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REVIEW ARTICLE

Cellular senescence as a source of SARS-CoV-2 quasispecies

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In-depth analysis of SARS-CoV-2 biology and pathogenesis is rapidly unraveling the mechanisms through which the virus induces all aspects of COVID-19 pathology. Emergence of hundreds of variants and several important variants of concern has focused research on the mechanistic elucidation of virus mutagenesis. RNA viruses evolve quickly either through the error-prone polymerase or the RNA-editing machinery of the cell. In this review, we are discussing the links between cellular senescence, a natural aging process that has been recently linked to SARS-CoV-2 infection, and virus mutagenesis through the RNA-editing enzymes APOBEC. The action of APOBEC, enhanced by cellular senescence, is hypothesized to assist the emergence of novel variants, called quasispecies, within a cell or organism. These variants when introduced to the community may lead to the generation of a variant of concern, depending on fitness and transmissibility of the new genome. Such a mechanism of virus evolution may highlight the importance of inhibitors of cellular senescence during SARS-CoV-2 clinical treatment.

Cellular senescence

Cellular senescence is a fundamental process through which a cell adopts a prolonged, generally irreversible, cell-cycle arrest accompanied by secretory activities termed senescence-associated secretory phenotype (SASP), upon damage or stress [1]. Senescent cells continually occur and are removed by immune cells, mainly macrophages and NK cells attracted by SASP, over the organism's lifespan. Transient incidence of

Abbreviations
ACE2, angiotensin-converting enzyme 2; ADAR, adenosine deaminases acting on RNA; APOBEC, apolipoprotein B mRNA-editing catalytic polypeptide-like; cGAS, cyclic GMP-AMP synthase; COVID-19, coronavirus disease 2019; CUL2, cullin-2; DAMP, danger-associated molecular pattern; E, envelope; HIF-1α, hypoxia-inducible factor 1a; HUVEC, human umbilical vein endothelial cell; IFN, interferon; M, membrane; N, nucleocapsid; NSP, nonstructural protein; ORF, open reading frame; RBD, receptor-binding domain; RdRp, RNA-dependent RNA polymerase; RNP, ribonucleoprotein; ROS, reactive oxygen species; S, spike; SASP, senescence-associated secretory phenotype; SCAP, senescent cell anti-apoptotic pathway; ssRNA, single-stranded RNA; STING, stimulator of interferon genes; UTR, untranslated region.

this dynamic cellular state plays beneficial roles in various physiological processes ensuring cellular and organismal homeostasis. During embryogenesis, senescence facilitates tissue development and morphogenesis while in the adult life apart from promoting tissue repair, it restrains the expansion of damaged cells. On the contrary, senescent cells can exert detrimental antagonistic effects through paracrine and/or systematic SASP, if not timely removed. Indeed, persistent senescence promotes tissue/organ dysfunction by reducing their regenerative potential and by promoting disruptive chronic inflammation and fibrosis, eventually leading the development of age-related or degenerative pathologies and aging [1].

The senescent phenotype is highly diverse, and mechanisms involved are not requisitely preserved among the senescence programs [1]. However, for conventional reasons, cellular senescence has been divided into two main categories. The term replicative senescence was initially proposed by *Leonard Hayflick and colleagues* in order to describe permanent arrest of human cells following a number of cell divisions in culture. Nowadays, it is known that replicative senescence is related to shortened telomere length and telomere dysfunction [1,2]. The second category represents stress-induced senescence, a wide group of senescence phenotypes which are triggered by a variety of insults regardless of telomeres [1,2]. Depending on the hallmarks of the stressogenic insult and the cell/tissue type, senescent cells can exhibit a wide spectrum of morphological, structural, and functional features [1,2]. Therefore, a multimarker approach for the precise determination of the senescence phenotype in biological and clinical specimens has been adopted [1,3]. Despite this heterogeneity, apart from cell-cycle withdrawal and secretory properties already mentioned, macromolecular damage, altered metabolism, and increased survival are among the characteristics constantly evident in senescent cells [1].

