CELLULAR AND MOLECULAR FEATURES OF SENESCENCE IN ACUTE LUNG INJURY

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Abstract

A wide range of insults can trigger acute injury in the lungs, which eventually may lead to respiratory failure and death of patients. Current treatment relies mainly on supportive measures and mechanical ventilation. Even so, survivors frequently develop important sequels that compromise quality of life. In the search for new approaches to prevent and treat acute lung injury, many investigations have focused on molecular and cellular pathways which could exert a pathogenic role in this disease. Herein, we review recent findings in the literature suggesting that cellular senescence could be involved in lung injury and discuss the potential use of senotherapies to prevent disease progression.

Keywords: acute respiratory distress syndrome, acute lung injury, senescence.

Abbreviations: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; CDK, cyclin-dependent kinases; COPD, chronic obstructive pulmonary disease; DDR, DNA damage response; ECM, extracellular matrix; IPF, idiopatic pulmonary fibrosis; ROS, reactive oxygen species; SA-β-gal, senescence-associated β-galactosidase; SAHF, senescence-associated heterochromatin foci; SASP, senescence-associated secretory phenotype; VILI, ventilator-induced lung injury; MV, mechanical ventilation.

1. Acute respiratory distress syndrome

The acute respiratory distress syndrome (ARDS), originally described by Ashbaugh and collaborators in 1967 (Ashbaugh et al., 1967), is a complex syndrome that leads to respiratory insufficiency and compromises patient survival. Despite the efforts to improve current therapies, ARDS is increasing in prevalence and is associated with high mortality and morbidity. Therefore, it represents an important health burden due to the elevated numbers of hospitalized, frequently critically ill patients, and entails significant costs to the total health-care budget worldwide (Matthay et al., 2012). There are diverse risk factors which could contribute to ARDS, of both pulmonary and extrapulmonary origin, as listed in Table 1. Additionally, socio-demographic and genetic factors come into play for determining ARDS development (Villar et al., 2003).

Table 1. Initiators of acute respiratory distress syndrome.

Lung-dependent	**Pulmonary contusion**
	Pneumonia

	Swine flu **Smoke inhalation** **Lung transplant** **Aspiration of fluids (gastric content, near drowning)**
Lung-independent	**Sepsis** **Multiple trauma** **Cardiopulmonary bypass** **Mechanical ventilation** **Oesophagectomy** **Acute pancreatitis** **Blood transfusion**

In general, ARDS confers to the patient a mixture of clinical symptoms that can contribute to long-term disabilities, especially in the aged population. Surviving patients remain with important sequels that compromise lung function as they may develop fibrosis of the lungs during the repair process. Other manifestations are beyond pulmonary location, and include neurological impairment, and muscle wasting and weakness. ARDS involves the manifestation of not only an alveolar diffuse injury but also other histological alterations like acute eosinophilic and organizing pneumonia, acute fibronous pneumonia, and diffuse haemorrhage with capillaritis. The process is characterized by an acute inflammation process (local and systemic) derived mainly from recruited neutrophils and also resident alveolar macrophages, lung edema, and surfactant dysfunction. The clinical manifestation involves decreased lung compliance, pulmonary infiltrates, and hypoxemia (Hughes and Beasley, 2017).

Shortly after an insult, neutrophils are recruited to the lungs where they release chemokines like CXCL-1/-5/-8, ENA-78 and CCL-2; pro-inflammatory cytokines, including IL-1, Il-6, IL-8 and TNFα; acute phase reactants (C-reactive protein and lipocalin); and matrix remodelling proteins as MMP-9. A prolonged state of this inflammatory response alters the alveolar-capillary barrier leading to non-cardiogenic pulmonary edema, the main cause of gas exchange impairment and, therefore, the main need for mechanical ventilation (MV). Occasionally, the edema is reabsorbed and the injured area of the alveolar epithelium can be repaired, allowing the patient to recover from the respiratory failure. On the contrary, if the edema persists, hyaline membranes start to appear, together with the fibrotization of the area and scarring of the tissue (Parekh et al., 2011).

There is still no effective pharmaceutical treatment for acute lung injury (ALI), being MV the main approach to prevent respiratory failure (Matthay et al., 2017), which in combination with critical care support, could improve health condition. However, the application of positive pressures on lung parenchyma can induce injury in healthy lungs (Wolthuis et al., 2009) or exacerbate pre-existing damage (Slutsky and Tremblay, 1998), the so-called ventilator-induced lung injury (VILI). In the last decades, research has focused on the identification of new routes

at cellular level which could provide a better understanding of the physiopathology of ARDS, but the precise cellular and molecular underlying mechanisms are still to be fully elucidated. Recent evidence points out to the activation of cellular senescence, opening a highly promising research field for lung disease therapies.

2. Cellular senescence

Cellular senescence was initially described by Hayflick and Moorehead as the finite lifespan that primary human fibroblasts derived from embryonic lung tissue have when culture *in vitro*, after a certain number of population doublings (Hayflick and Moorhead, 1961). Since then, the concept of senescence has evolved significantly and it is now considered as a cellular programme that involves a stable cell cycle arrest, which can be induced by a varied range of stimuli including: telomere attrition (replicative senescence; (Hayflick and Moorhead, 1961)), oncogene activation (oncogene-induced senescence (Bartkova et al., 2006)), and other stressors (premature senescence) like DNA damage (Di Micco et al., 2006), oxidative stress (Lopez-Diazguerrero et al., 2006), hyperoxia (Parrinello et al., 2003), ionizing irradiation (Lee et al., 2011), chemotherapy (Ewald et al., 2010), proteasome inhibition (Chondrogianni et al., 2003), autophagy impairment (Kang et al., 2011), mitochondrial dysfunction (Schuliga et al., 2018) or endoplasmic reticulum stress (Liu et al., 2014).

