Review

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The struggle of a good friend getting old: cellular senescence in viral responses and therapy

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Abstract

Cellular senescence is a state of stable cell cycle arrest associated with macromolecular alterations and secretion of pro-inflammatory cytokines and molecules. Senescence-associated phenotypes restrict damage propagation and activate immune responses, two essential processes involved in response to viral infections. However, excessive accumulation and persistence of senescent cells can become detrimental and promote pathology and dysfunctions. Various pharmacological interventions, including antiviral therapies, lead to aberrant and premature senescence. Here, we review the molecular mechanisms by which viral infections and antiviral therapy induce senescence. We highlight the importance of these processes in attenuating viral dissemination and damage propagation, but also how prematurely induced senescent cells can promote detrimental adverse effects in humans. We describe which sequelae due to viral infections and treatment can be partly due to excessive and aberrant senescence. Finally, we propose that pharmacological strategies which eliminate senescent cells or suppress their secretory phenotype could mitigate side effects and alleviate the onset of additional morbidities. These strategies can become extremely beneficial in patients recovering from viral infections or undergoing antiviral therapy.

Keywords ageing; antiretroviral therapy; cellular senescence; immunosenescence; viral infection

Subject Categories Autophagy & Cell Death; Immunology; Microbiology, Virology & Host Pathogen Interaction

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See the Glossary for abbreviations used in this article.

Introduction

The ongoing coronavirus disease 2019 (COVID-19) pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2), has highlighted how ageing and age-associated disease, such as diabetes or cardiovascular disease, are primary leading causes for the development of severe symptoms and death (Jordan *et al*, 2020). Together with this, infections might predispose to develop long-term health consequences and secondary morbidities. SARS-CoV and MERS-CoV have been associated with increase susceptibility to fibrotic disease.

Meta-analyses have reported that human cytomegalovirus virus (HMCV), Epstein–Barr virus (EBV) or influenza can increase the risk of cardiovascular disease (Warren-Gash *et al*, 2009; Wang *et al*, 2017), idiopathic pulmonary fibrosis (Sheng *et al*, 2020) and frailty (Wang *et al*, 2010). Although some infections such as human immunodeficiency virus (HIV) can now be treated with antiretroviral therapy, these individuals still suffer from an earlier onset of the same age-associated diseases. Overall lifespan is still shorter than HIV-negative individuals, suggesting that the HIV virus and/or antiretroviral medication are detrimentally modulating ageing and longevity (Smith *et al*, 2013). Identification of the mechanisms linking viral infection and antiretroviral therapy to age-associated morbidities might represent an important predictor and potential target for the consequences of long-term damages.

Cellular senescence as an antiviral response

Cellular senescence is a state of generally irreversible cell cycle arrest which cells can enter upon exposure to stress-inducing stimuli (Calcinotto *et al*, 2019). Originally, cellular senescence was defined as the mechanism regulating the finite replicative lifespan of cultured cells, also known as replicative senescence (Hayflick, 1965), and the consequence of progressive telomere attrition and eventual unwinding of the telomere cap. The exposed telomere end is recognised by DNA damage response (DDR) proteins γ-H2AX and 53BP1, which activate cell cycle inhibitors p16 and/or p21 to halt proliferation (Calcinotto *et al*, 2019). In recent years, it became evident that various events leading to genomic instability and DDR activation, such as activated oncogenes (Serrano *et al*, 1997) or direct genotoxic stress (Demaria *et al*, 2017), can prematurely induce senescence independently from telomere shortening. Besides the expression of cell cycle inhibitors and DNA damage proteins, senescent cells are characterised by increased lysosomal activity, exemplified by induction of the lysosomal enzyme senescence-associated β-galactosidase (SA-β-gal) (Dimri *et al*, 1995), and activation of a complex pro-inflammatory phenotype, called the senescence-associated secretory phenotype (SASP), which is typically mediated by NF-kB activity (Coppé *et al*, 2008; Chien *et al*, 2011).

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Glossary

Aβ	Amyloid beta
A-SAAs	Acute-phase serum amyloids
ARDS	Acute respiratory disease syndrome
AVTIS	antiviral therapy-induced senescence
ACE2	Angiotensin-converting enzyme 2
BMD	Bone mineral density
BM-MSCs	Bone marrow mesenchymal stem cells
cGAS	Cyclic GMP-AMP synthase
cGAMP	Cyclin GMP-AMP
COVID-19	Coronavirus disease 2019
DDR	DNA damage response
EBV	Epstein–Barr virus
e-NOS	Endothelial nitric oxide synthase
HAD	HIV-associated dementia
HAND	HIV-associated neurocognitive disorders
HAART	Highly active antiretroviral therapy
HBV	Hepatitis B
HMCV	Human cytomegalovirus virus
HPV	Human papillomavirus
HRSV	Human respiratory syncytial virus
HIV	Human immunodeficiency virus
HUVEC	Human umbilical vein endothelial cells
IKK	IκB kinase
IFN-β	Interferon-β
IRF3	Interferon regulatory factor 3
KSHV	Kaposi sarcoma-associated herpesvirus
MEF	Mouse embryonic fibroblasts
NAC	N-acetyl-L-cysteine
NO	Nitric oxide
NRTIs	Nucleoside reverse transcriptase inhibitors
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
ROS	Reactive oxygen species
SA-β-gal	Senescence-associated β-galactosidase
SARS-Cov-2	Severe acute respiratory syndrome coronavirus 2
SASP	Senescence-associated secretory phenotype
SCAPs	Senescent cell anti-apoptotic pathways
STING	Stimulator of interferon genes
TBK1	TANK-binding kinase 1
TERT	Telomerase reverse transcriptase
TLR2	Toll-like receptor 2
VCAM-1	Vascular cell adhesion molecule 1
VIS	Virus-induced senescence
VSV	Vesicular stomatitis virus

Innate immune signalling pathways, which are usually activated in response to invading pathogens, are also stimulated in senescent cells. Cyclic GMP-AMP synthase (cGAS) is a DNA sensor which binds cytosolic viral DNA, upon which it becomes active and synthesises the nucleotide messenger cyclin GMP-AMP (cGAMP). cGAMP translocates to the endoplasmic reticulum where it binds and activates stimulator of interferon genes (STING) (Sun *et al*, 2013; Motwani *et al*, 2019). As a result, STING moves to the Golgi apparatus and recruits TANK-binding kinase 1 (TBK1) and IκB kinase (IKK) to phosphorylate interferon regulatory factor 3 (IRF3) and the NF-kB inhibitor IκBα. Activated IRF3 localises into the nucleus and transcribes type I interferons including interferon-β (IFN-β), while phosphorylated IκBα allows NF-kB to transcribe pro-inflammatory molecules (Ishikawa *et al*, 2009; Corrales *et al*, 2017; Motwani *et al*, 2019).