Increased viability of senescent cells stems from the fact that they are more resistant to apoptosis in relation to normal proliferating (nonsenescent) cells [4]. Their lifetime can extent up to several weeks compared to the few days of their proliferating counterparts [5]. Apoptotic tolerance has been related to a plethora of insults (UV, ionizing radiation, oxidative stress, drugs, serum starvation) and a variety of cell types [fibroblasts, endothelial cells/human umbilical vein endothelial cell (HUVECs), and keratinocytes], denoting the role of cellular senescence as an adaptive response [4,6,7]. Accumulating evidence from proteomic and transcriptomic investigations supports that the extended lifespan of senescent cells depends on the activation of various, multilevel regulated, pro-survival pathways cumulatively described as senescent cell anti-apoptotic pathways (SCAPs) [4,6–8]. The pathways include networks related to p53/p21/serpins, BCL-2/Bcl-XL, PI3K/AKT/ceramide signaling, the hypoxia-inducible factor 1a (HIF-1α) pathway, or HSP90-dependent cascades, which are closely interconnected [4,6]. SCAPS are directly related to many features of the senescent phenotype and seem to protect senescent cells from their own pro-inflammatory secretome [7]. Given that they have emerged as essential targets for the development of senotherapeutic—particularly senolytic—strategies they are described as the Achilles' heel of senescent cells [7]. Indeed, inhibition of pro-survival pathways and a decrease in the expression of SCAP mediators can promote elimination of senescent and virus-infected cells, at least in some cell types [8]. In some instances, more than one SCAP pathways must be targeted to eliminate senescent cells, suggesting redundancy of cell defenses against apoptosis [9].

SARS-CoV-2: structure—life cycle—variants

In December 2019, many cases of a novel type of pneumonia were reported in Wuhan, Hubei Province, in China. This phenomenon rapidly evolved as a global pandemic which was named coronavirus disease 2019 (COVID-19) and was attributed to a new beta-coronavirus termed SAR-CoV-2 [10]. SAR-CoV-2 belongs to the family of *Coronaviridae* that are known to cause respiratory and neurological diseases.

SAR-CoV is an enveloped, nonsegmented, positive sense RNA virus. SARS-CoV-2 genome exhibits high homology, up to 80%, with SARS-CoV and MERS-CoV, at phylogenetic level [11]. Its genome is single-stranded and RNA-positive, with a size between 26.4 and 31.7 kb and a diameter of about 65–125 nm. The genome is composed of two untranslated regions (UTRs) at the 5′ and 3′ ends and a variable number (6–11) of open reading frames (ORFs) which encode 27 different proteins responsible for the replication and infection of the virus [12].

During the infection of the cells, the genomic RNA is translated from two ORFs, ORF1a and ORF1b, which encode nonstructural proteins (nsps). The subgenomic region comprising these ORFs is highly conserved between Coronavirinae subfamily members and consists of pp1a and pp1b genes encoding the nsps polyprotein 1a and b, respectively. Both polyprotein 1a and b are cleaved producing 11 and 16 nsps, respectively, by a reaction which is catalyzed by viral proteases nsp3 and nsp5 [12,13]. Nsp12 is a key producing molecule which

acts as an RNA-dependent RNA polymerase (RdRp) indicating that the viral RNA is used as template for virus replication. Downstream of this conserved region the remaining subgenomic region encodes genes for structural viral proteins. Using cell's own machinery, SARS-CoV-2 synthesizes its positive sense genomic and subgenomic RNA, the latter encoding conserved structural proteins (spike protein [S], envelope protein [E], membrane protein [M], and nucleocapsid protein [N]), and 6 several accessory proteins [14].