Cell cycle blockade involves the activation of cyclin-dependent kinase (CDK) inhibitors such as $p16^{INK4a}$ and $p21^{CIP1}$, key members of tumor-suppressor pathways which are orchestrated by p53 and pRb proteins, respectively (Campisi and d'Adda di Fagagna, 2007). Alongside the proliferation cease, senescent cells typically secrete inflammatory mediators, collectively known as the senescence-associated secretory phenotype, or SASP (Coppe et al., 2008), that has both autocrine and paracrine effects. Senescent cells exhibit a distinct phenotype characterised by cell flattening and enlargement, and chromatin rearrangements which generally lead to the formation of senescence-associated heterochromatin foci, or SAHF (Narita et al., 2003). They also experience metabolic changes like an increased lysosomal activity, represented by the positive staining for senescence-associated β-galactosidase (SA-β-gal) (Dimri et al., 1995) and a persistent activation of the DNA damage response (Fumagalli et al., 2014). Nevertheless, the nature of the stimulus and the cell type cause heterogeneous senescent phenotypes which could explain the functional heterogeneity of senescence (Kirschner et al., 2020).

Senescence is involved in normal embryonic development and morphogenesis (Rajagopalan and Long, 2012; Munoz-Espin et al., 2013; Storer et al., 2013). In adult tissues, senescence contributes to the maintenance of its homeostasis and repair (Demaria et al., 2014), and the prevention of fibrosis (Krizhanovsky et al., 2008; Jun and Lau, 2010). This could be partly due

to the fact that senescence may contribute to a reprogramming-like cellular plasticity in response to tissue damage *in vivo* (Mosteiro et al., 2016). Senescence also acts as a barrier to the proliferation of damaged or transformed cells (Ventura et al., 2007; Xue et al., 2007). Hence, it has been proposed as a potent anti-tumor mechanism alternative to apoptosis.

However, persistent accumulation of senescent cells in adult tissues can also be detrimental for the organism, since it could promote tumorigenesis or tissue dysfunction (Munoz-Espin and Serrano, 2014). Milanovic and colleagues have shown how chemotherapy –induced senescence may generate cancer stem cells within the tumor, which exhibit hallmarks of senescence and own the ability to drive tumor progression once they escape from senescence itself (Milanovic et al., 2018). There is also strong evidence for the contribution of cellular senescence to aging, and efficient removal of senescent cells in mice prolonged life expectancy and delayed age-associated disorders (Baker et al., 2011; Baker et al., 2016).

3. Converging paths of acute lung injury and cellular senescence

Many of the triggering events in ARDS lead to the activation of cell signalling cascades that resemble those described in senescence. During ARDS, the injured lung generally faces three phases: the exudative phase, characterized by diffuse alveolar damage in which epithelial and endothelial cells release surfactants and pro-inflammatory mediators among others that lead to an exudate that impairs gas exchange; the proliferative phase, which is initiated after absorption of the edema, to restore the alveolar epithelium; and finally, the fibrotic phase, which involves collagen deposition in the alveolar space but may be reached only in some patients and its severity correlates with mortality rates (Schwarz, 2001). Notably, inflammation, proliferation and fibrosis may be either causes or consequences of senescent responses. Nevertheless, literature establishing a link between ARDS and cellular senescence is scarce. Naturally occurring senescence in early stages of acute injury in other tissues has been shown to be beneficial, limiting apoptosis and favouring tissue repair. In heart and liver, inactivation of fibroblast senescence exacerbated fibrosis and contributed to organ dysfunction, suggesting that the senescence program could limit the fibrogenic response to acute damage (Krizhanovsky et al., 2008; Meyer et al., 2016). This is in contrast with the large body of evidence supporting a key role for senescence in the pathogenesis of age-related or chronic lung diseases like IPF, idiopathic pulmonary fibrosis, (Waters et al., 2018), and COPD, chronic obstructive pulmonary disease (Zhou et al., 2011; Sagiv et al., 2018). When no longer required, senescent cells are cleared by the immune system, but its decline, associated with aging and certain diseases, causes the inefficient removal of senescent lung cells. The accumulation of senescent cells overtime disrupts tissue structure and function. In general lines, a senescent alveolar epithelium

is unable to repair injured tissue while prolonging a pro-inflammatory state. In fibroblasts, senescence drives myofibroblasts differentiation leading to an irreversible fibrotization of damaged areas which eventually impedes gas exchange.

In the specific case of the lung, mechanical forces play an essential role in the maintenance of its function and structure. Breathing cycles expand the lung parenchyma and type II alveolar cells respond with surfactant secretion, differentiation to type I alveolar cells and apoptosis. This response not only occurs during lung development and maturation but also after injury (Edwards, 2001). Unfortunately, the excessive stretch of the lungs beyond the tolerance threshold worsens lung function, as exemplified by VILI. Despite being a life-saving intervention commonly used in the intensive care unit for patients with acute respiratory failure or compromised lung function, the application of positive pressures on the lung parenchyma can initiate damage (Wolthuis et al., 2009) or exacerbate pre-existing injuries (Slutsky and Tremblay, 1998).