Recent work has demonstrated a critical role for the cGAS-STING pathway in senescence induction and maintenance of the SASP. Host-derived cytoplasmic DNA can be observed in senescent mouse and human cells, and cGAS- or STING-depleted cells could not be induced to senescence with DNA damaging stimuli and p16, p21 and the SASP could not be upregulated (Yang *et al*, 2017; Glück *et al*, 2017; Dou *et al*, 2017). Cytoplasmic DNA in senescent cells originates from the derepression of LINE-1 retrotransposable elements mediated by upregulation in the FOXA1 transcription factor and a downregulation in RB (De Cecco *et al*, 2019). Cytoplasmic DNAses TREX1 and DNase2, which promote degradation of LINE-1 elements, are also downregulated in senescent cells and dependent on loss of E2F activity. Accumulated LINE-1 DNA is then recognised by cGAS to promote IFN-β expression (Takahashi *et al*, 2018; De Cecco *et al*, 2019). FOXA1 also promotes senescence via transcriptional activation of p16 (Li *et al*, 2013). Silencing IFN-β leads to a bypass of the growth arrest (Yu *et al*, 2015). Overall, these results suggest the cGAS-STING pathway regulates both growth arrest and inflammation in senescent cells through IFN-β activity. cGAS-STING also regulates innate immunity in senescent cells through NF-kB-mediated transcription of Toll-like receptor 2 (TLR2) and acute-phase serum amyloids (A-SAAs). A-SAAs are proteins which are recognised by TLR2 to further activate NF-kB and expression of pro-inflammatory SASP factors in a positive feedback loop. Overexpressing TLR2 induces cell cycle arrest and SA-β-gal, while silencing blunts p16, p21 and SASP expression (Hari *et al*, 2019).

Evidence of virus-induced senescence (VIS)

Although a small relatively number of studies are available, mounting evidence suggests the activation of senescence responses upon viral infections. Human respiratory syncytial virus (HRSV) was shown to induce senescence in A549 lung cancer cells and HEp-2 epithelial laryngeal carcinoma cells *in culture*, as well as mouse epithelial lung cells *in vivo* (Martínez *et al*, 2016). Similarly, measles virus and HCMV induced senescence in normal human lung fibroblasts (Noris *et al*, 2002; Chuprin *et al*, 2013), while influenza A virus subtype H7N9 induced senescence in Neuro2a mouse neuroblast cells (Yan *et al*, 2017). Infected cells upregulated various senescence markers including SA-β-gal, p16, p21 and pro-inflammatory SASP molecules IL-6 and IL-8 (Noris *et al*, 2002; Chuprin *et al*, 2013; Martínez *et al*, 2016; Yan *et al*, 2017). VIS IMR-90 fibroblasts also upregulated MICA and ULBP2 ligands (Chuprin *et al*, 2013), which were previously found to be important for modulating NK mediated cell killing of senescent cells (Sagiv *et al*, 2016). HIV proteins Tat and Nef induce the same markers of senescence (SA-β-gal, p21, IL-6 and IL-8) in human microglia and bone marrow mesenchymal stem cells (BM-MSCs) (Beaupere *et al*, 2015; Chen *et al*, 2018; Thangaraj *et al*, 2021). A recent study demonstrated that Tat protein induces senescence in microglial cells by increasing reactive oxygen species (ROS) levels through downregulation of SIRT3, a mitochondrial NAD^+-dependent deacetylase responsible for maintaining oxidative stress (Thangaraj *et al*, 2021).

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Potential mechanisms behind VIS are illustrated in Fig 1. A DDR could be detected in VIS cells and reported to be caused by an increase in mitochondrial ROS; treating infected cells with the ROS inhibitor N-acetyl-L-cysteine (NAC) was sufficient to prevent senescence induction (Beaupere *et al*, 2015; Martínez *et al*, 2016; Chen *et al*, 2018). It is possible that oxidative stress in VIS cells also leads to activation of cGAS-STING pathways as previously mentioned (FOXA1 activation, LINE-1 depression, etc.). The mechanism of ROS production in VIS cells remains poorly understood, although it has been reported that some viruses including influenza and HRSV stimulate upregulation of ROS-generating enzymes including NADPH oxidases and xanthine oxidase (Khomich *et al*, 2018).

Interestingly, senescent cells have themselves been shown to inhibit virulence. Lower titres of vesicular stomatitis virus (VSV) are obtained when infected into mouse embryonic fibroblasts (MEFs) induced to senescence with the chemotherapeutic bleomycin, compared to non-senescent MEFs. These results could be repeated with bleomycin-treated A549 and $HRAS^{V12}$-expressing MCF7 breast cancer cells (Baz-Martínez *et al*, 2016). Similar results were

