression of the mitochondrial NAD-dependent deacetylases Sir2 (SIRT3), increasing thereby MnSOD acetylation [73]. Conversely, SIRT3 overexpression in these cells decreased OGG1 acetylation, mtDNA damage and apoptosis, while its inhibition had the opposite effect. Finally, AEC mtDNA damage and apoptosis, as well as lung fibrosis induced by bleomycin and asbestos, were amplified in SIRT3 deficient mice [73]. As expected, mice over-expressing in their mitochondria antioxidant proteins such as the \(H_2O_2\) detoxifying enzyme catalase are resistant to mtROS production, mtDNA damage, AECII apoptosis and lung fibrosis development induced by bleomycin [74].

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In addition to these molecular actors, mediators associated with IPF and experimental lung fibrosis such as the “antiaging” molecule α-Klotho [75] and the bioactive lipid sphingosine-1-phosphate (S1P) [76] have been shown to influence mtROS production/mtDNA damage, AECII apoptosis and lung fibrosis. Thus, α-Klotho, whose expression is decreased in IPF patients’ serum, could protect mice from asbestos-induced lung fibrosis by reducing AEC mtDNA damage and apoptosis. Mechanistic insights about α-Klotho’s protective effect suggest it is mediated by fibroblast growth factor receptor 1 (FGFR1) and AKT activation [77]. On the other end, S1P, whose concentration is increased in IPF serum, could have the opposite effect. Indeed, pharmacological inhibition of its producer, the Sphingosine Kinase 1 (SPHK1), during experimental lung fibrosis induced by asbestos, reduced S1P concentration and lung mtDNA damage, an effect also observed in AEC in vitro [76].

It is clear from these data that mitochondrial dysfunctions, particularly in AECII, plays a critical role in lung fibrosis initiation and progression. Therefore, future therapies should aim to prevent mitochondrial dysfunction. As shown by PINK1’s example, targeting cross-talks between the endoplasmic reticulum and mitochondria might represent a good strategy to achieve this goal.

Endoplasmic reticulum stress

In addition to DNA damage, protein damage induced by ROS can lead to protein misfolding, endoplasmic reticulum stress (ER stress) and the activation of an unfolded protein response (UPR) [78]. Prolonged or excessive ER stress in AEC can induce apoptosis through the induction of CHOP, the activation of the ER-bound caspase (i.e., Caspase-4), and the activation of the JNK MAP Kinase pathway [78].

The link between AECII dysfunction, ER stress and pulmonary fibrosis has been particularly enlightened in the past decades by the discovery of genetic forms of interstitial pulmonary fibrosis caused by mutations in surfactant proteins A and C [78]. As a result of these mutations, surfactant proteins are misfolded, leading to ER stress and the activation of an UPR [78]. The link between these mutations, ER stress, and lung fibrosis has been studied using transgenic mice in which SFTC mutant expression could be induced specifically in AECII. While the mutant expression alone was not sufficient to induce epithelial cells death or lung fibrosis, these mice had, in response to bleomycin challenges, increased lung fibrosis, greater AEC apoptosis, and fibroblasts density. This suggests that in sporadic forms of IPF, ER stress could favour pulmonary fibrosis by interacting with other IPF risk factors/stressors [79].

In sporadic forms of IPF, Lawson, et al., using immunohistochemistry staining of IPF lung sections, reported increased ER stress markers expression (i.e., BiP, EDEM and XBP) mainly in AECII [80]. In the absence of SFTC proteins mutations, the authors suggested that ER stress was in these patients induced by herpesviruses infections. Indeed their proteins could be detected in 65% of the sporadic IPF lung samples and colocalised in AEC with ER stress markers [80]. Moreover, Korfei, et al., also reported in AECII from IPF patients increased expression of additional ER stress markers (i.e., ATF4, ATF6 and CHOP) in association with apoptotic markers such as cleaved caspase-3 [25].

Besides viral infections, Tagawa, et al., showed that cigarette smoke induces in human bronchial epithelial cells a ROS-dependent expression of ER stress markers such as CHOP and GRP78 as well as the activation of caspase-3 and -4 [81].

Finally, hypoxia could be an additional promotor of ER stress in IPF lungs’ AEC. Indeed, hypoxia markers, such as increased expression of the transcription factors HIF-1α and its target genes, have been reported in IPF and the bleomycin model of lung fibrosis at both the mRNA and protein level [82]. An early study by Krick, et al., showed that hypoxia induces primary rat AECII apoptosis through HIF-1α-dependent activation of the pro-apoptotic member of the Bcl2 family Bnip-3L [83]. In vitro, both hypoxia-induced Bnip-3L expression and the subsequent apoptosis of primary rat AECII could be prevented by administering the anaesthetic drug and HIF-1α inhibitor Propofol [84]. Moreover, hypoxia has been shown to exacerbate ER stress and fibrosis in the bleomycin lung fibrosis model [85,86]. More recent data revealed a co-expression of the hypoxia marker HIF-1α, and the ER stress marker CHOP in AEC from IPF patients and fibrotic lungs from bleomycin treated mice [87]. Interestingly, the authors showed in vitro that HIF-1α triggers AEC apoptosis by inducing the pro-

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apoptotic ER stress marker C/EBP homologous protein (CHOP) [87]. Consistently, CHOP–/– mice were resistant to bleomycin-induced AEC apoptosis and lung fibrosis but not mice with conditional deletion of HIF-1/2 in epithelial cells [85]. These discrepancies between the in vivo and in vitro results are not well understood but suggest that hypoxia might induce ER stress in AEC through interactions with other cells type. Further in vitro experiments identified the inositol requiring enzyme 1a (IRE1a) and the PKR-like ER kinase (PERK) pathway as downstream effectors of CHOP in AEC apoptosis induction by hypoxia and ER stress [85]. Finally, Shi, et al., observed in a model of obstructive sleep apnoea a concomitant increase in ER stress markers expression, AEC apoptosis and profibrotic markers expression (i.e., TGF-β1 and thrombospondin). Interestingly, all these effects were prevented by administering the ER stress inhibitor tauroursodeoxycholic acid (TUDCA) [88].