VILI is characterised by the activation of a molecular cascade that causes inflammation. Since inflammatory cytokines and chemokines are integral components of the SASP, we have hypothesized that cellular senescence could be one of the underlying pathological mechanisms of VILI. Recent research carried out by our group reveals that the impairment of the nuclear envelope caused by Zmpste24 inhibition triggers a pro-senescent response that, in turn, ameliorates VILI (Lopez-Alonso et al., 2018). Recently, we have shown in a clinically relevant murine model of lung injury and MV that mechanical stretch alone and in combination with acid instillation upregulated key senescence indicators and prevented damage in the lungs (Blázquez-Prieto et al., 2020).

It is of vital importance to identify the stage at which senescence goes from homeostatic to pathogenetic, so that it is no longer favourable but detrimental for the organism. Induction of senescence would be beneficial before reaching this threshold, while the inhibition would be favourable beyond it. Both strategies could actually be implemented to prevent the transition from ARDS into to irreversible chronic lung diseases, and to better determine the best approach to apply specific therapies, including MV, in order to improve the outcome of the patient. The notion that senescence in the lungs could have the potential to be a double-edged sword is supported by the fact that this concept has already been proposed in the context of aging and cancer (Campisi, 1997). Herein, we review current knowledge on cellular and molecular mechanisms of ALI which are indicative of senescence, and could provide a new perspective to be explored in the clinical practice.

3.1. Cell cycle arrest

The inability of senescent cells to proliferate is one of their main features. Cell cycle blockade usually underlies activation of CDKi such as $p16^{INK4a}$ and p21, key members of tumor-suppressor pathways which are orchestrated by p53 and pRb proteins, respectively (Campisi and d'Adda di Fagagna, 2007). Damage sensor proteins, like ataxia telangiectasia mutated (ATM) protein in replicative senescence, respond to stressors leading to the activation of p53 by phosphorylation. In turn, phosphorylated p53 activates downstream targets like p21 which induce cell cycle arrest early at G1, preventing cells to enter the S-phase. Cells could re-start proliferation if the damage is resolved. On the contrary, when damage persists, the arrest could lead to an upregulation of $p16^{Ink4a}$, which further activates Rb and eventually induces permanent cell cycle arrest in the G0/G1 stage.

During hyperoxia, oxidative stress or bleomycin-induced lung injury, p21 presence rapidly increased at both mRNA and protein levels, exerting a protective role against apoptosis, inflammation, fibrosis, and alveolar destruction (O'Reilly et al., 1998; Mishra et al., 2000; O'Reilly et al., 2001; Plataki et al., 2005). This upregulation of p21 has been reported to be either dependent or independent of p53 (Mishra et al., 2000; Blundell et al., 2004), and possibly as a result of TGF-β1 stimulation and via TNFα (Yamasaki et al., 2008). Our work showed that mechanical stress induced p21 and p53 upregulation in lung cells, and an increase in the proportion of cells in the G0/G1 phase. However, in p21 null mice, this insult led to a general decay in senescence markers and a more severe lung injury than their wild type counterparts (Blázquez-Prieto et al., 2020). P21 is also elevated in bronchial epithelium of patients with severe asthma, where it has been associated with abnormal repair responses that contribute to inflammation and remodelling (Puddicombe et al., 2003). Genetic variations of single nucleotide polymorphisms TP53 and CDKN1A (p21) cell cycle genes are associated with IPF progression, as they impair damage response and increase the loss of alveolar epithelial cells (Korthagen et al., 2012). Restoring p21 protein in a murine model of bleomycin-induced lung injury had anti-apoptotic and anti-fibrotic effects and ameliorated pulmonary fibrosis (Inoshima et al., 2004).

The INK4/ARF locus encodes for three tumor suppressor genes, including $p16^{INK4a}$ and ARF (CDKN2A), and $p15^{INK4b}$ (CDKN2B). Both $p15^{INK4b}$ and $p16^{INK4a}$ are CDKi, and can induce cell cycle arrest by directly inhibiting CDK. ARF targets MDM2, interfering with the p53/p21 pathway. $P16^{INK4a}$ activation, one of the most commonly used biomarkers for senescence, is even dispensable in some occasions (Munoz-Espin et al., 2013; Storer et al., 2013), including models of COPD/emphysema (Sundar et al., 2018).

Direct measurement of proliferation markers such as Ki67 and BrdU can also be used to assess cell cycle exit. However this may not be very informative in postmitotic or quiescent cells. Regarding ARDS and the fact that there is a proliferative phase to re-populate the damaged

epithelium (Gonzalez-Lopez et al., 2011), the question that arises is whether there could be cell to cell variability within the population of lung alveolar type II cells when deciding their fate as senescent or proliferating ones. Senescent and proliferating cells could co-exist to a certain extent but in order to proceed with the proliferative phase required in the repair process, the senescent cells need to be efficiently removed. Otherwise, its limited cell proliferation capacity would then interfere with the renovation of the injured epithelium.