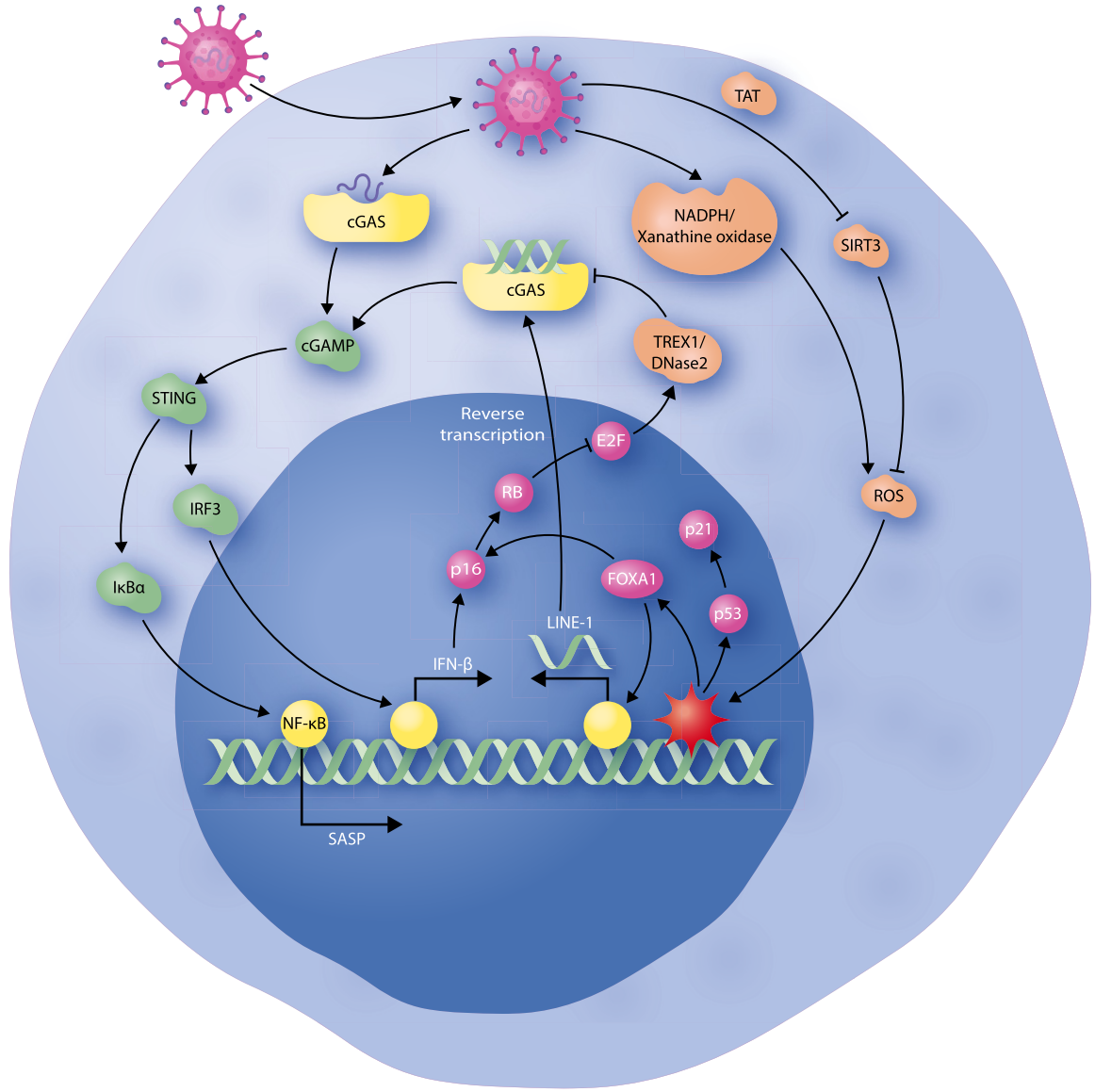

Figure 1. Potential mechanisms behind VIS in response to viral infections.

Viral entry into cells results in release of viral DNA into the host cytoplasm. Viral DNA may be recognised by cGAS, resulting in activation of the cGAS-STING immune signalling pathway and downstream activation of NF-κB and p-IRF3. NF-κB transcribes pro-inflammatory SASP genes while p-IRF3 can induce transcription of IFN-β. IFN-β can activate p16 and p21 tumour suppressors to induce growth arrest. Viruses may themselves induce activation of NADPH and xanthine oxidases, resulting in ROS accumulation. ROS can induce DNA damage and expression of FOXA1 to induce p16 transcription of LINE-1 elements. LINE-1 retrotransposons are reverse transcribed, and DNA elements are recognised by cGAS. E2F inhibition by RB in senescent cells also results in downregulation of TREX1 and DNase2, allowing DNA fragments to accumulate in the cytoplasm.

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independently observed with dengue virus and replicative senescent human umbilical vein endothelial (HUVEC) cells (Abubakar *et al*, 2014). In addition, senescence seems to control viral replication *in vivo*. When VSV was intranasally administered into mice, titres could not be obtained in lungs from mice treated intratracheally with bleomycin, a method to promote lung-specific senescence (Aoshiba *et al*, 2003). NK and dendritic cell infiltrates were also found in these animals, suggesting a role for the SASP in mediating immune-mediated viral clearance (Baz-Martínez *et al*, 2016).

Overall, these results suggest innate immune pathways enforce both growth arrest to inhibit viral replication and prevent further spread of pathogens, as well as expression of SASP molecules and NK cell ligands to promote immunosurveillance and clearance of infected cells. It is possible that senescence is an evolutionary mechanism to promote antiviral defence. This also ponders the question on whether oncoviruses are tumorigenic because they have evolved mechanisms to bypass the antiviral properties of senescent cells, in order to increase their infection efficiency.

Evidence of senescence evasion by oncoviruses

Human papillomavirus (HPV) infections most notably lead to the development of cervical cancer. E6 and E7 are two gene products expressed by the virus which are reported to interact with a range of targets to promote various aspects of tumorigenesis including inflammation, invasion and apoptotic resistance. However, E6 and E7 are also known to interfere with the p53 and pRB pathways, respectively, to destabilise senescence (Estêvão *et al*, 2019). E6 targets p53 by first binding to the E3 ubiquitin ligase UBE3A (this interaction is known as E6-AP). E6-AP subsequently ubiquitinates p53 to initiate its proteasomal-mediated degradation (Scheffner *et al*, 1990; Thomas *et al*, 1999). E7 binds to pRB, preventing the tumour suppressor from repressing E2F-mediated transcription of genes required for the G1-S transition in the cell cycle (Münger *et al*, 1989; Gonzalez *et al*, 2001). Repressing E6 or E7 in human cervical cancer cells can induce senescence through subsequent activation of p53 or p16/RB pathways (DeFilippis *et al*, 2003; Hall & Alexander, 2003).

Chronic hepatitis B (HBV) infections can lead to the development of hepatocellular carcinoma. HBx is one gene product expressed by HBV and is reported to be an important promoter of oncogenesis (Liang, 2009), possibly through HBx-mediated suppression of senescence in infected cells. Hbx has been reported to bypass senescence induced by oncogenic RAS, most likely via reducing expression of p16, p53 and p21 tumour suppressors (Oishi *et al*, 2007). Another study demonstrated that HBx bypasses senescence in HepG2 liver cancer cells by inducing methylation of the *p16* promoter, preventing binding of Ets1 and Ets2 transcription factors (Kim *et al*, 2010).

Kaposi sarcoma-associated herpesvirus (KSHV) can lead to the development of Kaposi's sarcoma. KSHV encodes LANA which inhibits senescence by binding to *p53* to inhibit its transcriptional activity (Friborg *et al*, 1999), or to pRB to allow E2F to transcribe genes required for cell cycle progression (Radkov *et al*, 2000). Two other KSHV gene products, v-cyclin and v-FLIP, have been shown to synergistically coordinate senescence bypass and tumorigenesis. v-cyclin is a homolog of mammalian cyclin D, whose expression by KSHV results in cell cycle dysregulation and senescence in fibroblasts, similar to what observed for RAS-induced senescence. However, v-FLIP can bypass v-cyclin-mediated senescence via inhibition of autophagy (Leidal *et al*, 2012). Since autophagy has been reported to be important for the generation of cytoplasmic DNA in senescent cells (Ivanov *et al*, 2013), a possibility is that v-FLIP inhibits the formation of cytoplasmic DNA fragments in KSHV-infected cells and prevents the subsequent engagement of the cGAS-STING signalling pathway.

The Epstein–Barr virus (EBV) most typically infects B lymphocytes, inducing their transformation into lymphoblastic leukaemic cells. The oncogenic potential of EBV results from its expression of LMP1, a viral protein which tethers to cell membrane of infected cells and induces the activation of proliferative and anti-apoptotic pathways (Young & Rickinson, 2004). However, LMP1 can suppress RAS-induced and replicative senescence by reducing promoter activity of *p16* (Yang *et al*, 2000) mainly by mediating the export of the Ets2 transcription from the nucleus into the cytoplasm (Ohtani *et al*, 2003). It should be noted that these findings were obtained in fibroblasts and need to be recapitulated in B cells.

Cellular senescence in response to antiviral therapy

Certain viral infections can be treated with the use of antiviral medicines. Interestingly, some of these drugs have been reported to be capable of inducing antiviral therapy-induced senescence (AVTIS) in mammalian cells *in vitro*. The majority of these studies have been carried out with antiretrovirals typically used against HIV: (i) entry inhibitors, (ii) nucleoside reverse transcriptase inhibitors, (iii) non-nucleoside reverse transcriptase inhibitors, (iv) integrase inhibitors and (v) protease inhibitors. HIV patients are typically administered drug combinations from these various categories in highly active antiretroviral therapy (HAART) in order to target multiple stages in a HIV's life cycle (De Clercq & Li, 2016). However, most of these investigations have used single agents, rather than combinations of drugs typically used in HAART. This may underestimate the possible senescence-inducing effect of these compounds in HIV patients. To our knowledge, only reverse transcriptase and protease inhibitors have been shown so far to have senescence-inducing properties (Fig 2).

