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 of the tyrosine kinase inhibitor Nintedanib and the anti-fibrotic drug Pirfenidone, which at best increases patient’s life expectancy by a few months/years. According to the current hypothesis on IPF pathogeny, repetitive injuries to the epithelial compartment play a critical role in the disease onset, by precipitating their death. On the long term, the cycles of repair and damages lead to the exhaustion of the alveolar epithelium regenerative capacities and the disruption of the epithelial/mesenchyme communication. As a result, mesenchymal cells such as fibroblasts proliferate and differentiate into myofibroblasts, become resistant to apoptosis and promote excessive extracellular matrix deposition and fibrosis development. In this article, we will review the significance and mechanism of alveolar epithelial cells death in IPF, their induction by IPF risk factors and pathologic cells interactions. Finally, we will review the effect of apoptotic cells efferocytosis by alveolar macrophages on their phenotype and fibrosis development as well as the role of necrotic cell-derived damage associated molecular patterns on 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; BCL-2: B-Cell Lymphoma 2; BID: BH3 Interacting Domain Death Agonist; BiP: Binding Immunoglobulin Protein; CDX: Cluster of Differentiation X; CHOP: C/EBP Homologous Protein; cNOS: Constitutive Nitric Oxide 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; EDEM: ER Degradation Enhancing α-Mannosidase-Like Protein; EMT: Epithelial to Mesenchymal Transition; ER stress: Endoplasmic Reticulum Stress; eROS: External Reactive Oxygen Species; FF: Fibroblastic Foci; FGFR1: Fibroblast Growth Factor Receptor 1; HGF: Haptocyte Growth Factor; HIF1-α: Hypoxia Inducible Factor 1-α; HMGB1: High Mobility Group Box 1; HSPX: Heat Shock Proteins X; IFNy: Interferonγ; IL-x: Interleukin-X; ILD: Interstitial Lung Diseases; iNOS: inducible Nitric Oxide Synthase; IPF: Idiopathic Pulmonary Fibrosis; iPSC: Inducible Pluripotent Stem Cells; IRE1α: Inositol Requiring Enzyme 1a; iROS: intracellular Reactive Oxygen Species; MD2: Myeloid Differentiation 2; MDM2: Mouse Double Minute 2;
MLKL: Mixed Lineage Kinase-Like; MMPX: Matrix metallopeptidase X; MnSOD: Manganese SuperOxide Dismutase; MOMP: Mitochondrial Outer Membrane Permeabilization; mtDNA: mitochondrial Deoxyribonucleic acid; mtROS: mitochondrial Reactive Oxygen Species; MUC5B: Mucin 5B; NF-κB: 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; PARN: Poly(A)-specific Ribonuclease; PDGF-BB: Platelet-derived growth factor-BB; PDGFRα: Platelet-Derived Growth Factor Receptorα; PERK: PKR-like ER Kinase; PGE2: Prostaglandin E2; PINK1: PTEN-Induced Putative Kinase 1; PTEN: Phosphatase and Tensin homolog; R-SMAD: receptor-activated SMADs; RIPKX: Receptor-Interacting Serine/Threonine-Protein Kinase X; RNA: Ribonucleic acid; RNS: Reactive Nitrogen Species; ROS: Reactive Oxygen Species; RTEL1: Regulator of Telomere Elongation Helicase 1; S1P: Sphingosine-1-Phosphate; SAP130: Spliceosome-associated protein 130; SFTPA2: Surfactant protein a2; SFTPC: Surfactant Protein C; SIRT3: Sir2 homolog 3; SPHK1: Sphingosine Kinase 1; TERC: Telomerase RNA Component; TERT: Telomerase Reverse Transcriptase; TF: Tissue Factor; TGFβX: Transforming Growth Factor beta receptor X; TOLLIP: Toll Interacting Protein; TRAIL: TNF-Related Apoptosis Inducing Ligand; TUDCA: TauroUrsodeoxyCholic acid; TLRX: Toll-like receptor X; TNF-α: Tumor Necrosis Factor-α; UPR: Unfolded Protein Response; XBP: X-box binding protein; α-SMA: α-Smooth Muscle Actin

Introducing idiopathic pulmonary fibrosis

Idiopathic Pulmonary Fibrosis (IPF) is a relentlessly progressive lung disease, with a life expectancy at diagnosis ranging 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 a decrease in lung function (i.e. forced vital capacity, total lung capacity and carbon monoxide diffusing capacity) manifested by exertional dyspnoea [4]. Additional symptoms include dry caught, 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 gas exchange capability of the lung [5,6]. The histopathological findings characteristic of IPF lungs pathologic remodelling 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 use of the term “Idiopathic”, IPF causes remain elusive. However, in the past decades, epidemiological studies of IPF patients highlighted in this population the prevalence of 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 some bacterial and viral infections were more frequent in IPF patients, suggesting their involvement in the disease onset [7,8]. Besides, genome-wide association studies also identified in IPF patients genetic risk factors, including mutations in genes relevant to lung epithelial cells functions (e.g. MUC5B, SFTPC, SFTPα2), telomere functioning (e.g. TERT, TERC, PARN, RTEL1) and innate immunity (e.g. TLR3, Tollip, IL-8) [9]. Thus, IPF results from the 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 nonimmune and immune 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 is two types of AEC, the type 1 (AECI) have a flat morphology are specialised in gas exchanges and represent around 40% of all AEC while occupying 90% of the alveolar...
space. AEC type 2 (AECII) on the other end, have a cuboidal shape and are involved in surfactant proteins secretion and serve as the stem cell compartment of the alveolar epithelium [14]. AECII, are located at alveolius’ 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 for the ACM and is constituted by extracellular matrix components (ECM) such as laminin, collagen IV, perlecan and entactin [12].