3.2. Senescence-associated secretory phenotype (SASP)

Apart from the permanent cell cycle arrest, cells undergoing senescence exhibit characteristic morphological features as well as metabolic alterations, which include a pro-inflammatory secretome known as SASP, the senescence associated-secretory phenotype (Coppe et al., 2008). SASP contains cytokines, chemokines, and extracellular matrix-degrading proteins which act locally but could also exert paracrine effects. Interestingly, the precise components of SASP may depend on the senescence-induction stimuli and the target cells (Maciel-Baron et al., 2016). The main function attributed to the SASP is to create a pro-inflammatory state and to promote the remodelling of the surrounding tissue. The SASP also attracts and activates cells of the immune system, which will ultimately kill senescent cells when they are no longer required (Krizhanovsky et al., 2008). When the immune system is compromised, as it occurs during aging, the inefficient removal of senescent cells turns SASP into a detrimental agent that modifies the tissue microenvironment and its structure, and enhances the appearance of chronic lung diseases like COPD, pulmonary fibrosis and cystic fibrosis (Chilosi et al., 2013; Hashimoto et al., 2016; Kumar et al., 2016; Bezzerri et al., 2019).

In spite of the heterogeneous nature of the critically ill patients with respiratory failure, they all generally show symptoms which involved an initial systemic inflammatory response syndrome, termed SIRS, mediated by the release of pro-inflammatory mediators. This is further alleviated by a compensatory anti-inflammatory response syndrome, CARS, to restore the homeostasia. It may well be the unbalance between SIRS and CARS that leads to an increase mortality rate (Xiao et al., 2011). Patients subjected to MV may show a release of inflammatory mediators from the lungs to the systemic compartment. This response has been termed biotrauma. Moreover, these patients exhibited a phenotype similar to the age-related decline of the immune system, known as immunosenescence (Dicarlo et al., 2009), which, in combination with the senescence program initiated in the lung parenchyma in response to injurious agents, can dramatically drive the patient to an even more life-threatening situation by exacerbating and prolonging inflammation, and therefore, altering the balance between SIRS and CARS.

NF-κB is a critical enhancer of the secretory activity of senescent cells (Acosta et al., 2008; Chien et al., 2011). Several pathways can regulate the SASP by modulating NF-κB activity, like

p38 mitogen-activated protein kinase (MAPK) (Freund et al., 2011) and mammalian target of rapamycin (mTOR) (Aarts et al., 2017). NF-κB is upregulated in aging and aging-related chronic diseases and also in acute injury. In a preclinical model, pretreatment with clarithromycin, an antibody belonging to the macrolides commonly used against pneumonia and septic shock, limits VILI probably due to a blockade in NF-kB signalling pathway (Amado-Rodriguez et al., 2013).

The JAK2/STAT3 route is another key regulator of the inflammation-senescence-SASP loop. In response to TGF-β1, IL-6 and IL-13, JAK2/STAT3 becomes activated in alveolar type II cells and lung fibroblasts and promotes epithelia to mesenchymal transition as well as differentiation of fibroblasts to myofibroblasts, respectively (Milara et al., 2018). Inhibitors of JAK2/STAT3 improve lung condition by preventing fibrosis, collagen deposition, senescence and an anti-apoptosic response (Salah et al., 2018).

The high mobility group box-1 protein, HMGB1, is a conserved non-histone nuclear protein associated with many DNA-related processes but, when extracellular secreted, it acts as alarmin to signal tissue damage. HMGB1 has been proposed as a prognostic biomarker in ALI, increased both at pulmonary and systemic level (Abraham et al., 2000), and also in cellular senescence, highly likely because it can trigger inflammation in a p53-dependent manner (Davalos et al., 2013). HMGB1 is expressed in response to lipopolysaccharide (LPS) and promotes the nuclear traslocation of NF-κB, with the consequent release of pro-inflammatory cytokines that exert a positive feedback loop on HMGB1 expression, reinforcing the inflammation cascade (Entezari et al., 2014). HMGB1 can also bind to TLRs and RAGE, activating MAP and ERK1/2 in addition to NF-κB and other downstream signalling pathways that contribute to tissue injury. To note, genetic or pharmacological inhibition of RAGE, a HMGB1 receptor, alleviated endotoxin-induced lung injury in mice (Achouiti et al., 2013). However, in the clinical practice, the benefits of interfering with inflammation are not as evident as those observed in experimental models.

3.3. Extracellular matrix remodelling

Matrix metalloproteinases, and other proteases are naturally secreted as SASP mediators, which contribute to tissue remodelling by producing or degrading extracellular matrix (ECM) proteins. The role of senescence in ECM remodelling is probably one of the most robust examples that support the benefits of the transient nature of senescent cells. In general, MMPs and other proteases limit fibrosis by degrading ECM, which, in combination with growth factors, favours wound healing (Krizhanovsky et al., 2008). However, permanent activity of MMPs can disrupt tissue structure (Elkington and Friedland, 2006).