Spike protein (S) consists of two subunits the N-terminal half (S1) and C-terminal half (S2). S1 domain also contains the receptor-binding domain (RBD) of the virus. Each domain is responsible for a different process. Angiotensin-converting enzyme 2 (ACE2) is the recognized receptor from virus and RBD is the key domain of S protein for the binding of virions to the receptor of host cells [15]. S2 subunit has a key role in the process of fusion between the virus and the cell membrane acting as a class I viral fusion protein [16]. Also, stabilization of fusion machinery complex is supported by the RBD, making it a crucial molecule for the infection and a potential therapeutic target.

The envelope protein is an oligomeric protein located at the membrane of SARS-CoV-2 acting as viroporin creating a pentameric protein–lipid ion transport channel [17]. Membrane protein (M) is a glycoprotein and the most abundantly expressed from the virus in host cells. It mediates the viral assembly and maturation via protein interaction with the structural viral proteins shaping the virions [18].

Nucleocapsid (N) protein is a dimeric protein which interacts directly with the RNA of virus and provides the genomic stability necessary for the RNA transcription and replication. The main role of N protein is the packaging of RNA into nucleocapsid structure or ribonucleoprotein (RNP) complex [19] promoting the viral release [20].

The emergence of SARS-CoV-2 variants has numerous implications in transmissibility, pathogenicity, immunity, and re-infection. Since December 2019, multiple variants and variants of concern have emerged worldwide, often prompting local or broad changes in public health management. To date, 5 major variants of concern have been identified thought worldwide SARS-Cov-2 complete genome sequencing. Variants B.1.1.7, B.1.1.7+E484K, B.1.351, P.1, and B.1.617.2 have significantly affected public health management due to the impact of their mutations on transmissibility immunity and disease severity [21–30]. In some cases, mutations such as N501Y [31] are recurring signifying the existence of hotspots within the genome that contribute to virus adaptation to humans.

These variants arise through the evolution of SARS-CoV-2 RNA genome mainly through two mechanisms: a virus polymerase-mediated and a cell-mediated. RdRp of RNA viruses such as nsp12 of SARS-CoV-2 (together with accessory subunits, nsp8 and nsp7) [32] have a high error rate, introducing random mutations. RdRp-mediated coronaviruses, in contrast to other RNA viruses, code for a proofreading exonuclease (nsp14) [33] that may correct such nucleotide misincorporations, probably as a requirement for the integrity of its large RNA genome, the largest among RNA viruses [34]. This proofreading mechanism minimizes the impact of polymerase-mediated genetic drift of coronavirus genomes. Moreover, the ubiquitin ligase complex could also play a role. Particularly, the interplay of ORF10 with the members of a cullin-2 (CUL2) RING E3 ligase complex may inactivate APOBEC (apolipoprotein B mRNA-editing catalytic polypeptide-like) by inducing ubiquitination and proteasomal degradation, similarly to the mechanism shown in HIV [35]. On the other hand, RNA-editing machinery may significantly contribute to viral genome evolution postgenome replication. Adenosine deaminases acting on RNA (ADAR) and APOBEC enzyme have been mainly implicated in SARS-Cov-2 evolution [36]. ADAR enzymes introduce adenosine-to-inosine changes while APOBEC enzymes introduce cytosine-to-uracil changes.

Both mechanisms introduce mutations across the genome, which are further subjected to natural selection in terms of viability and fitness. Most of the amino acid changes in virus genomes are lethal or significantly reduce virus fitness by disturbing important structures and interactions [37]. Mutations that constitute now variants of concern used to be preexisting variant genome quasispecies that rose in a single patient (patient 0 of each variant). However, as stated previously it is possible that a certain mutation may occur more than once independently. Increased fitness of mutant quasispecies may lead to domination within a patient, while increased transmissibility may lead to community domination [38].