As suggested by epidemiological studies, and shown in figure 1, micro-injuries to the alveolar epithelium by noxious agents (e.g. cigarette smoke, dust, viruses) are likely initiating events in IPF onset. The resulting epithelial inflammatory response (i.e. cytokines and chemokines secretion) lead to AM and lung microvascular endothelial cells activation as well as the recruitment of inflammatory cells such as neutrophils, monocytes and lymphocytes [12,16]. While this initial response is physiologic, the recurrence of these micro-injuries might prevent the inflammatory response termination and the subsequent tissue repair. Instead, the resulting chronic inflammation causes further tissue damages (e.g. Epithelial and Endothelial cells death, basement membrane degradation) that in a vicious cycle contributes to the exacerbation of inflammation [12,16]. On 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 alveoli 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 scarring is impaired, leading to excess scar formation [11]. Molecular pathways favouring scars formation such as the TGF-β pathway, become overwhelmingly prevalent leading to fibroblasts proliferation, differentiation in myofibroblasts, Epithelial/Endothelial to mesenchyme transition and further destruction of the alveolar structure by the formation of FF [11]. In late stages of fibrosis, the pathologic ECM can drive fibrosis independently of any external signals by promoting myofibroblastic differentiation of fibroblasts in a positive feedback loop which supports the relentless progression of fibrosis in IPF [17].

**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. Inflammatory cells by secreting pro-inflammatory and apoptotic factors such as cytokines, ROS and proteases 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. After years of chronic exposure to noxious agents and continuous inflammatory cells recruitment, further damage to the basement membrane is associated with the loss of Epithelial cells regulation of mesenchyme cells proliferation, leading to fibroblasts proliferation, myofibroblastic differentiation, apoptosis resistance and ECM deposition. Besides, AECII and endothelial further support the mesenchyme expansion by undergoing respectively epithelial and endothelial to mesenchyme transition, leading to the constitution of fibroblastic foci.
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Epithelial cells death in IPF: Significance, mechanisms and contribution to lung fibrosis

Although there is no certainty about 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 agent they are exposed to, the intensity and duration of this exposure as well as their internal capacities (e.g. ATP availability…) to cope with the damages suffered, cells will either recover or die [18]. Previously, the scientific community distinguished two types of cell death; accidental cell death (i.e. necrosis), and programmed cell death (i.e. apoptosis) [19]. However, further studies revealed that accidental cells 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 and results in the release in the environment of their intracellular molecules (e.g. mtDNA, HSP, HGMB1) which acts as damage-associated molecular patterns (DAMP) [19]. The recognition of these DAMPS by surroundings cells’ Danger receptors (e.g. TLR), triggers their expression of inflammatory cytokines and chemokines, and the subsequent recruitment of immune (e.g. neutrophils, monocytes) and mesenchymal cells (e.g. fibrocytes) in the damaged tissue [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 controlled death process, often happening in physiological responses such as normal wound healing [21], which does not result in significant DAMPS release and therefore regarded as less immunogenic than necrosis [19]. Whereas necrosis is characterised by organelles swelling and limited chromatin condensation, apoptotic cells shrink, condensate their chromatin and form apoptotic bodies which facilitate their phagocytosis by phagocytes (e.g. macrophages) 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 cells surface of death receptors by immune cells or inflammatory mediators such as the TNF-related apoptosis-inducing ligand receptor (TRAIL) and FAS [22]. The intrinsic pathway, on the other hand, is triggered by intracellular toxicity signals such as DNA damage, ER stress or Oxidative stress [22]. Both pathways result in the activation of a family of protein central to apoptosis regulation, named caspases [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 responsible for caspases activation and apoptosis [23].
Epithelial damages in IPF

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

Significance of epithelial cells death in IPF pathogenesis

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 myofibroblasts staining (i.e. α-SMA staining) on paraffin sections, reported the presence in IPF lungs of necrotic and apoptotic AEC particularly in cells immediately adjacent to α-SMA positive cells of the FF [27]. Both, this study, and a later one by Barbas-Filho., et al using the same technics, identified the dying cells as being 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 BCL-2 [28]. The same way, Nakashima., et al. showed in AEC from IPF samples increased expression and phosphorylation of p53 along with increased MDM2 and p21 expression. Interestingly, most of those markers except the senescence-associated marker p21 were more expressed in IPF than in non-specific interstitial pneumonia, another interstitial lung disease with a better prognosis than IPF and responsive to anti-inflammatory treatments [29]. The significance of AEC death contribution to IPF pathogenesis is further supported by experiments showing an abrogation of lung fibrosis development by administration of mediators having anti-apoptotic/pro-proliferative effects in AEC. 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, recent experiments showed that intratracheal administration of induced pluripotent stem cells (iPSC)-derived AECII of human origin, to rats 15 days after lung fibrosis induction by bleomycin instillation, could reduce TGF-β1 and α-SMA expression as well as pulmonary fibrosis and alveolar structure damages [34].

Conversely, intratracheal administration of apoptotic cells in rats induces secondary apoptosis, inflammation and pulmonary fibrosis [35], while caspase inhibitor attenuates 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. reported in a 2018 article, an increased expression of the necroptosis inducer RIPK3 in AEC from IPF and experimental lung fibrosis samples [38]. In this model, RIPK3 deficiency, as well as intranasal administration of the necroptosis inhibitor necrostatin, protected mice from lung fibrosis development [38]. Consistently, the protective role of necrostatin administration in experimental lung fibrosis has recently been shown by a second research team [39]. Moreover, using primary mouse cells, the authors highlighted in vitro, the role of necroptosis in AEC cells 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 not known; 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 IPF one [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].

Mechanisms of Epithelial cells death in IPF

In this section, we 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 focus on 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 [42].

In IPF early investigations revealed an increased level of oxidised methionine in patients’ Bronchoalveolar Lavage Fluid (BALF) [43] while the cells recovered from these samples (e.g. neutrophils, AM), were found to be releasing abnormally high levels of ROS such as superoxide [44,45]. Similarly, Psathakis., et al. found an increased concentration of hydrogen peroxide and 8-isoprostane in the expired breath condensate of IPF patients [46], while increased lipid peroxidation has been observed in the BALF and plasma of IPF patients [47,48].