Elevated expression of different MMPs, MMP-2, MMP-8, MMP-9, has been reported in mouse models of ALI of pulmonary or extrapulmonary origin, and also in patients suffering from ALI or ARDS (Fligiel et al., 2006; Santos et al., 2006; Albaiceta et al., 2008). Particularly after VILI, MMP-9, presumably released by neutrophils recruited at damaged sites, is significantly increased and the disruption of the gene encoding for this gelatinase led to a more severe injury compared to their wild type counterparts (Albaiceta et al., 2008). Similarly, MMP-8 knockout mice presented resistance to bleomycin-induced fibrosis (Garcia-Prieto et al., 2010). Apart from the MMPs secreted by immune cells to promote inflammation and stromal function, alveolar epithelial cells and fibroblasts undergoing senescence are also involved in the process. Excessive ECM production by activated fibroblasts in response to SASP released by epithelial senescent cells can lead to the fibrotization at sites of epithelial cell loss within the alveolar space (Chen et al., 2019), and this has been considered as a prognostic factor of IPF which correlates with survival (Nakagawa et al., 2019). Treatment with senolytics attenuated experimental lung fibrosis *ex vivo* by reducing the expression of senescence biomarkers and SASP in alveolar cells (Lehmann et al., 2017).

3.4. Metabolic activity: senescence-associated β- galactosidase

Despite the cell cycle blockade, senescent cells remain metabolically active. During many years, SA-β-gal has been considered as the gold standard senescence biomarker for both *in vitro* and *in vivo* assays (Dimri et al., 1995). Its activity, detectable at pH 6, is increased in senescent cells due to an enhanced lysosomal biogenesis (Kurz et al., 2000). Bleomycin, an anti-cancer drug known to cause lung fibrosis, is able to induce senescence in alveolar cells. A significant increased in the percentage of SA-β-gal positive alveolar cells was observed after 24h of bleomycin intratracheal administration, a percentage that dramatically increased in a dose- and time-dependent manner, reaching its maximum after 7 days of treatment. These cells co-localized with fibrotic areas, suggesting once again that senescence may be one of the pathological mechanisms involved in pulmonary fibrosis (Aoshiba et al., 2003). However, there could be more mechanisms other than senescence responsible for increased SA-β-gal activity (Severino et al., 2000). Remarkable, macrophages can naturally exhibit high levels of SA-β-gal activity as an indicator of the maturation stage (Bursuker et al., 1982). Additionally, it is not so trivial to detect increased SA-β-gal activity immediately after acute injury in young or adult individuals in comparison with aged one (Dimri et al., 1995). Its accumulation overtime hinders its potential as an early biomarker of senescence.

3.5. DNA damage response

Apart from telomere erosion, acute exposure to oxidants, γ-irradiation and/or UV-B light or cytotoxic agents can drive cells into senescence by activating the DNA damage response (DDR). In general, the DNA damage sensor ATM detects uncapped telomeres or DNA breaks and becomes activated by autophosphorylation. The activation is only complete when the MRN complex (MRE11-RAD50-NBS1) is assembled at the damage site and active ATM phosphorylates NBS1 and histone H2AX in Ser 139 (γH2AX) on the flanks of the damaged site (Rogakou et al., 1998; Berkovich et al., 2007). γH2AX can also be deposited by ATR (Ataxia- and Rad3-related), another PI3K-related kinase, in non-cycling cells (Matsumoto et al., 2007). This is followed by the recruitment of the MDC1 mediator that has affinity for γH2AX and acts as a platform for the recruitment of other DDR mediators and effectors, as well as complex chromatin remodelers that allow interaction with factor 53BP1 (p53- binding protein) at DNA injured sites. Concomitantly with DNA repair, ATM induces the activation of cell cycle control factors, particularly of the checkpoint-kinases Chk1 and 2, p53 and p16-RB, which induce cell cycle arrest and activate DNA repair, apoptosis or senescence depending on the magnitude of the DNA damage (Childs et al., 2014). In oncogene-induced senescence, the activation of DDR is also responsible for the cell cycle arrest to face the cellular proliferation promoted by oncogene activation (Bartkova et al., 2006; Di Micco et al., 2006).

Apart from the upregulation of key proteins in the DDR, γH2AX is a DNA damage marker typically observed in SAHF. Lung cells under mechanical stress exhibit structural changes in chromatin that could activate DDR pathways even in the absence of DNA breaks, by promoting the dissociation of inactive ATM dimers that are activated by adopting the monomeric configuration, favouring the binding of DDR proteins to chromatin. For example, inhibition of histone deacetylases, which are capable of modulating chromatin structure, can activate the ATM pathway (Bakkenist and Kastan, 2003). In the DDR canonical pathway, the chromatin flanking DNA breaks undergoes enrichment in H3K9me3 that contributes to the formation of transient heterochromatin domains that promote the binding of TIP60 acetyltransferase with consequent phosphorylation, activating ATM (Ayrapetov et al., 2014). Remarkably, the induction of chromatin condensation regardless of the existence of DNA breaks is capable of triggering DDR (Burgess et al., 2014).

Hyperoxia-induced acute lung injury triggers H2AX phosphorylation perceptible even after 12h of exposure (Sauler et al., 2015). This is in part mediated by the release of reactive oxygen species (ROS) within the lung due to pathological conditions which include not only hyperoxia but also sepsis (Stephens et al., 2015), mitochondrial dysfunction (Correia-Melo et al., 2016) among others. During inflammation, there is a significant release of ROS in lungs and cells of the immune system which can further generate direct damage onto DNA or act as second messengers, altering the expression of specific genes.