SARS-CoV-2 RNA editing—APOBEC enzymes

Expression of RNA-editing enzymes is induced during infection, usually downstream of innate immunity pathways [39,40]. It has been hypothesized that these enzymes have evolved from ancient intracellular innate immune mediators against viruses [40]. APOBEC enzymes, among other RNA-editing enzymes, act as suppressors of either RNA virus replication through

the introduction of debilitating hypermutations within the genome [41], or act as silencers of endogenous retrovirus expression and function [42]. APOBEC enzymes are thought to have co-evolved with viruses increasing in complexity after the waves of retrovirus colonization of the eukaryotic genomes [42]. Despite the fact that APOBEC enzymes have been mainly studied in conjunction with retrovirus pathogenesis and integration, a significant part of the literature has focused on APOBEC effect on the replication of positive and negative strand RNA viruses without DNA intermediate. APOBEC3G inhibited efficiently the replication of measles, mumps, and respiratory syncytial viruses [43]. The presence of APOBEC3G affected several steps in viral RNA synthesis, resulting in impaired viral transcription and increased genome mutation frequency [43]. On the other hand, APOBEC3G did not affect influenza virus replication although it is greatly upregulated during influenza virus infection [44]. An APOBEC3G inducing antiviral drug (IMB-Z, a N-phenylbenzamide derivative) led to inhibition of enterovirus 71 through interaction with viral 3D RdRp and viral RNA and incorporation into progeny virions [45]. Intriguingly, this report claimed that APOBEC3G antiviral activity was not associated with its cytidine deaminase activity.

Early during SARS-CoV-2 pandemic, the accumulation of complete genomes worldwide highlighted a possible relationship between C to U transitions and APOBEC activity [36,46,47]. C to U transitions were observed to preferentially occur downstream of uridines and adenosines in -1 and -2 positions for the transition point resembling to APOBEC1 consensus target sequence [48]. Contrary, a study that screened viral genomes failed to identify APOBEC3 footprints in MERS-CoV, SARS-CoV, and SARS-CoV-2 coronavirus genomes, although endemic coronaviruses were significantly footprinted [49]. The activity of APOBEC could be exerted in both positive and negative strands during RNA virus replication, leaving, however, a different footprint in each hypothesized model. A model of APOBEC enzyme intervention 'early' during viral replication on the SARS-CoV-2-positive strand could better fit to the observed high rate of C to U transitions in contrast to G to A transitions that correlate with negative strand processing [42]. Accumulation of random hypermutations within SARS-CoV-2 nascent genomic RNAs may undermine viability or replication efficiency of a significant proportion of these genomes. While in theory, SARS-CoV-2 hypermutation would result in virus evolutionary counteraction, previous work on SARS-CoV revealed that the virus may actively package APOBEC3G inside virus particles, similarly to HIV particles [50], through direct interaction with SARS-CoV N protein [51]. Packaging of APOBEC in HIV and SARS-CoV may result in in-virion APOBEC activity or an 'early' action upon viral RNA release into the cytoplasm during infection. Vif protein of HIV is a well-known inhibitor of APOBEC3G that inhibits its interaction with N protein and targets it for degradation through the proteasome [52]. N protein is the structural protein of coronaviruses that coats viral RNA within the virion, while in parallel protects viral RNA from host posttranscriptional control machinery, such as RNA [53]. A different opinion was put forward during the assessment of coronavirus hypermutation rate after overexpression of different APOBEC enzymes. While APOBEC3C, 3F, and 3H were able to inhibit virus proliferation, screening for APOBEC-related footprints did not reveal any effect, with the authors speculating that an unknown virus or virus-induced factor may counteract APOBEC activity [41]. They further speculated that the N protein interaction with APOBEC is a possible mechanism.