Consistent with the neutrophilia observed in IPF, the epithelial lining fluid of patients also presented higher concentrations of myeloperoxidase, a protein released by activated neutrophils and transforming hydrogen peroxide into the highly toxic hypoalde anion [44].

In addition to ROS, there is in IPF pieces of evidence of molecular damages induced by RNS. Indeed, IPF is associated with an increased formation of NO and peroxynitrite which correlates with a shift in nitrite oxide synthases expression in IPF lungs (i.e. downregulation of cNOS and upregulation of iNOS) [49]. Consistently, Saleh., et al. found in IPF lungs an increased level of Nitrotyrosine, a marker of protein nitration by peroxynitrite [49].

Conversely, several investigators found a decreased level of antioxidants in IPF patients. Thus, Rahman., et al. observed a marked reduction in antioxidant capacities (i.e. Trolox equivalent), within the plasma and BALF of IPF patients [48], which correlates with decreased glutathione concentration in the epithelial lining fluid [50], sputum and plasma of IPF patients [51]. Altogether, these observations reveal a strong imbalance between oxidants and antioxidants production in IPF lungs which have locals as well as 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 [52]. The same way, internal production of H$_2$O$_2$ following catalase inhibition in these cells, increased ceramides and induced apoptosis, a cascade that could be blocked by treatment with the antioxidant N-acetylcysteine [52].

Interestingly, many risk factors potentially associated with IPF onset such as cigarette smoking, or exposure to dust (i.e. Silica) can directly induce oxidative stress [53]. Moreover, experimental pieces of evidence suggesting that bleomycin-induced lung fibrosis involved AEC apoptosis by a ROS-dependent activation of the intrinsic pathway [54]. These initial source of oxidative stress, by damaging nucleic acids, proteins and lipids impair the functioning of subcellular organelles such as the nucleus, the endoplasmic reticulum and the mitochondria [42]. To manage those dysfunctions, cells activate several cellular processes, including, the DNA damage responses (DDR), the unfolded protein response (UPR) and autophagy. Failure to repair these damages can lead to excessive production of mitochondrial ROS, further molecular damages, higher activation of inflammatory pathways and ultimately cells death [42,55].

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 in avoiding genes loss of function, cell transformation and ultimately, death. The integrity of DNA is threatened by many agents, including ultraviolet, ionising radiation, as well as ROS [56]. DNA damaging agents can induce chemical modifications of nucleotides, DNA strand breaks (i.e. Single or Double) and interstream cross-linking [56]. 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 [56].

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) [57]. 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 [58]. Also, 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 [59]. Conversely, these cells present a decreased activation of the pro-proliferative and pro-survival MAP Kinase, ERK1/2 [59]. 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 [60, 61].

Mitochondrial dysfunction

Mitochondria are maternally inherited intracellular organelles, often considered as the cell’s powerhouse as they produce life’s molecular fuel adenosine triphosphate (ATP); [62]. As by-products of their respiration, mitochondria also produce ROS (mtROS) such as superoxide anion (O₂⁻), hydrogen superoxide (H₂O₂) and hydroxyl radical (OH•), which at physiologic concentrations activates antioxidant pathways in a process called mitohormesis [63].

The number of mitochondria per cell is variable across tissues, and for a given tissue across individuals depending on parameters such as age and physical activity, for instance [62]. 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[62]. Most of the mitochondrial proteins (~ 1200) are coded by nuclear DNA, produced in the cytoplasm and imported in the mitochondria [62]. In addition to the production of ATP, mitochondria play a significant role in cell fate through regulation of cellular processes such as apoptosis, cell cycling or differentiation [62].

In IPF, several studies reported in AECII an accumulation of dysmorphic mitochondria with a swollen appearance and disorganised cristae [64,65]. 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 is due to the activation of its transcriptional repressor ATF3 in response to ER stress [64,66]. Consistently, PINK1 deficiency aggravated lung fibrosis induced by bleomycin, whereas mice with a conditional deletion of ATF3 in AEC were protected [65,66]. Whereas 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 [65]. Interestingly, AEC derived from PINK1 deficient mice have increased ROS production and are more sensitive to TGF-β1-induced apoptosis [65].

However, the primary cause of mitochondrial damage might be excessive exposure to ROS. Indeed, heightened 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 [63]. Consistently, AEC from IPF lungs presents a reduced activity of the electron transport

chain complex I and IV [64], which can lead to increased mtROS production. Interestingly, in vitro experiments showed that TGF-β1 stimulation induces a reduction in ETC complex four activity and increased mtROS production in AEC [67]. 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 [68]. 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) [69]. 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 [70].

Moreover, recent data showed that mtOGG1 overexpression mitigates, mtDNA damage and AECII apoptosis associated with PINK1 deficiency [71]. Additional data, suggest the importance of post-translational modifications such as acetylation in regulating the activity of mitochondrial antioxidants proteins including OGG1 and the manganese superoxide dismutase (MnSOD) whose acetylation is increased in AECII from IPF patients [72]. In vitro, treatment of AEC with asbestos or H₂O₂ diminished the expression of the mitochondrial NAD-dependent deacetylases Sirtuin 3 (SIRT3) resulting in increased MnSOD acetylation [72]. Its 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 [72]. As expected, mice over-expressing in their mitochondria antioxidant proteins such as the H₂O₂ detoxifying enzyme catalase, are resistant to mtROS production mtDNA damage, AECII apoptosis and lung fibrosis development induced by bleomycin [73].

In addition to these molecular actors, mediators associated with IPF and experimental lung fibrosis such as the “antiaging” molecule α-Klotho [74] and the bioactive lipid sphingosine-1-phosphate (S1P) [75] 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, protect mice from asbestos-induced lung fibrosis supposedly by reducing AEC mtDNA damage and apoptosis by a mechanism involving fibroblast growth factor receptor 1 (FGFR1) and AKT activation [76]. On the other end, S1P, whose concentration is increased in IPF serum, has been shown to have the opposite effect. Indeed, pharmacological inhibition of its producer, the Sphingosine Kinase 1 (SPHK1) in experimental lung fibrosis induced by asbestos, reduced S1P concentration and lung mtDNA damage, an effect also observed in AEC in vitro [75].