Cyclic stretch can cause DNA strand breaks in alveolar epithelial cells *in vitro* via MAPK activation (Upadhyay et al., 2003). Despite the fact that there is scarce literature referring to physical breaks in DNA or repair mechanisms in *in vivo* models of mechanical stress, Burguess and Misteli proposed the activation of non-canonical routes that occur with the activation of the ATR by mediation of the nuclear lamina (Burgess and Misteli, 2015). It is important to note that results published recently demonstrated how the lung parenchyma of mice undergoing MV showed a drastic reorganization of chromatin, which was not observed in the absence of the mature form of lamin A (Lopez-Alonso et al., 2018). Likewise, an increase in ɣH2AX levels has been observed in nuclear extracts from lungs of mice undergoing MV (with a previous injury induced by acid inhalation) compared to mice that remain with spontaneous breathing or those with just the previous injury (Blázquez-Prieto et al., 2020). Both events, the rearrangement of chromatin and the accumulation of ɣH2AX, could be closely related to the damage observed in the lung parenchyma in response to mechanical stress, and indicate senescence.

3.6. Nuclear lamina

The nuclear lamina, located at the inner side of the nuclear envelope, is a meshwork of A- and B-type nuclear lamins including lamin A, C, B1 and B2. The nuclear lamina regulates the mechanical properties of the nucleus, the spatial organization of chromosomes, playing a key role as anchorage points at the nuclear periphery; and gene transcription (Harr et al., 2015). Abnormalities in the nuclear envelope can activate premature senescence, as observed in cells obtained from progeroid patients which exhibit mutations in the Lamin A gene (Kandert et al., 2009; Liu et al., 2011). Not only mutations but also ROS-induced oxidative damage to Lamin A predisposes cells to entry into a state of senescence (Pekovic et al., 2011). Also, cells experience a down-regulation of the Lamin B1 both at mRNA and protein levels in spontaneous and oncogene-induced senescence (Shimi et al., 2011; Shah et al., 2013).

Since the nuclear envelope has been recently proposed as one of the key players of mechanotransduction processes in the cell, the lamins have earned particular interest in the study of VILI. In a double hit model of MV in previously injured lungs, mechanical stretch alone and in combination with acid instillation upregulated key-senescence indicators, like p53 and p21, SAHF and γH2A.X (Blázquez-Prieto et al., 2020) and down-regulated Lamin A. Interestingly, Lamin B1 loss has been proposed has a senescence-associated biomarker since it has been observed in different *in vitro* and *in vivo* models of cellular senescence (Freund et al., 2012). By contrast, in Ataxia-Telangiectasa, a DDR syndrome, Lamin B1 accumulation mediates entry into oxidative stress-induced premature senescence, independent of ATM mutations (Barascu et al., 2012). In our system, Lamin B1 remained constant, suggesting that its role in senescence could depend on the context. Even though changes in nuclear lamins depend

on the nature of the stimuli or the tissue, it is reasonable to believe that the Lamin A/ Lamin B1 ratio, an indicator of the nuclear stiffness, is more informative about the predisposition of cells to enter into senescence than just the abundance of each of the lamins separately.

3.7. Chromatin architecture

Closely related to the changes at the nuclear lamina, senescent cells undergo chromatin remodelling. SAHF are domains of facultative heterochromatin that harbour silent proliferation-related genes, like E2F targets, in senescent cells (Narita et al., 2003). SAHF are characterized by hypoacetylation of histones, tri-methylation of histone H3 on lysine 9 (H3K9me2/3) and the presence of heterochromatin proteins like heterochromatin protein 1 (HP1). Also, the canonical histone H2A is replaced by its variant macroH2A (Zhang et al., 2007). This composition is distinct from other regions of constitutive or even facultative heterochromatin, like the inactivated X chromosomes in female mammalian cells, enriched for H3K27me3 (Narita et al., 2003). Narita and co-workers found that High-Mobility Group A (HMGA) proteins accumulate on senescent fibroblasts, where they play a structural role in SAHF, by interaction with $p16^{INK4a}$ (Narita et al., 2006). It seems that SAHF formation is dispensable for cellular senescence in some occasions, depending on the cell type and triggering event (Kosar et al., 2011).

A body of literature robustly supports the idea that chromatin alterations in lung diseases trigger an inflammatory response. Disruption of the acetylation:deacetylation balance in histones in response to cigarette smoke exposure promotes transcription of a pro-inflammatory signature in lungs that could contribute to the progression of COPD (Marwick et al., 2004) or asthma (Ito et al., 2002). Even in models of acute injury, the alveolar epithelium in mice exhibited chromatin rearrangements in response to injurious mechanical ventilation (Lopez-Alonso et al., 2018) or endotoxemia, and the use of histone deacetylase inhibitors attenuated the outcome (Ji et al., 2013; Chen et al., 2014; Samanta et al., 2018). To note, hypoacetylation per se can also drive cellular senescence via replication stress that leads to G2/M cell cycle arrest without DDR activation or p53, p21 or p16 upregulation (Prieur et al., 2011).