Changes in APOBEC expression may directly affect variant emergence. Thus, monitoring of mRNA abundance in conjunction with disease progression and severity may provide insights into virus evolution either within a host or the community. Analysis of differentially expressed mRNAs in the blood of COVID-19 patients revealed that APOBEC3G mRNA together with other type I interferon signaling-related mRNAs was upregulated in patients with mild and severe COVID-19 [53]. Intriguingly, APOBEC3G was less upregulated in severe disease group in the report [54]. Possibly, changes in blood cell populations during severe COVID-19 [55] may account for the observed reduction of APOBEG3G as it is predominantly expressed in lymphocytes [56] within blood cell populations. Stimulating cells with single-stranded RNAs (ssRNAs) that represented SARS-CoV-2 genome stretches before and post-APOBEC action provided evidence of APOBEC-mediated transitions involvement in COVID-19 pathology. In this *in vitro* study, stimulation of cells with RNAs with higher uracil content led to higher TNF-α and IL-6 expression [57]. Such an increase in pro-inflammatory status may have implications in COVID-19 cytokine storm initiation.

Cellular senescence and SARS-CoV-2 quasispecies generation

The knowledge linking cellular senescence with viral infection is limited and stems mainly from indirect observations [3]. Viral infection has been associated

with DNA damage and cell fusion, both well-known inducers of senescence [58,59]. On a non-cell-autonomous basis, viral infection elicits antiviral responses mediated by the release of pro-inflammatory factors (interferon I and III, interferon-γ, and IL-6) and the activation of the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) signaling pathway [60–62]. These processes allow infected cells to induce senescence, through a bystander effect, in other noninfected cells within the adjacent environment [63,64]. Interestingly, a sufficient number of cytokines/chemokines implicated in the highly lethal 'cytokine storm' related to COVID-19 can trigger senescence on a long-term basis in a paracrine manner [63–65]. Viral-mediated interferons (IFNs) may also promote senescence via inflammatory-mediated cell death mechanisms (e.g., necroptosis, pyroptosis), which results in release of molecules termed danger-associated molecular patterns (DAMPs) [66]. The latter can trigger interferon signaling cascades, which in turn can induce senescence [64,67–70]. Regarding the relationship of SARS-CoV-2 infection with senescence, only hypotheses without proof have been speculated.

Recent evidence from our group in the lower respiratory system of COVID-19 patients suggests that cellular senescence is directly induced by SARS-CoV-2, providing the first worldwide demonstration of viral-induced senescence [71]. By applying the same established senescence detecting methodology (GL13-SenTraGor™), these findings were confirmed in experimental models and in the upper respiratory track of clinical settings [72]. It was demonstrated that the virus highjacks several host mechanisms of the senescent cell, including the mitochondrion. Specifically, dysfunctional mitochondria release reactive oxygen species (ROS) that shorten telomere length, inducing cellular senescence [72]. Based on these findings, senolytic drugs, including Quercetin, have been proposed for the treatment of COVID-19. SARS-CoV-2-mediated senescence is accompanied by increased expression of SASP factors as well as elevated APOBEC enzyme expression [71]. The latter is in line with our recent findings supporting abundance of the APOBEC enzymes, especially APOBEC G and H, in cells undergoing stress-induced senescence [73,74]. Based on these data and the fact that infected senescent cells exhibit prolonged survival, we hypothesized that cellular senescence could contribute to the emergence of APOBEC footprints in the viral genome (Fig. 1). In this cellular context, the virus can be hosted for longer periods compared to other cells with higher proliferation rates, rendering its genome prone to host-mediated editing