Nucleoside reverse transcriptase inhibitors (NRTIs)

As HIV is a retrovirus, it must transcribe its RNA genome into DNA before being integrated into the cellular genome. HIV encodes a reverse transcriptase enzyme, transcribed from the *pol* gene, to carry out this essential step (Hu & Hughes, 2012). NRTIs competitively bind the reverse transcriptase enzyme at the catalytic site, thus inhibiting its enzymatic function (de Béthune, 2010). NRTIs stavudine and zidovudine as single agents induce various markers of senescence in human fibroblasts including SA-β-gal, p16 and p21, as well as a reduction in the number of proliferating cells. Levels of p16 and p21 are also higher in abdominal fat from HIV patients treated with HAART regiments that contained these two drugs, indicating they can induce AVTIS *in vivo* (Caron *et al*, 2008). The number of SA-β-gal-positive cells is also increased in zidovudine-treated human aortic endothelial cells (Chen *et al*, 2019). NRTIs tenofovir and emtricitabine induce AVTIS in human lung and cardiac fibroblasts as well as HUVECs when administered together, as judged by the increase in number of SA-β-gal-positive cells, p16 and p21, as well as by elevated expression of various pro-inflammatory SASP genes (Nacarelli *et al*, 2016; Cohen *et al*, 2018). Combination treatment of the NRTIs abacavir and lamivudine can

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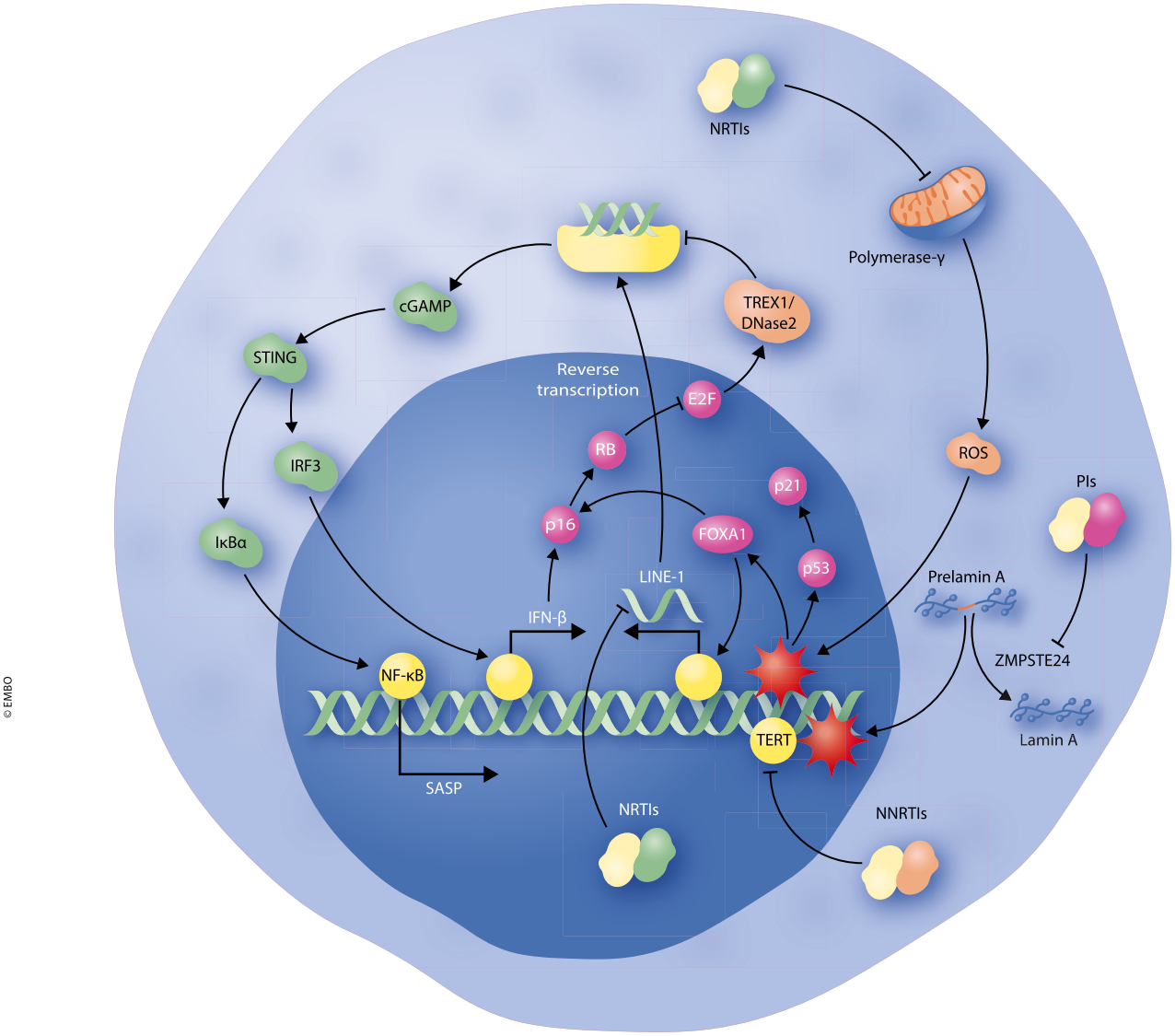

Figure 2. Cellular pathways involved in AVTIS in response to HIV antiretroviral compounds.

Protease inhibitors (PIs) inhibit the activity of ZMPSTE24, resulting in an accumulation of prelamin A. Prelamin A induces DNA damage at telomeres and induction of senescence by mechanisms described in the text. Nucleoside reverse transcriptase inhibitors (NRTIs) induce mitochondrial ROS and DNA damage through inhibition of polymerase-γ, although NRTIs may also inhibit the SASP through repression of LINE-1 elements. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) may induce senescence via inhibition of TERT, resulting in shortened telomeres and DNA damage.

induce SA-β-gal and high IL-6 secretion in cultured microglia (Cohen *et al*, 2017). Interestingly, these two drugs could not induce senescence as single agents in fibroblasts *in vitro*, and HIV patients treated only with lamivudine do not display increased p16 or p21 expression in abdominal fat (Caron *et al*, 2008).

Interestingly, the NRTIs lamivudine and stavudine has also been shown to repress the SASP as they can inhibit the activation of LINE-1 elements. Importantly, these drugs improve various aspects of mouse health lifespan where the SASP plays a detrimental role such as skeletal atrophy, bone density and muscle mass (De Cecco *et al*, 2019; Simon *et al*, 2019). Further work is clearly required to delineate the role of NRTIs in senescence and ageing. It is possible that the effect of NRTIs on senescence and the SASP is required on a range of factors including cell type, dosage or whether drugs are administered together or as lone agents.

Nevertheless, when AVTIS could be induced in cells *in vitro*, mitochondrial ROS levels were increased (Caron *et al*, 2008; Nacarelli *et al*, 2016; Cohen *et al*, 2017; Cohen *et al*, 2018; Chen *et al*, 2019), indicating NRTIs induce oxidative stress as a by-product. NRTIs-mediated ROS induction might by due to direct inhibition of mitochondrial enzymes or polymerase-γ, an enzyme responsible for replication and repair of mitochondrial DNA (Smith *et al*, 2017).