It is clear from these data that mitochondrial dysfunction, particularly in AECII plays a critical role in lung fibrosis initiation and progression. Future therapies should aim to prevent mitochondrial dysfunction. As shown by PINK1 regulation, cross-talks between the endoplasmic reticulum and mitochondria might be a good target to achieve this goal. In the next section, we will focus on the role of ER stress in AEC death associated with IPF.

**Endoplasmic reticulum stress**

In addition to DNA damage, protein damages induced by ROS can lead to protein misfolding, endoplasmic reticulum stress (ER stress) and the activation of an unfolded protein response (UPR) [77]. 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 [77].

The role of ER stress in AECII dysfunction and pulmonary fibrosis has been particularly enlightened in the past decades by the discovery of a genetic form of interstitial pulmonary fibrosis linked to mutations in surfactant proteins A and C [77]. As a result of these mutations, surfactant proteins are misfolded, leading to ER stress, and the activation of a UPR [77]. The link between these mutations, ER stress, and lung fibrosis has been studied using transgenic mice expressing an inducible mutant of SFTC in AECII. While the expression of the
Alveolar Epithelial Cells Death in Idiopathic Pulmonary Fibrosis: Significance, Mechanisms and Contribution to Disease Progression

mutant was not sufficient to induce epithelial cells death or fibrosis, these mice had increased lung fibrosis associated with greater AEC apoptosis and density of fibroblasts in response to bleomycin challenges, suggesting that in IPF, ER stress could favour pulmonary fibrosis by interacting with other stressors [78].

In sporadic forms of IPF, Lawson, et al. reported using immunohistochemistry staining of IPF lung sections, increased ER stress markers expression (i.e. BiP, EDEM and XBP) mainly in AECII [79]. In the absence of SFCT proteins mutations, the authors suggested that ER stress was in these patients induced by herpesviruses infections as its viral proteins could be detected in 65% of the sporadic IPF lung samples and colocalised in AEC with ER stress markers [79]. 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-induced in human bronchial epithelial cells, a ROS-dependent induction of ER stress markers such as CHOP and GRP78 as well as the activation of caspase-3 and -4 [80]. Finally, hypoxia could be an additional factor inducing AEC ER stress in IPF. Indeed, markers of hypoxia 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 [81]. An early study by Krück, et al. showed that hypoxia-induced primary rat AECII apoptosis through HIF-1α-dependent activation of the pro-apoptotic member of the Bcl2 family Bnip-3L [82]. Both, hypoxia-induced Bnip-3L expression and subsequent primary rat AECII apoptosis could be prevented by pre-treatment with the anaesthetic drug Propofol due to its inhibition of HIF-1α [83]. Moreover, hypoxia has been shown to exacerbate ER stress and fibrosis in the bleomycin lung fibrosis model [84,85]. 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 bleomycin-induced lung fibrosis [86]. Interestingly, the authors showed in vitro that HIF-1α triggers AEC apoptosis by inducing the pro-apoptotic ER stress marker C/EBP homologous protein (CHOP) [86]. Consistently, CHOP⁻/⁻ mice were resistant to bleomycin-induced AEC apoptosis and lung fibrosis but not mice with conditional deletion of HIF1/2 in epithelial cells [84]. The reason for these discrepancies between the in vivo and in vitro results is not well understood but suggest that hypoxia might induce ER stress in AEC through interaction with other cells type. Further in vitro experiments, established the inositol requiring enzyme 1a (IRE1a) and the PKR-like ER kinase (PERK) pathways as downstream effectors of CHOP in AEC apoptosis induction [84]. Finally, Shi, et al. showed using a model of obstructive sleep apnoea (i.e. Chronic intermittent hypoxia) an increased in ER stress markers expression, AEC apoptosis and pro-fibrotic markers expression (i.e. TGF-β1 and thrombospondin) that could be prevented by treatment with the ER stress inhibitor tauroursodeoxycholic acid (TUDCA) [87].

TGF-Beta-induced alveolar epithelial cell death

Transforming growth factor-beta 1 (TGF-β1) is a multifunctional mediator known to be a 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 [88,89]. TGF-β1 has been shown to elicit growth inhibition and apoptosis in multiple epithelial cells type while stimulating the proliferation of mesenchymal cells [88].

TGF-β1 signal through its membrane-bound receptor TGF-β Receptor II (TGF-βRII), triggering the formation of a heterotetrameric complex at parity with TGF-β Receptor I (TGF-βRI) [88]. TGF-βRII then phosphorylate and activate TGF-βRI, which in turn activates receptor-activated SMADs (R-SMAD) also known as SMAD2 and 3 [88]. 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 [88, 90]. 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 [88].

Investigation of the TGF-β1 pathway actors’ in IPF revealed an increased concentration of active TGF-β1 in their BALF [91] while conflicting results exist regarding its serum concentration with one study reporting an increase [92], and another no change [93]. Im-

munohistochemical studies revealed an increased expression of TGF-β1 in AEC and macrophages from IPF lungs [94], and lung fibrosis induced by bleomycin instillation [95].

Interestingly, BALF from IPF patients was found to induce AEC apoptosis in vitro, an effect blocked by the adjunction of anti-TGF-β1 blocking antibody [96]. In vitro, TGF-β1 has been shown to induce normal alveolar epithelial cells apoptosis through activation of SMAD3 [97] and downregulation of the cell cycle regulator p21 [96]. 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 [98]. 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 [99]. 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 [100].