TGF-Beta-induced alveolar epithelial cell death

Transforming growth factor-beta 1 (TGF-β1) is a multifunctional mediator and master regulator of wound healing and tissue remodelling. Its central role in wound healing is supported by its ability to regulate a wide range of cellular processes including, cell proliferation, differentiation, migration and apoptosis [89,90]. In particular, TGF-β1 stimulation elicits growth inhibition and apoptosis in multiple epithelial cells type while promoting the proliferation of mesenchymal cells [89].

TGF-β1 signal through and bind to its membrane-bound receptor TGF-β Receptor II (TGF-βRII), an interaction that triggers the formation of a heterotetrameric complex at parity with the TGF-β Receptor I (TGF-βRI) [89]. TGF-βRII then phosphorylate and activate TGF-βRI, which in turn activates receptor-activated SMADs (R-SMAD), also known as SMAD2 and 3 [89]. Once activated, R-SMADs form heteromeric complexes with the co-SMAD (i.e., SMAD4), which translocate to the nucleus and interact with co-activators and co-repressors to regulate genes expression [89,91]. Among the targets positively regulated by TGF-β1 signalling, we find inhibitory SMAD proteins (i.e. SMAD6 and 7), which prevent the activation of R-SMADs and constitute a negative feedback loop of this pathway [89]. Investigation of the TGF-β1 pathway in IPF revealed an increased concentration of its active form in their BALF [92]. However, conflicting results exist regarding its serum concentration, with one study reporting an increase [93] and another no change [94]. However, immunohistochemical studies revealed an increased expression of TGF-β1 in AEC and macrophages from IPF lungs [95] and experimental lung fibrosis (i.e. bleomycin instillation) [96].

Interestingly, BALF from IPF patients was found to induce AEC apoptosis in vitro, an effect blocked by the adjunction of blocking anti-TGF-β1 antibodies [97]. In vitro, TGF-β1 has been shown to induce normal alveolar epithelial cells apoptosis through activation of SMAD3 [98] and downregulation of the cell cycle regulator p21 [97]. Moreover, in vivo, bioactive TGF-β1 overexpression in the lung induces a wave of epithelial cells apoptosis followed by the recruitment of mononuclear cells and tissue fibrosis [99]. Besides, Kang., et al., showed that apoptosis-associated with TGF-β1 overexpression in mice lung required activation of the pro-apoptotic factors Bid and Bax [100]. Consistently, AEC from BID null mice are resistant to TGF-β1-induced AEC apoptosis and BID null mice are protected against bleomycin-induced lung fibrosis [101].

Moreover, gene transfer of soluble hence inhibitory TGF-βRII 3 days before or four days after bleomycin instillation in mice is associated with decreased cellular apoptosis rate and tissue fibrosis [102]. However, unlike what was observed in bronchiolar epithelial cells in vitro, pulmonary overexpression of TGF-β1 increased p21 expression in epithelial cells and macrophages in a TNF-α-dependant manner [103]. Moreover, p21 expression acted as negative feedback of TGF-β1-induced TNF-α expression, and its deletion aggravated cell apoptosis, inflammation and fibrosis induced by TGF-β1 overexpression [103]. Conversely, in experimental lung fibrosis, adenoviral expression of human p21 predominantly in the epithelium seven days after bleomycin instillation decreased apoptosis, inflammation and fibrosis at day 14 [104]. Since p21 is an essential regulator of cell senescence, it seems there is, in vivo, a trade-off between apoptosis and senescence that limit early tissue damages and later fibrosis development.

Besides, stimulation of the lung epithelial cell line Mv1Lu by TGF-β1 inhibited the mitochondrial complex IV resulting in prolonged

mtROS production [68]. Thus, TGF-β1 could also induce AEC apoptosis in IPF through a mtROS-dependent pathway.

(Myo) Fibroblasts as drivers of AEC death in IPF

As observed in early and more recent research, AEC death often occurs in FF’s vicinity, which contains myofibroblasts, suggesting a possible implication of these cells in AEC’s death. Interestingly, Whagray.,., et al., showed that fibroblasts from IPF lungs primed with TGF-β1 released hydrogen peroxide and induced small airway epithelial cells death in a co-culture system [105]. However, this response is not specific to IPF fibroblasts since earlier works showed an NADH-dependent production of hydrogen peroxide by normal human foetal lung fibroblasts in response to TGF-β1 stimulation [106]. In addition to hydrogen peroxide, another study by Wang.,., et al., showed that fibrotic lungs fibroblasts released six times more angiotensin II than normal fibroblasts. Moreover, Angiotensin II blocking in fibrotic lungs fibroblasts conditioned medium impaired their ability to induce AEC apoptosis [107].

IPF fibroblasts and myofibroblasts do not only secrete factors inducing AEC’s apoptosis, but they also downregulate their secretion of factors supporting AEC’s proliferation and survival. Indeed, IPF-derived fibroblasts secrete lower level of the AEC’s anti-apoptotic factor Prostaglandin E2 (PGE2), whose autocrine action in these cells also stimulate the expression and activation of hepatocyte growth factor (HGF), another AEC pro-survival mediator [108,109]. Despite a lower expression of HGF by IPF-derived fibroblasts, HGF’s level in the BALF and serum of IPF patients is higher than in normal subject, suggesting higher production by other cellular sources [110].