Non-nucleoside reverse transcriptase inhibitors

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) function in a similar manner to NRTIs to inhibit HIV replication. The major

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difference is that they bind the enzyme in a non-competitive manner at an allosteric site, inducing a conformational change in the enzyme that results in reduced catalytic activity (de Béthune, 2010). NNRTIs nevirapine and efavirenz have been reported to induce AVTIS *in vitro*. However, these studies have only used immortalised cancer or stem cell lines, making it difficult to assess if NNRTI-mediated senescence is solely due to inhibition of telomerase reverse transcriptase (TERT). Treatment with NNRTI induced SA-β-gal, reduced number of cycling cells, upregulation in p16 or p21, and presence of a DDR (Stefanidis *et al*, 2008; Fang & Beland, 2013; Jin *et al*, 2016; Hecht *et al*, 2018). Whether NNRTIs can induce AVTIS in primary cells is a question that should be investigated.

Protease inhibitors

Newly synthesised HIV DNA becomes integrated into the host genome by the HIV integrase enzyme, where it is then transcribed to synthesise HIV mRNA. This product is then translated by the host machinery to form an immature polyprotein. HIV protease cleaves at specific sites to yield newly matured HIV reverse transcriptase and integrase products and a subsequent restart of the viral replication cycle. Protease inhibitors interfere with this essential step (Lv *et al*, 2015). Protease inhibitors lopinavir and atazanavir independently induce SA-β-gal, p16 and p21, as well as lamin B1 loss in human BM-MSCs. Although ritonavir, another protease inhibitor currently used as a booster of other retrovirals, is not sufficient to induce AVTIS in these cells, its administration with lopinavir and atazanavir synergised to promote senescence-inducing capabilities (Hernandez-Vallejo *et al*, 2013). A lopinavir/ritonavir combination also induced these markers in human arterial endothelial cells as well as production of IL-6 and IL-8, indicating presence of a pro-inflammatory SASP (Lefèvre *et al*, 2010; Auclair *et al*, 2014; Afonso *et al*, 2017). Finally, the protease inhibitors nelfinavir and indinavir could individually induce AVTIS in human fibroblasts as shown by elevated p16 and p21 levels, SA-β-gal activity and reduced proliferative rates (Caron *et al*, 2007).

A potential mechanistic explanation of the senescence-inducing potential of protease is accumulation of prelamin A. Prelamin A is the precursor of the nuclear lamina protein lamin A and undergoes a series of post-translational modifications to produce the mature protein. One of these steps involves the conversion of farnesyl-prelamin A to lamin A by the zinc metalloprotease ZMPSTE24 (Clarke, 2007). Prelamin A accumulation is observed in AVTIS cells induced by protease inhibitors but senescence induction was less efficient when cells were also treated with protein farnesylation inhibitors pravastatin or zoledronic acid (Caron *et al*, 2007; Lefèvre *et al*, 2010; Hernandez-Vallejo *et al*, 2013; Auclair *et al*, 2014; Bonello-Palot *et al*, 2014; Afonso *et al*, 2017). Therefore, AVTIS induction by protease inhibitors is dependent upon immature processing of lamin A. Lopinavir and atazanavir inhibit the activity of ZMPSTE24 as an off target, which explains how prelamin A can accumulate (Coffinier *et al*, 2007). It is speculated this off target effect occurs because ZMPSTE24 and HIV protease share sequence and conformation in regards to their enzymatic site (Clarke, 2007). Interestingly, the protease inhibitor darunavir does not inhibit ZMPSTE24 (Coffinier *et al*, 2008), which may explains why this drug does not induce senescence in human arterial endothelial cells (Auclair *et al*, 2014).

It should be noted that *de novo lamin A* mutations are commonly associated with people suffering from Hutchinson–Gilford progeria syndrome. This mutation results in the formation of a splice site and an in-frame deletion of 50 amino acids near the C-terminus. However, this abnormal product called progerin is not processed by ZMPSTE24, resulting in the accumulation of an immature farnesylated product (Gonzalo *et al*, 2017). Progerin can induce telomere-associated DNA damage, leading to activation of a DDR and entry into senescence (Benson *et al*, 2010). It is therefore possible that, in cells treated with protease inhibitors, accumulated prelamin A can also induce senescence via this mechanism.

Viral infections, antiretroviral therapy and immunosenescence: a role for cellular senescence?

Aged individuals are more prone to suffer from infections as the consequence of a general functional decline of innate and adaptive immunity—a phenomenon termed "immunosenescence" (Aw *et al*, 2007). Immunosenescence is generally associated with decreased ratio of $CD4^+$ to $CD8^+$ and of naïve to memory T cells, reduced total number of phagocytes and of antibody-producing B cells and diminished NK cells cytotoxicity. Various intrinsic and extrinsic factors are reported to influence immunosenescence including genetics, hormones, nutrition, physical activity, but also chronic viral infections (Aiello *et al*, 2019).

HIV-infected individuals, even in children and young adults, display an immunosenescent phenotype (Chiappini *et al*, 2018). Patients display decreased ratios of $CD4^+/CD8^+$ T cells, as well decreased ratios of naïve/effector memory T or B cells (Mansoor *et al*, 2009; Díaz *et al*, 2012; Sainz *et al*, 2013; Rinaldi *et al*, 2017). Many of these studies included patients undergoing HAART therapy but studies have determined whether HAART can solely modulate immunosenescence. NRTIs and NNRTIs may inhibit TERT activity in hematopoietic stem cells, which could diminish their differentiating capacity and limit the generation of new immune cells. Therefore, it is difficult to delineate to what extent the HIV virus or antiretroviral drugs are contributing to the immunosenescent phenotype.

It is currently debated whether immunosenescent cells in the aged environment should be defined senescent *per se.* T-cell telomere length declines with age (Rufer *et al*, 1999), suggesting they have undergone replicative senescence from repetitive divisions. p16 levels also positively associate with age in this cell type (Liu *et al*, 2009). Interestingly, certain hallmarks of cellular senescence are also found in T cells from patients with HIV (Effros *et al*, 1996; Wolthers *et al*, 1996; Palmer *et al*, 1997; Nelson *et al*, 2012; Pereira Ribeiro *et al*, 2016). Telomere lengths of $CD8^+$ T cells are shorter in HIV patients compared to healthy individuals, but this observation was not found in $CD4^+$ T cells. These finds were also independent on whether HIV patients were on antiretroviral therapy (Effros *et al*, 1996; Wolthers *et al*, 1996; Palmer *et al*, 1997). However, p16 levels are increased in $CD4^+$, but not $CD8^+$, T cells in HIV patients untreated with HAART, compared to uninfected individuals (Nelson *et al*, 2012; Pereira Ribeiro *et al*, 2016). As HIV is only capable of infecting $CD4^+$ T cells (Wilen *et al*, 2012), it is possible that the virus directly induces VIS in these cells. $CD8^+$ T cells can still proliferate resulting in an overall reduction in the $CD4^+/CD8^+$ ratio.