Moreover, gene transfer of soluble hence inhibitory TGF-βRII 3 days before or four days after bleomycin instillation in mice, is associated with a decreased in cellular apoptosis and tissue fibrosis [101]. However, in this model of TGF-β1 overexpression in the lung, unlike in bronchiolar epithelial cells in vitro, TGF-β1 was found to increase p21 expression in epithelial cells and macrophages in a TNF-α-dependent manner [102]. Moreover, p21 acted as negative feedback of TGF-β1-induced TNF-α expression, and its deletion aggravates cell apoptosis, inflammation and fibrosis induced by TGF-β1 overexpression [102]. Conversely, human p21 adenoviral expression predominantly in lung epithelial cells seven days after bleomycin instillation in mice resulted in decreased apoptosis, inflammation and fibrosis at day 14 [103]. 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 fibrosis.

Also, TGF-β1 stimulation in the lung epithelial cell line Mv1Lu is associated with a decrease in mitochondrial complex IV activity resulting in prolonged mtROS production [67]. Thus, TGF-β1 could induce AEC apoptosis in IPF through a mtROS-dependent pathway.

(Myo)Fibroblasts as drivers of AEC death in IPF

As noticed in early and more recent observations, AEC death often occurs in the vicinity of FF containing myofibroblasts, suggesting a possible role of these cells in this phenomenon. 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 [104]. 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 [105]. 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 and that Angiotensin II blocking in fibrotic lungs fibroblasts conditioned medium impaired their ability to induce AEC apoptosis [106].

Not only IPF fibroblasts and myofibroblasts secrete factors inducing AEC apoptosis, but they also downregulate the secretion of factors known to support AEC proliferation and or survival. Thus, IPF-derived fibroblasts secrete lower level of the AEC 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 [107, 108]. Despite this decreased expression of HGF by IPF-derived fibroblasts, the level of HGF in the BALF and serum of IPF patients is higher than in normal subject, suggesting increased production by other cellular sources [109].

Interestingly, PGE2 inhibits lung fibroblasts proliferation and collagen expression while increasing their apoptosis in response to FASL [110,111]. AEC is a source of PGE2 and contributes to the control of lung fibroblast function; however, AEC from IPF lung present a reduction in their expression of cyclooxygenase-1 and -2 (COX-1 and -2), the enzymes involved in PGE2 synthesis [112]. Thus, PGE2 downregulation in IPF lungs has been proposed to be responsible for the apoptosis paradox (i.e. increase AECII apoptosis and fibroblast resistance to apoptosis) observed in IPF [111]. More recent researches identified epigenetic repression of genes involved in PGE2 synthesis as the cause of its decreased production in IPF lungs [113,114].

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 aggravate the epithelial cell death tall.

In IPF, the recruitment of inflammatory cells is associated with a doubling in BALF cellularity, with a massive increased in the number of neutrophils and eosinophils, and in a lesser extent in that of lymphocytes and macrophages [10]. Cytotoxic lymphocytes are known to express the transmembrane death receptor activator FAS Ligand (FASL) whose cleavage by MMP results in the release of a soluble form known as soluble FAS Ligand (sFASL) [115]. Interestingly Kuwano, et al showed that FASL expression was increased in BALF cells of IPF patients while being absent from control [116]. RT in situ PCR and immunohistochemistry identified Lymphocytes and granulocytes as FASL producers in IPF [116], a finding supported by the correlation between the degree of lymphocytes alveolitis in IPF BALF and the concentration of sFASL [115].

Besides, bronchiolar and alveolar epithelial cells from IPF lungs present an increased expression of the death receptor FAS as detected by immunohistochemistry [116]. Interestingly Hagimoto, et al demonstrated that BALF from IPF was able to induce bronchiolar epithelial cells apoptosis [96]. The authors also suggested a synergy between FASL and TGF-β1 in the induction of bronchiolar epithelial cells apoptosis [96].

Similar to IPF, bleomycin-induced lung fibrosis is associated with increased expression of FASL and AEC apoptosis which can be prevented by the administration of FAS antigen or FASL blocking antibodies [117]. Conversely, repeated inhalations of an agonistic anti-FAS antibody by mice is associated with a dose-dependent increased in bronchiolar and AEC apoptosis, lung inflammation and lung fibrosis as measured by lung hydroxyproline content [118]. 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 cell types, including AEC and fibroblasts in the lung [119]. 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 [119]. In addition, the angiotensin system comprises two receptors named AT1 and AT2 expressed across several cell types [119]. More recently, the ACE homologue ACE-2 which transform ANGII into ANG1-7 have been discovered. ANG1-7 has been shown to elicit different and often opposite cellular function through activation of the receptor mas [119].

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 [120], while ACE concentration is increased in their BALF [14]. Finally, the mRNA expression of both angiotensin receptors (i.e. AT1 and AT2) are increased in IPF lungs [121]. Moreover, IPF patients bearing the G-6A polymorphism of ANG associated with higher mRNA expression and protein production, have faster disease progression [122]. Li, et al. also showed by immunohistochemistry that apoptotic AEC from IPF samples expresses ANG peptide suggesting a possible causal link [120]. Consistent with this hypothesis, inhibition of local ANGII production by intratracheal instillation of antisense nucleotide prevented AEC apoptosis and fibrosis induced by bleomycin instillation [123].

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 [124]. Moreover, mice overexpressing ANG ubiquitously develop lung fibrosis with sub-optimal doses of bleomycin. At the same time, intratracheal administration of an adenovirus expressing ANG led to its preferential expression by AEC,
which was associated with their apoptosis and the development of lung fibrosis [124]. 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 [124].

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 [125-127]. Interestingly, subsequent investigations made in various AEC models (i.e. primary rat AEC, MLE-12, A549) revealed that the ACE-2 product ANG1-7, protect them from bleomycin or ANGII-induced apoptosis by inhibiting JNK activation in a mas and MAP Kinase phosphatase-2 dependent pathway [135, 136]. In addition to bleomycin, ER stress and the subsequent UPR induced by chemicals or the SP-C BRICHOS domain mutant G100S in AEC (i.e. primary human AEC and A549) has also been shown to induce AEC death through autocrine ANGII stimulation [137]. 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 [137]. Moreover, ANG1-7 administration could prevent AEC apoptosis induced by ER stress, an effect abrogated by the mas inhibitor A779 [138].