Interestingly, PGE2 inhibits lung fibroblasts proliferation and collagen expression while increasing their apoptosis in response to FASL [111,112]. AEC is a source of PGE2 and contributes to the control of lung fibroblast function; however, AEC from IPP lung present a reduction in their expression of cyclooxygenase-1 and -2 (COX-1 and -2), the enzymes involved in PGE2 synthesis [113]. Thus, PGE2 downregulation in IPF lungs has been proposed to be responsible for the apoptosis paradox (i.e., increased AECII apoptosis and fibroblast resistance to apoptosis) observed in IPF [112]. More recent research identified epigenetic repression of genes involved in PGE2 synthesis as the cause of its decreased production in IPF lungs [114,115].

Inflammation as a driver of AEC death in IPF

In addition to toxic agents primarily involved in IPF onset and AEC damage (e.g., cigarette smoke, viruses, mineral/metal/wood dust etc.), inflammatory mediators (e.g., cytokines, chemokines, DAMP) released by damaged cells and newly recruited inflammatory cells can worsen epithelial cell death.

In IPF, inflammatory cells’ recruitment results in a doubling of BALF cellularity mainly due to increased neutrophils and eosinophils numbers, and to a lesser extent in that of lymphocytes and monocytes [10]. Cytotoxic lymphocytes are known to express the transmembrane death receptor activator FAS Ligand (FASL), whose cleavage by Matrix metallopeptidase (MMP) results in the release of a soluble form known as soluble FAS Ligand (sFASL) [116]. Interestingly Kuwano.,., et al., showed that FASL expression was increased in BALF cells of IPF patients while being absent from control [117]. In situ Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and immunohistochemistry identified Lymphocytes and granulocytes as the primary FASL producers in IPF [117]. This finding is supported by correlations between the degree of lymphocytes alveolitis and sFASL concentration in the BALF of IPF patients [116].

Besides, bronchiolar and alveolar epithelial cells from IPF lungs present an increased expression of the death receptor FAS as detected by immunohistochemistry [117]. Interestingly, Hagimoto.,., et al., demonstrated that BALF from IPF patients induced bronchiolar epithelial cells apoptosis [97]. The authors also suggested a synergy between FASL and TGF-β1 in the induction of bronchiolar epithelial cells apoptosis [97].

Like in IPF, there is in bleomycin-induced lung fibrosis an increased expression of FASL and AEC apoptosis which can be prevented by
administering FAS antigen or FASL blocking antibodies [118]. Conversely, repeated inhalations by mice of an agonistic anti-FAS antibody induced a dose-dependent increased in bronchiolar and AEC apoptosis, lung inflammation and lung fibrosis [119]. Altogether these data suggest a prominent role for the FAS/FASL pathway in AEC apoptosis associated with IPF.

The angiotensin system

Angiotensin II is a mediator initially discovered for its role in blood pressure regulation. The angiotensin system includes the angiotensin precursor angiotensinogen, produced by various cells types, including AEC and fibroblasts in the lung [120]. The system also includes an aspartyl protease such as renin or cathepsin D, which transform angiotensinogen into Angiotensin I (ANGI) and an ANGI converting enzyme (ACE), which convert ANGI into ANGII [120]. Finally, the angiotensin system comprises two receptors named AT1 and AT2 expressed across several cell types [120]. More recently, the ACE homologue ACE-2, which transform ANGII into ANGI-7, have been discovered. Further experiments showed that ANGI-7 activated the receptor mas and elicited responses different and often opposite to that of ANGII [120].

Interestingly, most members of the angiotensin system are increased in IPF. Thus, angiotensinogen mRNA and protein expression are increased by around 20 and 4 fold, respectively in IPF lung biopsies [121], while ACE concentration increases in their BALF [14]. Finally, in IPF lungs, both ANGII receptors expression are increased at the mRNA level [122]. Moreover, IPF patients bearing the G-6A polymorphism of ANG associated with higher mRNA expression and protein production have faster disease progression [123]. Li, et al, also showed by immunohistochemistry that apoptotic AEC from IPF samples expresses ANG peptide suggesting a possible causal link [121]. Consistently, inhibition of local ANGII production by intratracheal administration of antisense nucleotide prevented AEC apoptosis and fibrosis induced by bleomycin instillation [124].

Conversely, administration of purified ANGII by aerosol (i.e., 30 minutes daily for 14 days) in rats was associated with increased hydroxyproline deposition in the lung [125]. Moreover, mice overexpressing ANG ubiquitously develop lung fibrosis with sub-optimal doses of bleomycin. At the same time, intratracheal administration of an adenosine expressing ANG led to its preferential expression by AEC, which was associated with their apoptosis and lung fibrosis [125]. Both AEC apoptosis and lung fibrosis induced by adenoviral expression of ANG could be prevented by the AT1 receptor antagonist Losartan or the caspase inhibitor ZVAD-fmk [125].

Previous in vitro studies using the human AEC line A549 and primary rats AEC showed that FAS and TNF-α induced apoptosis in these cells by stimulating ANG expression and autocrine binding to the AT1 receptor [126-128]. Finally, using the same ACE model in vitro, researchers showed that cells pre-treatment with the ACE inhibitor captopril or the AT1 antagonist Losartan inhibited their apoptosis induced by bleomycin treatment or FAS activation [129,130]. Consistently, in the bleomycin model of lung fibrosis, AT1 inhibition by Losartan or its genetic deletion resulted in decreased ACE apoptosis and lung fibrosis [131]. In contrast, AT2 receptors’ activation by the selective compound 21 inhibited bleomycin-induced lung inflammation and fibrosis [132]. Although AT2 is mainly express by AEC and fibroblasts/myofibroblasts in the lung, its selective activation did not inhibit AEC apoptosis [132].