HAART-treated HIV patients displayed p16 levels comparable to uninfected controls in T cells (Nelson *et al*, 2012; Pereira Ribeiro *et al*, 2016). However, $CD4^+/CD8^+$ ratios are still low in patients

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receiving HAART (Serrano-Villar *et al*, 2014). Although HAART may limit viral replication in $CD4^+$ T lymphocytes and limit ageing of these cells, they may promote immunosenescence through other aspects, e.g. by exhausting the hematopoietic stem cell pool, as mentioned previously (section 2). Since senescent cells are capable of inducing paracrine senescence via the SASP (Acosta *et al*, 2013), it is possible that AVTIS induction in somatic cells *in vivo* promote senescence-like phenotypes in immune cells.

$p16^+$ T cells are likely to have a reduced immune capacity as specific deletion of *p16* in these cells enhances immune function in aged mice (Liu *et al*, 2011). Therefore, one mechanism by which viruses may induce the premature onset of various age-related diseases is by compromising the immune-mediated clearance of senescent cells. Mice engineered with an impaired capacity to eliminate senescent cells display increased natural accumulation of senescent cells, premature signs of ageing and shortened lifespans compared to wild-type animals (Ovadya *et al*, 2018).

Viral infections, antiretroviral therapy and age-associated diseases

The natural accumulation of senescent cells in mammals during ageing contributes to the development of various age-associated diseases (Baker *et al*, 2016; Calcinotto *et al*, 2019; Borghesan *et al*, 2020). Although immunosenescence may play a role in the development of these diseases, as previously mentioned, this section will discuss mechanisms behind how VIS and AVTIS could be linked to premature ageing in HIV patients (Fig 3).

Bone disease

Decreased bone mineral density (BMD), otherwise known as osteopenia, is associated with increased age in humans. Low BMD results from impaired bone mineralisation and decreased bone strength. This results in a greater chance of suffering a bone fracture, otherwise known as osteoporosis. Bone structure is mediated by a tightly controlled balance between bone synthesis by osteoblasts and bone resorption by osteoclasts. However, this balance shifts towards bone resorption in osteoporotic patients (Tu *et al*, 2018). In aged mice, the SASP promotes decreased BMD from inhibition of osteoblasts-mediated bone mineralisation and stimulation of osteoclast differentiation from progenitor cells (Farr *et al*, 2017).

HIV patients are consistently reported to display reduced BMD and increased risk of developing osteoporosis, although there is some divergence as to whether this phenomenon occurs due to the HIV retrovirus or protease inhibitor therapy (Tebas *et al*, 2000; Bruera *et al*, 2003; Amiel *et al*, 2004; Brown & Qaqish, 2006). These conflicts are possibly reconciled by reports that find both HIV proteins Tat and Nef and protease inhibitors lopinavir and atazanavir are capable of inducing senescence in BM-MSCs. Importantly, senescent BM-MSCs do not be effectively differentiated into osteoblasts and deposit reduced levels of calcium *in vitro* (Hernandez-Vallejo *et al*, 2013; Beaupere *et al*, 2015). Overall, these results suggest that VIS and AVTIS of BM-MSC in HIV patients compromise their osteoblastic potential, leading to reduced BMD and increased risk of osteoporosis.

Cardiovascular disease

Atherosclerosis is an age-associated vascular disease characterised by the formation of plaques in arterial vessels, which can lead to the development of various cardiovascular disorders including myocardial infarction, stroke and sudden cardiac death (Tabas *et al*, 2015). Endothelial dysfunction plays a critical role in disease development. Although a healthy endothelium maintains vascular homeostasis through regulated secretion of various dilators such as

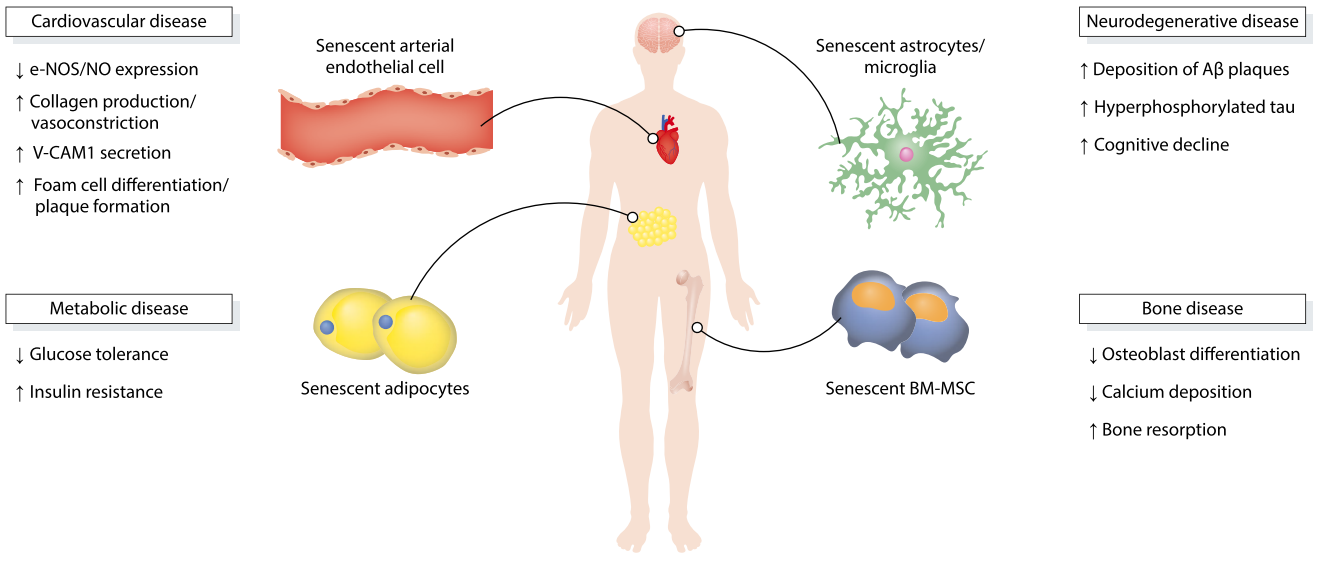

Figure 3. Implications of cellular senescence in age-related diseases commonly associated with HIV patients undergoing HAART therapy.

Potential mechanisms behind VIS and AVTIS in age-associated diseases in HIV patients. See text for further details.

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nitric oxide (NO), endothelial cells in an atherosclerotic lesion produce less NO, resulting in vascular smooth muscle cell proliferation, collagen production and vasoconstriction. Importantly, a fraction of dysfunctional endothelial cells in atherosclerotic plaques is senescent (Minamino *et al*, 2002) and secretes vascular cell adhesion molecule 1 (VCAM-1) to recruit and differentiate monocytes into foam cell macrophages (Davignon & Ganz, 2004; Childs *et al*, 2018). These foamy macrophages also display senescence-associated features and secrete VCAM-1 to further promote monocyte differentiation and plaque formation. Matrix metalloproteinase secretion from these cells also trigger plaque degradation (Childs *et al*, 2016).