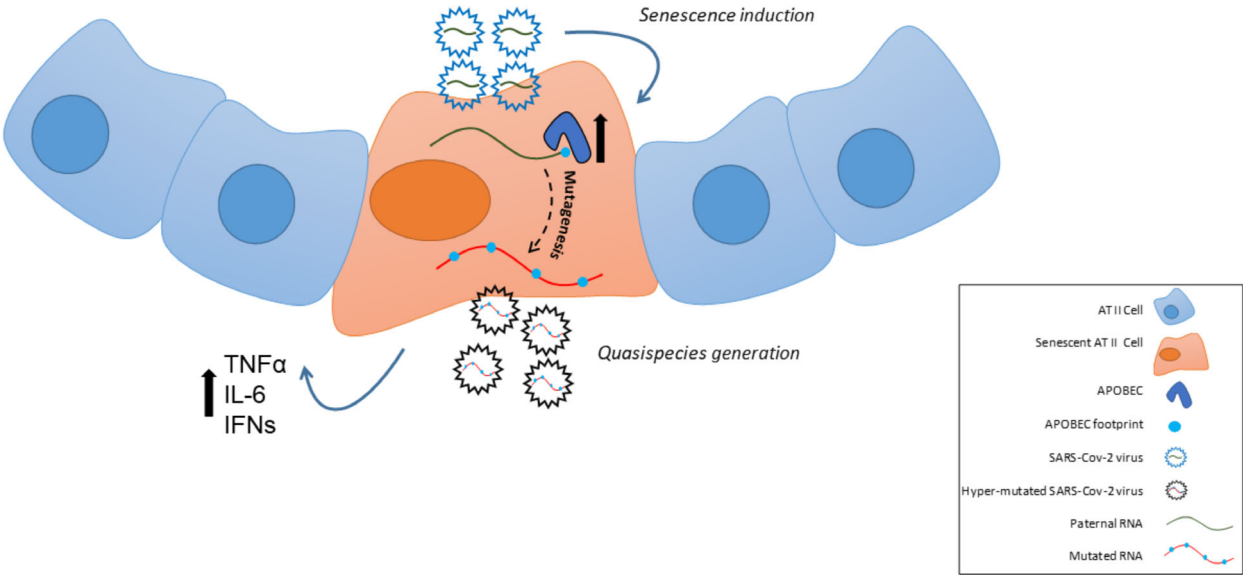

Fig. 1. Proposed model for viral-induced senescence as a 'fertile' environment for SARS-CoV-2 quasispecies generation. In brief, SARS-CoV-2 triggers senescence in infected alveolar type II (ATII) cells that is accompanied by an inflammatory phenotype. Given that senescent cells are resistant to apoptosis, the virus can be harbored for prolonged periods, making thus its genome more vulnerable to host-mediated editing. High levels of the APOBEC enzymes, that are responsible for virome editing, occur in senescent cells. Eventually, APOBEC-mediated phenomena include the following: (a) nucleotide substitutions in the progeny derived from the 'paternal' strain take place and (b) further increase of released pro-inflammatory factors.

(Fig. 1). Further supporting our case, by analyzing a large cohort of SARS-CoV-2 strains available in the GISAID database, we showed that APOBEC signatures potently determine the mutational profile of the SARS-CoV-2 genome [71]. Indeed, by comparing the viral sequence at the onset and following prolonged infection in an *in vitro* setting, an APOBEC-driven mutational signature in the virome of the progeny was identified [71].

Conclusion

In the current review, we discuss the hypothesis linking viral-induced cellular senescence and SARS-Cov-2 virus mutagenesis. We suggest that application of senolytic drugs that eliminate senescent cells should be taken into consideration in the context of COVID-19 disease. Firstly, senolytic drugs might prevent the potential adverse effects of SASP, improving the clinical outcome of patients, as supported by recent findings in experimental models [75]. Secondly, they could confer in neutralizing a fertile cellular environment for the emergence of SARS-CoV-2 quasispecies (Fig. 1) that has direct implication in vaccine effectiveness. In this context, a new perspective is provided in the field of virology, exploiting the potential of senolysis and hence providing an additional therapeutic approach.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

KE and VGG contributed to conceptualization; KE, IK, and NL contributed to methodology; IK, NL, and KE involved in writing—original draft preparation; KE and VGG involved in writing—review and editing; KE prepared the figure; KE, IK, and NL contributed to literature search; KE and VGG supervised the study; VGG acquired the funding. All authors have read and agreed to the published version of the manuscript.

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