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 [139]. 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 leucocytes elastase**

The activation of the coagulation cascade is an early response to tissues damages that facilitate the recruitment of inflammatory cells and the repair process [140]. In IPF lungs, Tissue Factor (TF) is upregulated in AECII in areas of important fibrin deposition [141]. The contribution of an excessive coagulation cascade activation to IFP pathogeny is suggested by the protective effect of the anticoagulant warfarin in patients experiencing acute exacerbations of the disease [142]. Among actors of the coagulation system, the serine protease thrombin has been involved in TGF-β1 activation by AEC through protease-activated receptor-1 (PAR-1) cleavage [143]. In addition, direct activation of PAR-1 by leucocytes elastase at AEC’s surface induces their apoptosis through the intrinsic apoptotic pathway [144,145].

Interestingly, thrombomodulin, a modulator of thrombin activity which bind to it and turn its activity from pro- to anticoagulant, has been shown to prevent lung fibrosis induced by TGF-β1 overexpression or bleomycin [146]. The authors also showed that recombinant human thrombomodulin administration prevented AEC apoptosis induced by bleomycin both *in vivo* and *in vitro* [146]. Similar to what
was observed with the anticoagulant warfarin, a meta-analysis of clinical trials testing the effect of recombinant human thrombomodulin on acute exacerbation of IPF concluded to its benefit and an increased patients’ survival [147]. However, conflicting results exist on this point since a recent randomised clinical trial showed no benefit of recombinant human thrombomodulin administration in acute exacerbation of IPF [148].

**Figure 2:** Key mechanisms involved in Alveolar epithelial cells type II death during IPF onset and progression. IPF risk factors such as cigarette smoke and viral infections have been shown to induce DNA damages and ER stress. The subsequent activation of a DNA damage response (DDR) and the unfolded protein response (UPR) can lead to activation of the caspases cascade and cell apoptosis. In IPF, persistent ER stress increases the expression of the transcription factor ATF3, which represses the expression of the mitophagy regulator PINK1. As a result, IPF AEC accumulates dysfunctional mitochondria leading to increased intracellular ROS (iROS) production, which increases DNA and proteins damage, and the response associated, reinforcing pro-apoptotic signals. Besides, newly recruited cells such as myofibroblasts and neutrophils or activated resident cells (i.e., alveolar macrophages) are sources of extracellular ROS (eROS) which also contributes to cells damage and apoptosis. Also, neutrophils and CD8+ lymphocytes through their secretion of FASL activate the extrinsic apoptotic pathway while neutrophils elastase and thrombin can activate AECII's protease-activated receptor 1 (PAR-1) to induce their apoptosis. In IPF, myofibroblasts alveolar macrophages and AECII themselves are important sources of TGF-β1 which triggers AECII apoptosis through TGF-βRII activation. Finally, increased angiotensinogen production by myofibroblasts and AECII themselves is responsible for increased angiotensin II production, and subsequent activation of AT1 receptors leading to activation of the caspases cascade and AECII apoptosis.

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Epithelial cells death contribution to fibrosis development in IPF

As all mucosal organs, the lung is exposed to constant aggressions from the external environment, and the ability of AECII to renew themselves and differentiate into AECI is crucial to alveolus homeostasis. However, as described earlier, IPF is characterised by important AECII 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 of professional phagocytes such as AM in IPF progression.

Apoptotic cells efferocytosis by alveolar macrophages

AM are by far the most abundant resident leucocyte in human lung and ensure several housekeeping functions such as surfactant protein recycling and cleansing of foreign particles [10]. As professional phagocytes, one of their essential functions is the phagocytosis of cell debris and apoptotic cells in a process called efferocytosis [149]. Efficient efferocytosis of apoptotic inflammatory cells recruited to an inflammatory site is critical to inflammation termination and the transition toward 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, 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) [149].

Consistent with a pro-repair phenotype of AM after efferocytosis, Wang., et al. showed that instillation of apoptotic BALF cells in rats’ lung (1x10^6 UV treated autologous BALF cells) induced secondary apoptosis of recipients’ cells, inflammation and the development of moderate fibrosis [35]. The authors observed in their BALF 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 [150]. 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 [150]. 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 [150]. Interestingly, the authors found that, in vitro, Jurkat cells efferocytosis by primary macrophages induced significantly less expression of TGF-β1 and arginase compared to that of epithelial cells efferocytosis, suggesting a possible cell type-dependent regulation of efferocytic macrophage phenotype [150].

On the other hand, in an interesting 2012 article, Jihee Lee Kang team demonstrated that intratracheal administration of apoptotic cells (i.e. 10x10^6 UV treated Jurkat cells) two days after bleomycin instillation in mice was able to attenuate lung injury. This effect was supposedly due to induction of HGF expression as this effect was blocked by the concomitant administration of blocking anti-HGF antibodies [151]. The authors identified AM as the source of the increased HGF expression up to 21 days post bleomycin treatment and sug-
gested that early AM efferocytosis was responsible for this persistent expression of HGF. Besides, apoptotic cells instillation also elicited anti-apoptotic action, which is consistent with HGF’s anti-apoptotic effect in epithelial and endothelial cells [152]. 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) [153]. 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 [154]. In a third study, the authors confirmed the existence of an autocrine positive feedback loop between COX-2/PGE2 and HGF expression induced by apoptotic cells efferocytosis in RAW 264.7 and primary mouse peritoneal macrophages [155]. However, in IPF, this positive feedback loop seems to be lacking since HGF concentration in BALF and serum is high [109,156], while PGE2 expression in IPF BALF is diminished [112]. The reason for such a disconnection between the expression of both mediators in IPF is not known. The team also showed a second administration of apoptotic Jurkat cells 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 PPARy activation [157]. Finally, the authors showed that inhibition of either, COX-2, EP2 or HGF after apoptotic cells transfer abrogated the inhibition of the late TGF-β expression and lung fibrosis induced by bleomycin [154]. Besides, the team also demonstrated that secretion of prostaglandins (i.e. PGE2 and PGD2) and HGF by macrophages following apoptotic cells efferocytosis could inhibit pathogenic mechanisms involved in IPF progression such as Epithelial to Mesenchymal Transition (EMT) in the epithelial cells line LA-4 [158], and primary mouse lung fibroblasts trans-differentiation and invasiveness induced by TGF-β1 or EGF [159].