HIV patients have been found to have a higher risk of cardiovascular events, which was even higher in patients treated with protease inhibitors (Friis-Moller *et al*, 2007; Islam *et al*, 2012; Shah *et al*, 2018). Protease inhibitors are reported to induce senescence in human arterial endothelial cells *in vitro*. Importantly, these senescent cells displayed reduced expression of endothelial nitric oxide synthase (e-NOS), an enzyme responsible for NO synthesis, and increased secretion of VCAM-1 (Lefèvre *et al*, 2010; Auclair *et al*, 2014; Afonso *et al*, 2017). Therefore, it is possible that protease inhibitor administration in HIV patients induces atherosclerosis through induction of vascular endothelial cell senescence, which leads to dysregulated endothelial health and foam cell formation. The HIV virus may also independently contribute to cardiovascular risk as HIV proteins gp120 and Tat can induce senescence in aortic endothelial cells (Hijmans *et al*, 2018).

Metabolic disease

Cohort studies have found that the incidence of type II diabetes is increased in individuals with HIV compared to the general population, but exposure to antiretroviral therapy is reported to drive this association, rather than HIV infection *per se*. Results were also independent on age or whether patients were obese, suggesting traditional risk factors are not involved (Tripathi *et al*, 2014; Samad *et al*, 2017; Hernandez-Romieu *et al*, 2017). Senescence is reported to detrimentally influence diabetes by inducing adipose tissue dysfunction. In mice, excessive caloric intake results in an accumulation of reactive oxygen species in adipose tissue and increased expression of various senescence and inflammatory markers such as *p21*, *TNF* and *CCL2* (Minamino *et al*, 2009). Senescent cells in adipose tissue contribute to metabolic dysfunction as they induce impairments in glucose homeostasis and insulin sensitivity (Palmer *et al*, 2019), although precise mechanisms are currently unknown. To our knowledge, no evidence yet exists on whether antiretroviral compounds can induce senescence in adipocytes *in vitro*, but it has been demonstrated that protease inhibitors and NRTIs can induce p16 and p21 expression in human adipose tissue (Caron *et al*, 2007, 2008). When mice fed on a high-fat diet were also administered with the NNRTI efavirenz and NRTI emtricitabine, there was a decrease in glucose tolerance and increase in insulin resistance (Pepin *et al*, 2018). Insulin sensitivity was also decreased in healthy individuals treated with the NRTI stavudine (Fleischman *et al*, 2007). Overall, these results suggest that AVTIS induction in adipose tissue induces metabolic dysfunction and onset of diabetes in HIV patients, but the exact role of senescence in promoting metabolic disease in this context remains to be further investigated.

Neurodegenerative disease

The term HIV-associated neurocognitive disorders (HAND) refers to a collection of neurological conditions which can occur in HIV patients. Although this condition is normally mild, some patients can develop a highly severe form of HAND known as HIV-associated dementia (HAD), associated with extreme motor and cognitive symptoms typical of Alzheimer patients (Kaul, 2009). Moreover, brains from post-mortem HIV patients display increased levels of deposited amyloid beta (Aβ) and hyperphosphorylated tau compared to age-matched controls, suggesting pathological links exist between Alzheimer's disease and HAD (Green *et al*, 2005; Anthony *et al*, 2006; Achim *et al*, 2009). Evidence accumulated in recent years has shown direct correlation exists between senescence in various brain cells and Alzheimer' disease. Increased p16 protein levels can be observed in astrocytes from Alzheimer patients compared to age-matched controls (Bhat *et al*, 2012), while tau-containing neurofibrillary tangles in neurons of Alzheimer patients also display increased expression of *p16* mRNA, along with upregulation of an inflammatory transcriptome (Musi *et al*, 2018). Senescent astrocytes and microglia accumulate in a tau-dependent mouse model of AD where they contribute to cognitive decline in these mice (Bussian *et al*, 2018; Zhang *et al*, 2019). The HIV virus has been reported to be capable of inducing senescence in human astrocytes and microglia *in vitro*, as well as rat astrocytes *in vivo* (Yu *et al*, 2017; Chen *et al*, 2018). This process is likely to be mediated by a Tat-dependent downregulation of SIRT3, as the enzyme is found to be downregulated in the prefrontal cortex of HIV patients, along with an upregulation of p16 and p21 (Thangaraj *et al*, 2021). Therefore, HIV-mediated senescence of these cells could lead to cognitive decline in HIV patients.

Conclusions and future questions

Potential roles for cellular senescence in COVID-19 pathology

The biological links between viral infections and ageing are still poorly characterised (see also Box 1). However, cellular senescence could play a key role, either because of the engagement of antiviral defence pathways or because different types of somatic cells can be reengaged to enter senescence upon exposure to viral infection. Most experimental evidence is based on the correlation between HIV VIS and the premature onset of various age-associated pathologies, but similar phenomena might well happen for other viruses, including SARS-Cov-2. This could be an important factor in how

Box 1. In need of answers

i Does senescence play a direct role in why HIV patients have shorter lifespans than people without HIV? To what extent does VIS or AVTIS play a role in each age-associated disease?
ii Can immunosenescent cells be classified as "senescent?" Can these cells be targeted by senolytic compounds and improve immune function?
iii Can senolytics or SASP modulators be used as novel therapies for people suffering from both acute and chronic viral infections.
iv What is the role of senescence in COVID-19 pathology and is there a link to why older patients have a higher chance of mortality?

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COVID-19 manifests, especially in the lungs, and why older people suffer from higher mortality (illustrated in Fig 4). In young individuals, SARS-Cov-2 entry into lung alveolar cells may induce senescence and a SASP, in order to limit viral infection and trigger immune-mediated clearance of infected cells. The SASP may additionally induce paracrine senescence in order to limit viral infection in neighbouring lung cells. However, inflammation may already be high in lungs of older individuals owing to an age-associated increase of senescent cells. Moreover, immune-mediated clearance of infected cells could also be compromised because of age-associated immunosenescence. SARS-Cov-2 infection induces senescence in lung cells but increased presence of inflammatory factors and failure to clear infected cells could lead to a hyperinflammatory environment (also referred to as a cytokine storm) and the onset of acute respiratory disease syndrome (ARDS) (preprint: Evangelou *et al*, 2021). Approximately 70% of COVID-19 deaths are reported to occur from ARDS with age being one of the most significant risk factors (Hojyo *et al*, 2020; Jordan *et al*, 2020).

Individuals who recover from SARS-Cov-2 display other damaging symptoms in various organs where senescence could play a detrimental role. For example, around 30% of SARS-Cov-2 patients in intensive care units display thrombotic complications (Klok *et al*, 2020), and senescence is reported to promote clotting via the secretion of certain SASP molecules (Wiley *et al*, 2019). Neurological and cardiovascular complications are also common observations in COVID-19 patients (Ellul *et al*, 2020; Zheng *et al*, 2020). Since the receptor for SARS-Cov-2, angiotensin-converting enzyme 2 (ACE2) is expressed in the brain and heart (Hamming *et al*, 2004), it is possible that viral-mediated entry of these cells induces VIS and localised inflammation leading to tissue dysfunction.