Unlike other angiotensin system members, ACE-2 expression and activity are reduced by 92 and 74% respectively in IPF lungs biopsies; an observation also made in the bleomycin model of lung fibrosis [133]. Moreover, molecular or pharmacological inhibition of ACE-2 increased ANGII and collagen accumulation in the lung, while systemic administration of recombinant ACE-2 or infusion of ANGI-7 attenuated bleomycin-induced lung fibrosis [134] by inhibiting the MAP Kinase and NF-κB pathways [133,135]. Interestingly, subsequent investigations made in various AEC models (i.e., primary rat AEC, MLE-12, A549) revealed that ANGI-1, the product of ACE-2, protects cells from bleomycin, or ANGI-induced apoptosis by inhibiting JNK activation in a mas and MAP Kinase phosphatase-2 dependent pathway [136,137]. In addition to bleomycin, ER stress and the subsequent UPR induced in AEC (i.e., primary human AEC and A549) by chemicals or the SP-C BRICHOS domain mutant G100S has also been shown to induce their death through autocrine ANGII stimulation [138]. Interestingly, UPR signalling in AEC led to the activation of the ACE-2 ectodomain shedding enzyme TACE, providing a rationale for the decreased activity of ACE-2 in IPF samples [138]. Moreover, ANGI-7 administration could prevent AEC apoptosis induced by ER stress, an effect abrogated by the mas inhibitor A779 [139].

The link between the renin-angiotensin system and IPF progression has recently been evaluated in a posthoc analysis of several IPF clinical trials cohorts. The authors found that those receiving ACE inhibitors to treat hypertension had slower disease progression, while ANGII receptor blockers did not significantly alter disease progression [140]. Therefore, prospective clinical trials are warranted to evaluate the benefit of angiotensin modulators in IPF.

In addition to AEC, myofibroblasts constitute a key source of AGNII in IPF lungs [14]. However, as opposed to its effect in AEC, angiotensin has been shown to stimulate lung fibroblasts proliferation as well as their production of TGF-β1 and pro-collagens [14]. Thus, ANGII is another contributor to IPF’s apoptosis paradox.

**PAR-1 activation by thrombin and leukocytes elastase.**

The coagulation cascade’s activation is an early response to tissues damages facilitating inflammatory cells recruitment and tissue repair [141]. In IPF lungs, Tissue Factor (TF) is upregulated in AECII in areas of significant fibrin deposition [142]. The protection against acute exacerbations of IPF conferred by administrating the anticoagulant warfarin supports the idea of excessive activation of the coagulation cascade in IPF [143]. Among the coagulation system actors, the serine protease thrombin has been involved in TGF-β1 activation by AEC through protease-activated receptor-1 (PAR-1) cleavage [144]. Besides, direct activation of PAR-1 by leukocytes elastase at AEC’s surface induces their apoptosis through the intrinsic apoptotic pathway [145,146].

Interestingly, thrombomodulin, a thrombin ligand that turns its activity from pro- to anti-coagulant, has been shown to prevent lung fibrosis induced by TGF-β1 overexpression or bleomycin [147]. The authors also showed that recombinant human thrombomodulin administration prevented AEC apoptosis induced by bleomycin both in vivo and in vitro [147]. Consistently a meta-analysis of clinical trials testing the effect of recombinant human thrombomodulin on acute exacerbation of IPF concluded to its benefit in increasing patients’ survival [148]. However, a recent randomised clinical trial did not confirm those benefits; therefore, additional clinical trials are warranted to clarify the potential benefits of thrombomodulin in IPF treatment [149].

**Epithelial cells death contribution to fibrosis development in IPF**

Like all mucosal organs, the lung is exposed to constant aggressions from the external environment. Therefore, AECII’s ability to renew themselves and differentiate into AECI is crucial to alveolus homeostasis. However, as described earlier, IPF is characterised by significant AECII’s death due to the activation of several cellular and molecular responses (Figure 2). In this section, we will focus on the impact of different cell death mechanisms (e.g., Apoptosis vs Necroptosis) on tissue inflammation and fibrosis development, as well as the role played by professional phagocytes such as AM in IPF progression.

![Figure 2: Key Mechanisms Involved in Alveolar Epithelial Cells Type II Death During IPF Onset and Progression.](image-url)
Apoptotic cells efferocytosis by alveolar macrophages

AM are the most abundant resident leucocyte in human lungs by far and ensure several housekeeping functions such as surfactant protein recycling and foreign particles’ cleansing [10]. One of their essential functions is the phagocytosis of cell debris and apoptotic cells in a process called efferocytosis [150]. Efficient efferocytosis of apoptotic inflammatory cells recruited to an inflammatory site is critical to inflammation termination and tissue repair. Not only efferocytosis remove pro-inflammatory signals released by apoptotic cells such as DAMP and alarmins, but it also favours a phenotypic shift of AM toward a pro-repair phenotype. This pro-repair phenotype is characterised by the secretion of anti-inflammatory and pro-repair molecules such as IL-10, TGF-β and pro-resolving lipids mediators (e.g., lipoxins, resolvins and maresins) [150].

Wang, et al, on the other hand, showed that instillation of apoptotic BALF cells in rats’ lung (1x10⁶ UV treated autologous BALF cells) induced secondary apoptosis of recipient’s cells, inflammation and the development of moderate fibrosis [35]. In their BALF, the authors observed a two-fold increase in TNF-α and TGF-β expression up to 4 weeks post instillation, with expression peaks at one day and one week for TNF-α and TGF-β, respectively [35]. The TNF-α producing cells were identified as AM. Moreover, the persistence of an increased rate of apoptotic cells and caspase-8 activation 28 days after apoptotic cells administration correlated with a two-fold increase in TGF-β expression [35].