The bottom line from these experiments is that instillation of apoptotic cells in healthy lungs can generate inflammation, apoptosis and fibrosis through efferocytic AM release 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 damages and 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 [154, 160, 161]. 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-scaring potential. Interestingly, a recent article suggests that in chronic rhinosinusitis, the balance between TGF-β1 and HGF expression regulate the remodelling pattern, with high TGF-β1/HGF ratio correlated with increasing α-SMA and collagen expression [162].

Also, it is not clear 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 the relative expression of PGE2/HGF and TGF-β expression by efferocytotic AM, and in 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 expression and release of free oxygen radicals [10]. Moreover, AM from IPF have been shown to have defective efferocytosis [163], a phenomenon that could be linked in IPF to RhoA activation by cigarette smoke-induced ER stress [164] or the engulfment of particles such as silica [165]. 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 [166]. In the next section, we will evaluate the potential impact of AEC necroptosis and release of DAMP on IPF progression.
Necroptosis, DAMPs 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 [167]. Unlike apoptosis, necroptosis is characterised by pore formation and cell swelling which favour the release by necroptotic cells of DAMP (e.g. HMGB1, mtDNA, ATP, RNA), that elicit strong pro-inflammatory responses [167, 168]. 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 [168].

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 [169]. Besides, DAMP molecules such as ATP, HMGB1, mtDNA and Spliceosome-associated protein 130 (SAP130), are increased in IPF patients' BALF and, or serum [68, 168, 170-172]. In this section, we will focus on recent researches highlighting the potential role of TLR9 and TLR2/4 activation by DAMP such as mtDNA and HMGB1, respectively, in IPF progression.

DAMP-dependent TLR9 activation in lung fibrosis

TLR9 is an endosomal TLR activated by unmethylated CpG nucleotides found in bacterial DNA [173]. TLR9 expression is increased in IPF patients' BALF at the mRNA level, despite decreased expression by lymphocytes, suggesting expression by newly recruited cells [174,175]. Unexpectedly, immunohistochemistry analysis of IPF lungs revealed strong TLR9 expression in stromal cells such as fibroblasts [176]. Indeed, fibroblasts isolated from IPF lungs present increased expression of TLR9, and its level of expression in the lung positively correlates with disease progression [68,177]. These observations are consistent with in vitro experiments showing increased expression of TLR9 by normal lung fibroblasts stimulated with TGF-β1 for three days [178].

Originally, TLR9 was believed to be exclusively activated by pathogens-associated molecular patterns (PAMP) such as bacterial and viral DNA [173]. However, later experiments showed that mammalian DNA in the form of immune complexes were potent TLR9 inducers [179]. Thus, in podocytes mtDNA, which contain unmethylated CpG rich motifs has been shown to bind to and to activate TLR9 contributing to cell apoptosis [180]. However, IPF fibroblasts are resistant to apoptosis, and TLR9 activation by its agonist CpG-ODN or mtDNA, but not nuclear DNA, resulted in primary human lung myofibroblastic differentiation as assessed by increased expression of α-SMA [68,170,176]. Also, chronic stimulation of normal lung fibroblasts' TLR9 by CpG-DNA in vitro, induce an IPF 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 [178].

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 [170]. 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 [68]. However, another team showed trough PINK1 knock-out or overexpression in the AEC line A549, that mitophagy rather than impaired mitophagy was associated with mtDNA releases in a model of cyclic stretching-induced lung epithelial cell injury [181]. Therefore, further experiments are required to determine the mechanism of mtDNA release by AEC. Regardless of its origin, stimulation of primary AEC with extracellular mtDNA resulted in its endocytosis, TLR9 activation and TGF-β1 secretion. The data also suggest that the pro-fibrogenic potential of mtDNA increases with its level of oxidative damage [68]. Besides, TLR9 stimulation by CpG-DNA in the AEC line A549 and blood monocytes led to EMT and differentiation into fibrocytes, respectively [177]. 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 through its recognition of bacterial lipopolysaccharides and viral motifs [179]. Later studies revealed its ability to recognise a large number of DAMP, such as the nuclear protein HMGB1, the AECII marker surfactant protein A, but also several heat shock proteins, and damaged ECM components [179].

Analysis of TLR 4 expression in UIP lungs, showed an increased expression mainly in bronchial epithelial cells, AEC and AM [182]. Whereas in this study, TLR4 has not been detected in fibroblast/myofibroblasts, other researcher showed using immunofluorescence and laser capture microdissection, an expression of TLR4 by myofibroblast (i.e. α-SMA+ cells) in FF. Analysis of TLR4 mRNA and protein expression in IPF-derived fibroblasts confirmed its expression in these cells at levels similar to that of normal human lung fibroblasts [183]. Interestingly, the authors showed that normal and IPF fibroblasts diverged in their response to stimulation by TGF-β1 and the TLR4 activator LPS. Indeed, TLR4 activation by LPS inhibited TGF-β1 stimulation of myofibroblastic markers expression (i.e. α-SMA, COL1A1 and CTGF) in normal human lung fibroblasts, but not in IPF-derived fibroblasts [183]. Further investigation revealed that LPS stimulation in IPF-derived fibroblasts failed to inhibit TGF-β Receptor type 1 (TGF-βR1) and subsequent SMAD3 activation [183]. Interestingly, toll-interacting protein (TOLLIP), a TLR binding protein whose single nucleotide polymorphisms are associated with IPF [9], has been shown to inhibit TGF-β1 response by interacting with SMAD7 and promoting TGF-βR1 degradation in various cell line including mouse embryonic fibroblasts [184]. 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 [185].