In contrast to the traditional view of chromatin as a nuclear entity, functionally active fragments of chromatin from the nucleus or the mitochondria have been found in the cytoplasm of senescent cells. cGAS-STING (cyclic GMP-AMP synthase-stimulator of interferon genes) is a cytosolic DNA sensor that activates innate immunity, which binds to extranuclear chromatin after DNA damage leading to SASP-mediated inflammation in primary human cells and mice (Dou et al., 2017). In fact, activation of cGAS seems to be essential for cellular senescence (Yang et al., 2017). Interestingly, during inflammation neutrophils release neutrophil extracellular traps in response to different stimuli. In models of chemically-induced lung injury,

the release of mitochondrial DNA triggers NETs formation via cGAS-STING and TLR9 pathways (Liu et al., 2019), which could be interpreted as a pre-senescent event. In this sense, cGAS deletion has been shown to accelerated spontaneous immortalization of mouse embryonic fibroblasts and abrogated SASP (Yang et al., 2017). Traditionally, cGAS activation was considered as a defence mechanism against microbial DNA. However, transfusion-related or trauma-induced lung damage to self nuclear or mitochondrial DNA can lead to its accumulation in the cytosol, resulting in cGAS activation and favouring the senescent phenotype.

3.8. Mitochondrial dysfunction

Recent evidence has established mitochondrial dysfunction as one of the effectors for cellular senescence. This was initially based on the ability of mitochondria to generate ROS, which would further activate the DDR. Also, cytosolic mitochondrial DNA, one of the so called damage-associated molecular patterns (DAMPs), activates the immune system and promotes inflammation (Zhang et al., 2010). But the role of mitochondria in senescence goes beyond this. The group led by Passos and co-workers has proved that the activation of senescence, mainly the pro-oxidant and the pro-inflammatory axes, is directly linked to the mitochondrial biogenesis mediated by PGC-1β in a pathway that also involves ROS and DDR (Correia-Melo et al., 2016).

Massive transfusions are associated with another form of acute lung injury, transfusion-related acute lung injury. It has been reported that transfusion products contain large amounts of extracellular mitochondrial DNA DAMPs that could contribute to the development of ARDS (Simmons et al., 2017). In addition, mitochondrial DAMPs are also released during trauma- and sepsis-induced lung injury (Faust et al., 2020). One hand, this triggers an inflammatory reaction, and on the other, correlates with a decay in mitochondrial mass, both effectors of senescence. Different strategies, from the transference of mitochondria to pharmacological interventions to regulate mitochondrial function and dynamics exerted protective effects on experimental models of sepsis-induced ALI (Islam et al., 2012; Dong et al., 2018; Shi et al., 2019).

4. From bench to bedside: senotherapies in ALI

One of the main challenges that arises when investigating senescence is the existence of robust and consolidated markers. *In vitro* studies, with homogeneous populations, may bring more consistency into specific pathways but when assessing senescence *in vivo* the guidelines are not so evident. An added difficulty is that senescent cells generally represent a small percentage of the total cell population within a tissue, and the levels of senescent biomarkers could be masked by the presence of neighbouring cells that do not senesce. In addition, depending on the triggering event or the cell type, there is additional cell-to-cell variability (Wiley et al., 2017)

which makes the use of a single marker inadequate. Furthermore, some of the most commonly used indicators the field involved its accumulation overtime, like SA-β-gal, hindering early detection of senescence in response to acute insults. In order to overcome this, the use of multiple senescence markers is becoming more and more popular, as well as its combination with functional studies, but these are not always possible in clinical samples. Nevertheless, cumulative evidence supports a role for senescence in chronic lung diseases like COPD, IPF (Zhou et al., 2011; Sagiv et al., 2018; Waters et al., 2018). In the case of acute or severe lung injury, some of these indicators have been found, and even thought they might have not been necessarily linked to a senescence program, they could clearly anticipate its onset and be considered as pre-senescent biomarkers.

In response to different stressors, cells could enter a senescence program which involves a permanent cell cycle arrest. Nevertheless, senescent cells remain metabolically active and can exert not only autocrine but also paracrine effects, mediated by the SASP, which allows them to communicate with distant cells and orchestrate a multicellular response. Physiological senescence occurs during embryonic development and in adult cells in a strictly controlled manner. But, unfortunately, the accumulation of senescent cells triggers a pathological response that has been associated with aging, cancer, and other diseases. Based on this, Childs and colleagues proposed the existence of two types of senescent cells, acute or chronic, according to their lifetime (Childs et al., 2014), which is lengthened with age (Karin et al., 2019). Acute senescent cells live for short periods of time, as the organism is able to clear them efficiently when they are no longer needed. Acute senescent cells would be those involved in numerous physiological processes which are not only restricted to embryonic development, like morphogen gradients, regulation of cellularity, placental angiogenesis, tumor suppression, wound repair, and tissue regeneration. In contrast to this, chronic senescent cells are those who persist in the organism for longer periods of time and their accumulation, enhanced with age due to immune system decline, is detrimental, as they could promote tumorigenesis and tissue dysfunction (Childs et al., 2014).

This dual nature of cellular senescence, beneficial or detrimental, seems applicable to pulmonary diseases. During acute injury, senescence would limit the extent of DNA damage, preserving cells as senescent and avoiding apoptosis. The SASP would contribute to create a pro-inflammatory state to recruit immune cells, and remodel the ECM. Once senescent cells have been cleared out, proliferation will proceed to regenerate injured areas. When the turnover of senescence cells slows down, they can become pathogenic as they may prevent recovery from lung injury, by decreasing proliferation rate and prolonging a pro-inflammatory status. The role of senescence in chronic or age-related lung diseases is abundantly illustrated in the literature, but less evidence exists regarding ALI.