Similar observations were made by Kim, et al, in a recent research article in which AECII apoptosis induction in vivo was associated with increased macrophage efferocytosis. In vitro, macrophage efferocytosis of apoptotic AECII promoted a profibrotic M2 phenotype characterised by increased arginase and TGF-β expression [151]. In vivo, repetitive instillation of apoptotic primary AECII, or MLE-12 (mouse epithelial cell line) but not Jurkat cells (human T Lymphocytes cell line) induced lung fibrosis development [151]. Furthermore, deficiency in the efferocytosis receptor CD36 attenuated lung fibrosis induced by apoptotic MLE-12 transfer suggesting a direct involvement of efferocytosis in fibrosis development by promoting profibrotic AM [151]. Interestingly, the authors found that, in vitro, Jurkat cells efferocytosis by primary macrophages induced significantly less expression of TGF-β1 and arginase than that of epithelial cells efferocytosis. This suggests a possible cell type-dependent regulation of efferocytic macrophage phenotype [151].

On the other end, Jihee Lee Kang's team demonstrated that intratracheal administration of apoptotic cells (i.e., 10x10⁶ UV treated Jurkat cells) two days after bleomycin instillation in mice was able to attenuate lung injury. This effect was supposedly due to HGF expression stimulation as this effect was blocked by the concomitant administration of blocking anti-HGF antibodies [152]. The authors identified AM as the source of the increased HGF expression up to 21 days post bleomycin treatment. They suggested that early AM efferocytosis was responsible for this persistent expression of HGF. Besides, apoptotic cells instillation also elicited anti-apoptotic action, consistent with HGF's anti-apoptotic effect in epithelial and endothelial cells [153]. However, the authors reported greater TGF-β expression during the acute phase (i.e., 2 hours and three days after apoptotic cells transfer) and lower levels during the chronic phase (i.e., 14 and 21 days after apoptotic cells transfer) [154]. Finally, in vitro, apoptotic Jurkat cells administration to the murine macrophage cell line RAW 264.7 blocked TNF-α expression induced by LPS or bleomycin challenge in an HGF-dependent way. In a second study the same team showed that apoptotic Jurkat cells efferocytosis increased lung and AM expression of COX-2 and PGE2, which increased HGF expression by stimulating the PGE2 receptor EP2. As long term HGF inhibition by PHA-665752 resulted in downregulation of COX-2 expression and PGE-2 production; the authors concluded that an autocrine positive feedback loop existed between PGE2 and HGF expression in the lung after apoptotic cells transfer [155]. In a third study, the authors confirmed in RAW 264.7 and primary mouse peritoneal macrophages the existence of an autocrine positive feedback loop between COX-2/PGE2 and HGF expression following apoptotic cells efferocytosis [156]. However, in IP, this positive feedback loop seems to be lacking since HGF concentration in BALF and serum is high [110, 157], while PGE2 expression in IP BALF is diminished [113]. The reason for this disconnection between the expression of both mediators in IP is not known. The team also found that the administration of apoptotic Jurkat cells a second time, seven days post-bleomycin instillation combined with the efferocytosis "enhancer" simvastatin further increased HGF and IL-10 expression in the lung and their secretion by efferocytosis AM through PPARγ activation [158]. Finally, the authors showed that COX-2, EP2 or HGF inhibition after apoptotic cells transfer abrogated the inhibition of the late TGF-β expression and lung fibrosis induced by bleomycin [155]. Besides, the team also demonstrated that prostaglandins (i.e., PGE2 and PGD2) and HGF could inhibit pathogenic mechanisms involved in IPF progression, such as Epithelial to Mesenchymal Transition (EMT) [159], and fibroblasts trans-differentiation and invasiveness induced by TGF-β1 or EGF [160].

The bottom line from these experiments is that apoptotic cells instillation in healthy lungs can generate inflammation, apoptosis and fibrosis through the release by efferocytic AM of mediators such as TNF-α and TGF-β. However, in an already injured lung, early apoptotic cells instillation could cut short the acute inflammatory phase by generating pro-repair macrophages expressing IL-10, HGF and PGE2, limiting the extent of acute damage consequently late tissue fibrosis.

The antagonism between HGF/PGE2 and TGF-β signalling in various cell types, including AM, AEC, and fibroblasts, is key to the contrasted contribution of efferocytic AM to lung fibrosis [155,161,162]. Indeed, efferocytic AMs have been identified as important sources of IL-10, HGF, PGE2, TGF-β and TNF-α in the lung, but the kinetic and level of their expression might be critical to the balance between their pro-repair and pro-scarring potential. Interestingly, a recent article suggests that the balance between TGF-β1 and HGF expression in chronic rhinosinusitis regulates the remodelling pattern. A high TGF-β1/HGF ratio correlated with increasing αSMA and collagen expression [163].

It is also unclear to what extent the apoptotic cells type (e.g., leucocyte or epithelial) influences efferocytic AM phenotype. Further experiments should be conducted to determine whether efferocytosis of different cell-type (e.g., epithelial vs immune cells) influences PGE2/HGF and TNF-α/TGF-β’s relative expression by efferocytotic AM, and to which extent this affects AECII repair and lung regeneration.

Finally, AM in IPF has been shown to have a monocytic-like phenotype characterised by the retention of monocytic markers such as CD14 and the increased production of free oxygen radicals [10]. Moreover, AM from IPF have been shown to have defective efferocytosis [164], a phenomenon that could be linked in IPF to RhoA activation by cigarette smoke-induced ER stress [165] or the engulfment of particles such as silica [166]. Defective efferocytosis in IPF could limit AM pro-repair phenotype (i.e., HGF and prostaglandins secretion) while contributing to the secondary necrosis of non-phagocytised apoptotic cells and the release of DAMP as observed in endotoxin-induced pulmonary inflammation [167]. In the next section, we will evaluate the potential impact of AEC necroptosis and DAMP release on IPF progression.