Moreover, Bhattacharyya, et al. identified a TLR4-responsive gene signature in systemic sclerosis skin biopsies, and skin fibroblasts treated with a selective inhibitor of the exclusive TLR4 co-receptor myeloid differentiation 2 (MD2) showed impaired myofibroblastic differentiation in vitro [186]. Importantly, systemic sclerosis is also associated with lung fibrosis and can be modelled by the subcutaneous injection of bleomycin. In this model, pharmacological inhibition of TLR4 or its conditional deletion in fibroblasts protected mice from lung fibrosis development [186]. 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 [187]. 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 triggers foreskin fibroblasts myofibroblastic differentiation in a TLR-4 dependent manner, while in vivo Tenascin-C deficiency protected mice from lung and skin fibrosis induced by repetitive subcutaneous injection of bleomycin [187].

However, in lung fibrosis induced by intratracheal administration of bleomycin, which is associated with acute injuries of AEC, TLR4 deficiency resulted in inflammation and fibrosis exacerbation [188], potentially because its activation by hyaluronan plays a critical role in AECII renewal [189].

A critical pathogenic feature of IPF is fibroblasts resistance to apoptosis potentially as a result of their premature senescence [190]. The radiation-induced lung fibrosis model shares many features of IPF pathogenesis, including DNA damage and its progressive and intractable fibrotic development. Hanson, et al. showed that in this model fibroblasts resistance to apoptosis precedes fibrosis development and that TLR4-/- mice resistance to radiation-induced lung fibrosis was associated with a loss of their fibroblast’s 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, but 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]. As often, pro-proliferative stimuli also elicit anti-apoptotic effects; these data support a role for HMGB1-induced TLR4 activation in IPF lung 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 not clear whether differences in TLR4 and TGF-β crosstalk in skin and lung fibroblasts in vitro, and in models of systemic sclerosis and IPF in vivo, reflect phenotypic or pathogenic differences, but both set of experiments highlight crosstalk between the TLR4 and TGF-β pathways in fibroblasts that can modulate fibrosis development. The role of TLR4 activation in AEC, on the other hand, seems to be more constricted due to its role in AECII renewal, explaining divergent results between lung fibrosis models involving or not acute AEC damages.

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

Similar to TLR4, TLR2 is an extracellular TLR involved in recognition of microbes-derived lipids. Also, TLR2 can be activated by several DAMP (e.g. HMGB1, HSP60 and 70, Hyaluronan) also recognised by TLR4 [173, 179]. TLR2 expression is increased mainly in bronchial epithelial cells, AEC and AM of fibrotic lungs with a UIP pattern characteristic of IPF [182]. Interestingly, hyaluronan fragments from the serum of individuals experiencing acute lung injuries have been shown to elicit chemokines releases by macrophage through TLR2 and 4 activations. Moreover, mice deficient in both TLR2 and 4 have impaired inflammatory cells recruitment an increased epithelial cells death in response to bleomycin instillation [193], reflecting their role in both chemokines signalling and AECII renewal [189, 193]. Later experiments, evaluating TLR2 deficiency alone suggest, its persistent activation by DAMP in the bleomycin model of lung fibrosis is associated with the recruitment of immunosuppressive cells (i.e. Regulatory T Cells, alternatively activated macrophages), that prevent DAMP and PAMP elimination and support fibrosis development [194]. Kim., et al. showed using mice deficient for TLR2 in the hematopoietic lineage, that activation of AEC TLR2 promoted an immunosuppressive environment through IL-27-dependent inhibition of IL-17 leading to heightened bleomycin-induced lung fibrosis [195]. Also, recent data 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 different 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 through the promotion of a TH2 immune response.

**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 [168,172]. For some of them there, is 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 and 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 [168]. Both IPF patients and mice undergoing experimental lung fibrosis, have an elevated level of ATP 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 pro-

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fibrotic action of eATP is elicited through the engagement of the P2X<sub>7</sub> receptor/pannexin-1 axis and the subsequent maturation of IL-1β. Consistently, P2X<sub>7</sub> deficient mice were protected from bleomycin-induced lung inflammation and fibrosis [199].

Finally, dying cells produce and release large amounts of the purine catabolite uric acid [168]. Thus bleomycin-induced lung fibrosis is associated with uric acid production, which activates the NALP3 inflammasome leading to IL-1β production. Moreover, exogenous uric acid administration recapitulates lung inflammation and repair in a MyD88, IL-1R and TLR2/TLR4 dependent pathway [200].

**Figure 2:** KPotential contribution of AECII death to fibrosis progression in IPF. 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 following TNF-R1 activation. Apoptotic cells efferocytosis by alveolar macrophages induces their acquisition of a pro-repair phenotype associated with increased expression of HGF/PGE2 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 defective 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 I 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 respectively the P2X7 receptor and the NALP3 inflammasome could contribute to chronic IL-1β expression, leading to further inflammation and fibrosis.

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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’ critical pathogenic mechanisms. However, we showed in this review the critical role played by AEC death, and in particular AECII death in IPF 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 creates further AEC apoptosis while favouring fibroblast proliferation, differentiation into myofibroblast and resistance to apoptosis. Moreover, AM efferocytosis of apoptotic cells induce a pro-repair phenotype characterised by the release of mediators favouring AEC repair (e.g. HGF, PGE2) or death (e.g. TGF-β and TNFα). The mechanisms regulating the balance in 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 the disease progression by fostering a chronic inflammatory and profibrotic milieu through the release of DAMP. Those DAMP through activation of danger receptors, including TLR have been shown to exert profibrotic actions in both AEC and lung fibroblasts, supporting a critical role of necroptosis to IPF progression. The existence of cells expressing both apoptotic and necroptotic markers suggest a possible evolution in the ratio between those populations during disease progression and acute exacerbation episodes. It is unclear, in which extent IPF AM impaired efferocytosis participate in necrotic cells accumulation in IPF. The data presented here support the development of treatment 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 as well as 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 thereby DAMP releases might also be an exciting strategy to slow-down IPF progression.

Bibliography


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