Targeting cellular senescence and the SASP to alleviate viral pathologies

There is currently much interest for the discovery and characterisation of novel compounds which can either specifically induce cell death in senescent cells (senolytics) or inhibit the SASP (SASP modulators). This does pend the question on whether these drugs could also be therapeutic approach against the detrimental pro-ageing sequelae of VIS or AVTIS in patients who suffer from HIV or other infections. Senolytics typically target senescent cell anti-apoptotic pathways (SCAPs), survival pathways utilised by senescent cells to maintain their viability (Soto-Gamez *et al*, 2019). ABT-263 is one senolytic and functions to inhibit the BCL-2 family of anti-apoptotic proteins, which are typically upregulated in senescent cells (Zhu *et al*, 2016; Chang *et al*, 2016). Interestingly, ABT-263 has been shown to sensitise retinal pigment epithelium cells and

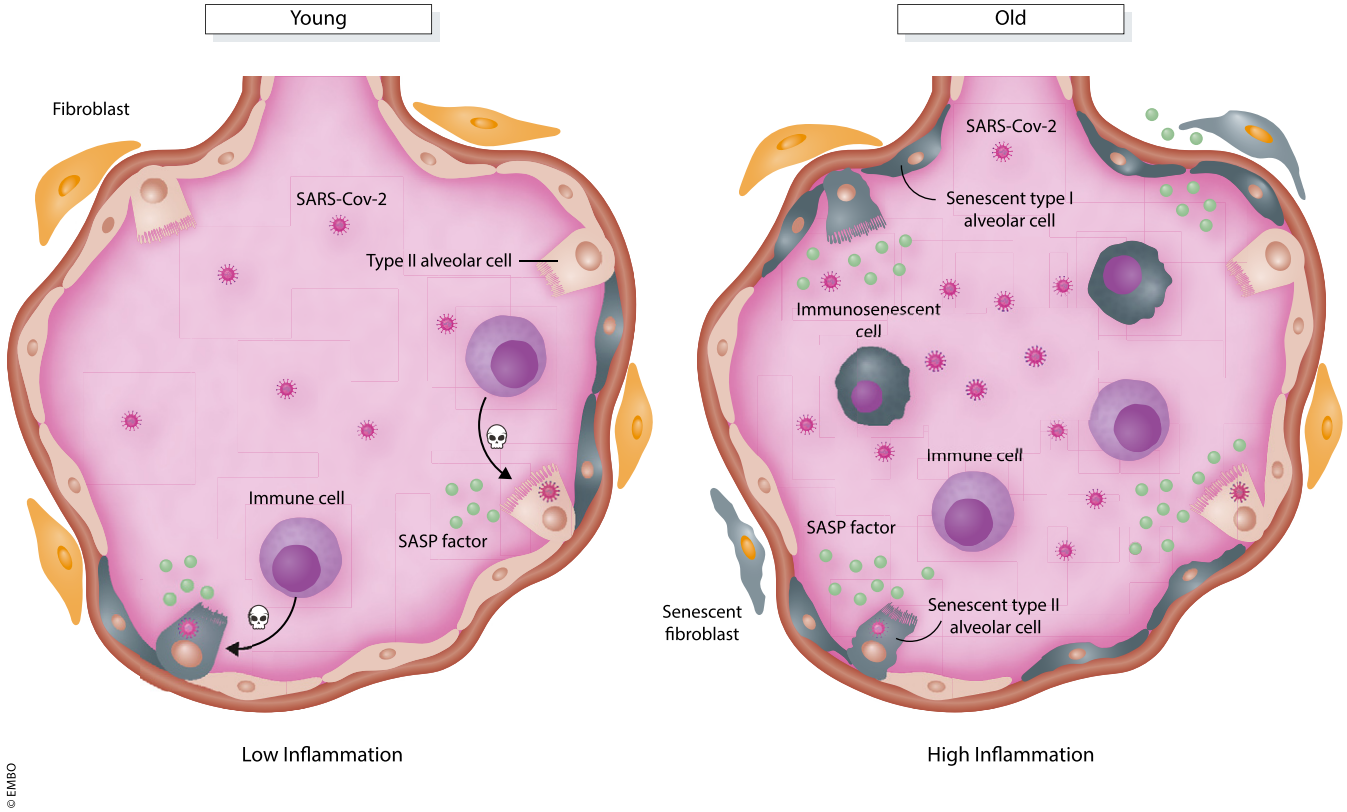

Figure 4. Potential roles for senescence in COVID-19 pathology in old individuals.

In young individuals, SARS-Cov-2 infection of lung alveolar cells may promote VIS and secretion of SASP molecules to promote paracrine senescence in neighbouring cells and immune-mediated clearance of infected cells. However, immune-mediated clearance is compromised due to immunosenescent cells in old individuals. SARS-Cov-2 infected cells, along with the presence of age-associated senescent lung cells, secrete hyper levels of SASP factors leading to a cytokine storm and immunopathology.

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macrophages infected with a variety of virus types (influenza, herpes simplex, measles) to undergo cell death (Kakkola *et al*, 2013). However, this study did not analyse whether infected cells were senescent. The AKT pathway is another SCAP used by senescent cells to maintain survival. HSP90 inhibitors such as geldanamycin, or natural flavonoids such as quercetin, have been demonstrated to exert senolytic properties by interfering with this pathway (Zhu *et al*, 2015; Fuhrmann-Stroissnigg *et al*, 2017; Kirkland *et al*, 2017). To our knowledge, there is no evidence on whether these senolytics can sensitise viral-infected cells to die and studies are warranted to answer this.

SASP modulators are actively being trialled to reduce mortality in hospitalised COVID-19 patients, owing to their potential in repressing the detrimental cytokine storm (Nehme *et al*, 2020). Some compounds are shown to be beneficial in improving mortality in severely ill patients, but more investigations are required to determine whether this occurs from any possible effect in repressing the SASP from senescent SARS-Cov-2 senescent cells in the lung. Glucocorticoids are a group of steroid hormones which have anti-inflammatory properties by repressing IL-6 secretion via inhibition of NF-κB activity (Laberge *et al*, 2012). Dexamethasone is an approved glucocorticoid (Dexamethasone in Hospitalized Patients with Covid-19—Preliminary Report, 2020) and has been demonstrated to blunt the SASP, albeit in models of senescent cancer cells (Ge *et al*, 2018; Buhl *et al*, 2019). Two inhibitors of the IL-6 receptor, tocilizumab and sarilumab, have also recently been approved for use against the disease after promising clinical results in severely ill patients (Wise, 2020).

Future studies are warranted to determine whether senolytics or SASP modulators can induce death or repress pro-inflammatory signalling in VIS and ATVIS cells respectively (see also Box 1). As well as benefiting patients suffering from acute viral infections like SARS-Cov-2, they may also be beneficial in alleviating onset of age-associated symptoms in patients with chronic viral infections, such as HIV patients on antiretroviral therapy. Moreover, HIV patients treated with protease inhibitors may also benefit from treatment with pravastatin or zoledronic acid, as they may prevent senescence induced by accumulation of prelamin A. Phase 2 trials have reported that zoledronic acid prevents bone loss in HAART-treated HIV patients (Ofotokun *et al*, 2016).

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Conflict of interest

M.D. is advisor and shareholder of Cleara Biotech.

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