Necroptosis, DAMP and TLR activation in IPF

As said earlier, recent pieces of evidence from IPF samples and experimental lung fibrosis suggest a role for AEC necroptosis in IPF pathogenesis [38]. Necroptosis is a recently discovered form of regulated cells death mediated by TNF receptor 1 activation [168]. Unlike apoptosis, necroptosis is characterised by pore formation and cell swelling, favouring the release of DAMP (e.g., HMGB1, mtDNA, ATP, RNA) by necroptotic cells [168,169]. Once released, DAMP binds to several danger receptors, including toll-like receptors (TLR), whose activation triggers inflammatory genes expression and activation of both the innate and adaptive immune response [169].

The significance of AECII necroptosis in IPF is supported by the presence in patients’ serum of elevated concentrations of AECII-derived molecules such as surfactant proteins A and D and the transmembrane mucin KL-6 [170]. Moreover, DAMP molecules such as ATP, HMGB1, mtDNA and Spliceosome-associated protein 130 (SAP130) are increased in IPF patients’ BALF and serum [69,169,171-173]. This section will focus on recent research highlighting the potential role of TLR9 and TLR2/4 activation by DAMP, such as mtDNA and HMGB1, in IPF progression.

DAMP-dependent TLR9 activation in lung fibrosis

TLR9 is an endosomal TLR activated by unmethylated CpG nucleotides found in bacterial DNA [174]. TLR9 expression is increased in IPF patients’ BALF at the mRNA level, despite a decreased expression by lymphocytes. This suggests an increased expression in other resident cells types or the recruitment of cells with a higher level of TLR9 expression [175,176]. Unexpectedly, immunohistochemistry analysis of IPF lungs revealed strong TLR9 expression in stromal cells such as fibroblasts [177]. Indeed, fibroblasts isolated from IPF lungs present increased expression of TLR9, and its level of expression in the lung positively correlates with disease progression [69,178]. These observations are consistent with in vitro data showing an increased expression of TLR9 in normal lung fibroblasts after three days of stimulation by TGF-β1 [179].

Originally, TLR9 was believed to be exclusively activated by pathogens-associated molecular patterns (PAMP) such as bacterial and viral DNA [174]. However, later experiments showed that mammalian DNA in the form of immune complexes were potent TLR9 inducers [180]. Thus, in podocytes, mtDNA, which contain unmethylated CpG rich motifs, has been shown to bind to and to activate TLR9 contributing to cell apoptosis [181]. However, IPF fibroblasts are resistant to apoptosis, and TLR9 activation by its agonist CpG-ODN or mtDNA, resulted in myofibroblastic differentiation as assessed by increased expression of α-SMA [69,171,177]. Also, chronic stimulation of normal lung fibroblasts’ TLR9 by CpG-DNA in vitro induce an IPF-derived fibroblast-like phenotype characterised by an invasive phenotype, resistance to hypoxia-induced apoptosis, inflammatory cytokines and growth factor secretion (e.g., IFNγ; PDGF-BB) and stable expression of α-SMA, PDGFRα, CD44 MMP14 and MMP2 [179].
Moreover, Ryu, et al., recently showed that normal human lung fibroblasts release an increased amount of mtDNA when stimulated by TGF-β1 or cultured on stiff hydrogel [171]. As said earlier, reduced mitophagy in AEC due to ER Stress-mediated repression of PINK1 expression is associated with increased mtDNA damage. Interestingly, the authors showed that these cells also released mtDNA, which could, in an autocrine fashion, stimulate TLR9 expression in primary AEC [69]. However, another team showed in a lung epithelial cell injury model induced by cyclic stretching that PINK1 knock-out was associated with mitophagy impairment, reduced mtDNA’s release, and milder inflammatory damage [182]. Therefore, further experiments are required to determine the mechanism of mtDNA release by AEC.

Regardless of its origin, primary AEC exposure to extracellular mtDNA results in its endocytosis, TLR9’s activation, and TGF-β1 secretion stimulation. The data also suggest that mtDNA’s pro-fibrogenic potential increases with its level of oxidation [69]. Moreover, TLR9 stimulation by CpG-DNA in the AEC line A549 and blood monocytes led respectively to EMT and differentiation into fibrocytes [178]. Thus, in IPF, secretion of mtDNA-dependent TLR9 activation could be added to the list of pathologic interactions between AEC and lung fibroblasts supporting fibrosis development.

DAMP-dependent TLR4 activation in lung fibrosis

Apart from TLR9, TLR4 has also been involved in IPF pathogenesis. Unlike TLR9, TLR4 is a transmembrane TLR, mainly known for eliciting antimicrobial responses by recognising bacterial lipopolysaccharides and viral motifs [180]. Later studies revealed its ability to recognise many DAMP, such as the nuclear protein HMGB1, the AECII marker surfactant protein A, and several heat shock proteins and damaged ECM components [180].