In any case, different routes to interfere with senescence have been explored and proven to beneficial in lung injury. More importantly, researchers in the field decided to join efforts to develop compounds which were able to specifically kill senescent cells, which are, by nature, resistant to apoptosis (Wang, 1995). The term senolytics was coined to designate these compounds, whose targets are senescent cells, not specific molecules or signalling pathways, and two of them, Dasatinib and Quercetin, served as proof of principle, improved tissue function in aged animals (Zhu et al., 2015). Regarding the lung, these two compounds prevented experimentally-induced pulmonary fibrosis (Lehmann et al., 2017; Hohmann et al., 2019) and emphysema (Ganesan et al., 2010). Navitoclax, which selectively inhibits Bcl-2 family members, was also able to reverse pulmonary fibrosis induced by irradiation in mice (Pan et al., 2017). There are many more examples this kind, but it is important to highlight that experimental models of ALI are frequently attributed to a single event, while this is not the case for ALI in humans. Nonetheless, the knowledge inferred can help to better characterized essential signalling pathways involved in disease and explore new therapeutic intervention. Hence, in 2019 a pilot study in humans suffering from IPF provided evidence that senolytics could improve their condition (Justice et al., 2019).

From a study carried out in a model of osteoarthritis, senescent cells were observed to reappear after cessation of senolytic treatment (Jeon et al., 2017). This could suggest that it may be preferable to address the triggering event that its output via senescence. Indeed, it could seem that the ideal approach to prevent ARDS and its more severe manifestations would be to administer an effective therapy for the underlying condition before the patient reaches respiratory failure. The feasibility of this rationale would strictly depend on the nature of the triggering which, in some occasions, could be relatively easy to achieve, like the administration of antibiotics to treat sepsis. However, the benefits are not so straightforward and ARDS may persist even after removal of the original insult.

Although a new era of senotherapeutic treatments is approaching, conventional drugs and natural compounds with anti-apoptotic, anti-inflammatory or anti-oxidant potential may be repurposed due to its potential to fine tune senescence regulation, emerging as efficient tools to treat acute lung injury and other pathways related to co-morbidities. Malavolta and colleagues described numerous dietary bioactive compounds, like curcumin, resveratrol, and genistein among others, which can induce senescence in cancer cells *in vitro* (Malavolta et al., 2018). This could be a promising research area, since these inducers of senescence could emerge as adjuvant treatments not only in cancer, but also in other pathologies, like ALI, to reinforce senescence at early stages to accelerate immune cells recruitment, tissue repair and regeneration. We have also recently found that drugs already available in the clinical practice, like the anti-retroviral lopinavir/ritonavir (a known ZMPSTE24 inhibitor) induced a protective

senescence-like phenotype in a model of acute lung injury (Blázquez-Prieto et al., 2020). However, inducers of senescence have to be utilised with caution, since they would need to target the cell type of interest, and also, the increase of senescence cells could represent a challenge for the immune system.

5. Concluding remarks

Probably one of the most disheartening aspects of studying cellular senescence is the diversity of mediators that could intervene, which may be closely related to the nature of the stimuli. In addition, senescence can have a very different meaning depending on the cell type, the developmental stage and the chronological age. This, in combination with the multi-factorial origin of ALI, makes it extraordinary complex to investigate its underlying mechanisms.

A few questions still remain open (Table **2**). Is it better to senesce or not? The answer is not trivial. There is evidence supporting a beneficial role of senescence cells in tissue repair, but also how senolytic agents, which induce apoptosis in senescent cells but hardly affect proliferating cells, limit injury, extend lifespan and delay the onset of age-related ailments. Many of the so-called senescence features have been thoroughly characterised in *in vitro* experiments using non-physiological stressors, but not in *in vivo* models. In addition, none of them seems to be exclusively specific of cellular senescence. How easy is to detect these senescence markers *in vivo*? A recent study showed activation of senescence in alveolar macrophages in LPS-induced ALI rather than a systemic activation, and that old mice exhibited attenuated adaptive immunity that worsened ALI prognosis compared to young mice (Brandenberger et al., 2018). How can we address the overall contribution of the aging process, or other factors such as sex or environmental cues, to senescence-mediated pathology? In the case of IPF, senescence has been reported in various cell types, including alveolar epithelial type II cells, fibroblasts and endothelial cells (Liu and Liu, 2020). Does senescence advance towards the same direction in every cell type? If the answer is not, could we induce or inhibit senescence in specific a cell type within a mixed population of cells?

Table 2. Some questions in the field of senescence thadt remain unanswered.

Is it better to senesce or no? Which factors really matter?
Would we be able to establish universal biomarkers to detect senescence *in vivo*?
How can we address the overall contribution of the aging process, or other factors such as sex or environmental cues, to senescence-mediated pathology?
Does senescence advance towards the same direction in every cell type?
Can we induce or inhibit senescence in a specific cell type within a mixed population of cells?

Definitely, further investigation at the cellular and molecular level of ALI is a road to drive along on the search for novel biomarkers that pursue early diagnosis of this life-threatening condition, which may be linked to cellular senescence.

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