Analysis of TLR 4 expression in UIP lungs showed an increased expression mainly in bronchial epithelial cells, AEC and AM [183]. Whereas in this study, TLR4 has not been detected in fibroblasts/myofibroblasts, other researcher using immunofluorescence and laser capture microdissection observed an expression of TLR4 in myofibroblasts (i.e. α-SMA+ cells) from FF. Later on, mRNA and protein expression analysis in IPF-derived fibroblasts confirmed TLR4 expression in these cells at levels similar to normal human lung fibroblasts [184]. Interestingly, the authors showed that normal and IPF-derived fibroblasts diverged in their responses to stimulation by TGF-β1 and the TLR4 activator LPS. Indeed, TLR4 activation by LPS inhibited myofibroblastic markers (i.e., α-SMA, COL1A1 and CTGF) expression induced by TGF-β1 stimulation in normal human lung fibroblasts, but not in IPF-derived fibroblasts [184]. Further investigations revealed that LPS stimulation in IPF-derived fibroblasts failed to inhibit TGF-β Receptor type 1 (TGF-βR1) and the subsequent activation of SMAD3 [184]. Interestingly, toll-interacting protein (TOLLIP), a TLR binding protein whose single nucleotide polymorphisms are associated with IPF development [9], has been shown to inhibit TGF-β1 response by interacting with SMAD7 and promoting TGF-βR1 degradation in various cell lines, including mouse embryonic fibroblasts [185]. In sharp contrast with those results, increased TLR4 expression in skin fibroblasts from patients with systemic sclerosis is associated in vitro with increased TGF-β response when cells are co-treated with the TLR4 ligand LPS [186]. Moreover, Bhattacharyya, et al., found in systemic sclerosis skin biopsy an increase expression of TLR4-responsive genes. Finally, pharmacological inhibition of TLR4’s co-receptor myeloid differentiation 2 (MD2) impaired myofibroblastic differentiation in vitro and skin fibrosis development in several preclinical models of systemic sclerosis[187]. Significantly, systemic sclerosis is also associated with lung fibrosis and can be modelled by subcutaneous injections of bleomycin. In this model, pharmacological inhibition of TLR4 or its conditional deletion in fibroblasts protected mice from lung fibrosis development [187]. Interestingly, increased expression of the ECM component and TLR4 ligand Tenascin-C has been found in the skin, serum and fibroblasts from systemic sclerosis patients and experimental model of the disease [188]. Tenascin-C is mainly expressed during embryogenesis and transitorily during wound healing. However, in chronic pathological conditions, Tenascin-C can act as DAMP through TLR4 activation. The authors showed that in vitro, Tenascin-C stimulation triggered foreskin fibroblasts’ myofibroblastic differentiation in a TLR-4 dependent manner, while in vivo, its deficiency protected mice from lung and skin fibrosis induced by repetitive subcutaneous injection of bleomycin [188]. However, in lung fibrosis induced by intratracheal administration of bleomycin, TLR4 deficiency was associated with inflammation and fibrosis exacerb-
tion [189]. As this model is associated with AEC’s acute injuries, the deleterious effect of TLR4 deficiency could be explained by its critical role in AECII renewal [42].

A critical pathogenic feature of IPF is fibroblast resistance to apoptosis, potentially due to their premature senescence [190]. Radiation-induced lung fibrosis models share many IPF pathogenesis features, including DNA damage and its progressive and intractable fibrotic development. Hanson, et al, showed in this model that fibroblasts resistance to apoptosis precedes fibrosis development and that TLR4-/- mice resistance to radiation-induced lung fibrosis was associated with a loss of their fibroblasts’ resistance to apoptosis in vitro [191]. Based on significant differences in plasmatic HMGB1 concentrations between WT and TLR4-/- mouse, the authors suggested that fibroblasts’ resistance to apoptosis could be mediated by HMGB1-induced TLR4 activation. However, they did not provide experimental data supporting this hypothesis [191]. Interestingly, in IPF and the late phase of experimental lung fibrosis induced by bleomycin, HMGB1 expression was increased in inflammatory cells and hyperplastic AECII.

Moreover, in vitro, stimulation of the human embryonic lung fibroblast cell line (WI-38) with HMGB1 induced their proliferation [192]. Because often, pro-proliferative stimuli also elicit anti-apoptotic effects, these data support a role for HMGB1-induced TLR4 activation in IPF-derived fibroblasts resistance to apoptosis. However, HMGB1 also binds to TLR2 and the advanced glycation product receptor RAGE [192]; therefore, further investigations are required to uncover the role of fibroblasts’ TLR4 activation by DAMP in their resistance to apoptosis.

It is unclear whether differences in TLR4 and TGF-β crosstalk in vitro (i.e. skin vs lung fibroblasts) and in vivo (i.e., systemic sclerosis vs IPF models) reflect phenotypic or pathogenic differences. However, both sets of experiments highlight crosstalk which can modulate fibrosis development. The contribution of TLR4 activation to AEC damage and dysfunction, on the other hand, seems to be more contrasted because of its role in AECII renewal. This critical function might explain divergent outcomes of its inhibition in experimental lung fibrosis models associated or not with acute AEC damages.

**DAMP-dependent TLR2 activation in lung fibrosis**

Similar to TLR4, TLR2 is an extracellular TLR involved in the recognition of microbes-derived lipids. Likewise, TLR2 can be activated by several DAMP (e.g., HMGB1, HSP60 and 70, Hyaluronan) also recognised by TLR4 [174,180]. TLR2 expression is increased mainly in bronchial epithelial cells, AEC and AM of fibrotic lungs with a UIP pattern characteristic of IPF [183]. Interestingly, hyaluronan fragments from the serum of individuals experiencing acute lung injuries have been shown to elicit chemokines releases by macrophage through TRL2 and 4 activations. Moreover, mice deficient in both TLR2 and 4 have impaired inflammatory cells recruitment and increased epithelial cells death in response to bleomycin instillation [193], reflecting their role in both chemokines signalling and AECII renewal [42,193]. Later experiments evaluating TLR2 deficiency alone in the bleomycin model of lung fibrosis suggest, its persistent activation by DAMP is associated with the recruitment of immuno suppressive cells (i.e., Regulatory T Cells, alternatively activated macrophages), which prevent DAMP and PAMP elimination and thereby favours fibrosis development [194]. Kim, et al, showed using mice deficient for TLR2 in the hematopoietic lineage, that TLR2’s activation in AEC promoted an immunosuppressive environment through IL-27-dependent inhibition of IL-17, leading to heightened bleomycin-induced lung fibrosis [195]. Recent data also showed that attenuation of bleomycin-induced lung fibrosis by the spirulina platensis-derived pigment phycocyanin was decreased in TLR2-/- mice [196]. Finally, TLR2 inhibitors prevented peritoneal dialysis solution-induced fibrosis potentially by blocking its activation by DAMP such as HSP70 and hyaluronan. However, in this model, TLR2 inhibition favoured Treg recruitment and inhibition of neutrophils and TH17 cells recruitment [197].

Overall TLR2 deficiency is associated with reduced fibrosis in various organs, including the lung. Moreover, in the lung, its expression by AEC and macrophages seems to play an essential role in the bleomycin model of lung fibrosis by promoting a TH2 immune response.

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Additional DAMP involved in lung fibrosis

In addition to TLRs ligands, other mediators released by dying cells such as ATP, uric acid, SAP130, and IL-33 are increased in the BALF and serum of IPF patients [169,173]. For some of them, there are pieces of evidence regarding their contribution to IPF or experimental lung fibrosis progression.

For instance, IL-33, a cytokine released by cells upon death and regarded as an alarmin, is constitutively expressed in AEC and up-regulated in macrophages after bleomycin instillation [198]. In the bleomycin-induced lung fibrosis model, IL-33 released by dying cells binds to its receptor ST2 on AM and triggers an M2 polarisation characterised by IL-13 and TGF-β1 secretion. Besides, IL-33 induces the expansion of type 2 innate lymphoid cells, leading to further IL-13 releases [198].

Similarly, extracellular adenosine triphosphate (eATP) is released by necrotic cells and act as a DAMP [169]. Both IPF patients and mice undergoing experimental lung fibrosis have an elevated ATP level in their BALF [199]. In vitro, pulmonary epithelial cells treated with bleomycin release ATP and in vivo, the addition of ATP aggravate bleomycin-induced lung fibrosis [199]. The pro-inflammatory and profibrotic action of eATP is elicited by engaging the P2X7 receptor/pannexin-1 axis leading to the subsequent maturation of IL-1β. Consistently, P2X7 deficient mice were protected from bleomycin-induced lung inflammation and fibrosis [199].

![Figure 3: Potential Contribution of AECII Death to Fibrosis Progression in IPF.](image)

In IPF, AEC exposure to a combination of external stressors (i.e., IPF risk factors) and pro-inflammatory/-apoptotic mediators lead to their death by apoptosis or necroptosis. Apoptotic cells efferocytosis by alveolar macrophages induces their acquisition of a pro-repair phenotype associated with increased expression of mediators such as HGF, PGE2 TNF-α and TGF-β. The balance between those mediator favours either alveolar regeneration or AECII death and lung scarring. Due to exposure to cigarette smoke and dust such as silica particles, IPF alveolar macrophages present deficient efferocytosis, which can lead to secondary necrosis of apoptotic cells. Necrotic cells release damage-associated molecular patterns such as mtDNA, HMGB1, extracellular ATP (eATP), and uric acid, which can further aggravate lung inflammation and fibrosis. Thus, lung fibroblasts’ TLR9 activation by mtDNA or TLR-4 activation by HMGB1 could induce their myofibroblastic differentiation, whereas, in AECII, TLR9 activation by mtDNA leads to TGF-β secretion and epithelial to mesenchymal transition (EMT). Moreover, TLR2 activation in AECII could stimulate IL-27 secretion leading to the recruitment of immune-suppressive and profibrotic immune cells. Besides, IL-33, through its receptor ST2, induces an M2 polarisation of macrophages that could favour fibrosis development. Finally, eATP and uric acid, through activation of the P2X7 receptor and the NALP3 inflammasome, respectively, could contribute to chronic IL-1β expression, leading to further inflammation and fibrosis.
Concluding remarks

IPF is a devastating fibrotic lung disease which evolves silently for decades before patient manifest symptoms in their later years. Preclinical models of the disease are partially relevant, making it challenging to identify the disease's specific pathogenic mechanisms. However, we showed in this review the critical role of AEC death, particularly that of AECII, in IPF’s onset and progression. AECII death in IPF occurs in response to extrinsic noxious agents (Cigarette smoke, Viruses, etc.), which induces cells dysfunction such as ER stress, mitochondrial dysfunction and DNA damage. Over the years, the chronic inflammatory and profibrotic milieu created by the lung response to AEC micro-injuries induces further AEC apoptosis while favouring fibroblasts' proliferation, differentiation into myofibroblast and resistance to apoptosis. Besides, apoptotic cells efferocytosis by AM induces their acquisition of a pro-repair phenotype characterised by the release of mediators favouring AEC repair (e.g., HGF, PGE2) or death (e.g., TGF-β). The mechanisms regulating the balance between the expression of those mediators by efferocytic macrophages are unclear but could play a critical role in IPF progression. Finally, necroptosis is a new form of regulated cell death recently identified in IPF lungs, which might play a critical role in its progression by fostering a chronic inflammatory and profibrotic milieu through DAMP release. Those DAMP, through the activation of danger receptors, have been shown to elicit profibrotic effects in both AEC and lung fibroblasts, suggesting that necroptosis could play a critical role in IPF progression. The existence of cells expressing both apoptotic and necroptotic markers suggests a possible evolution in the ratio between those populations during disease progression and acute exacerbation episodes. It is unclear to which extent the impaired efferocytosis observed in IPF AM participates in necrotic cells accumulation during the disease progression. Data presented here support the development of treatments targeting the apoptosis paradox observed in IPF by restoring PGE2 and ACE-2 expression in AEC, fibroblasts and AM. Also, drugs able to relieve mitochondrial dysfunctions, ER stress, DNA damages, and those able to boost AM efferocytosis and secretion of HGF/PGE2 might be useful in IPF treatment. Finally, administration of drugs inhibiting necroptosis and preventing DAMP releases might also be beneficial to slow-down IPF progression.

Bibliography

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