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Hallmarks and detection techniques of cellular senescence and cellular ageing in immune cells

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Abstract

The ageing of the global population brings about unprecedented challenges. Chronic age-related diseases in an increasing number of people represent an enormous burden for health and social care. The immune system deteriorates during ageing and contributes to many of these age-associated diseases due to its pivotal role in pathogen clearance, tissue homeostasis and maintenance. Moreover, in order to develop treatments for COVID-19, we urgently need to acquire more knowledge about the aged immune system, as older adults are disproportionally and more severely affected. Changes with age lead to impaired responses to infections, malignancies and vaccination, and are accompanied by chronic, low-degree inflammation, which together is termed immunosenescence. However, the molecular and cellular mechanisms that underlie immunosenescence, termed immune cell senescence, are mostly unknown. Cellular senescence, characterised by an irreversible cell cycle arrest, is thought to be the cause of tissue and organismal ageing. Thus, better understanding of cellular senescence in immune populations at single-cell level may provide us with insight into how immune cell senescence develops over the life time of an individual. In this review, we will briefly introduce the phenotypic characterisation of aged innate and adaptive immune cells, which also contributes to overall immunosenescence, including subsets and function. Next, we will focus on the different hallmarks of cellular senescence and cellular ageing, and the detection techniques most suitable for immune cells. Applying these techniques will deepen our understanding of immune cell senescence and to discover potential druggable pathways, which can be modulated to reverse immune ageing.

KEYWORDS
ageing markers, immunosenescence, methods

1 | INTRODUCTION

Organismal ageing is caused by the progressive deterioration of most cells, tissues or organs of the body. The immune system is not an exception. Its dysregulation and deterioration, so-called 'immunosenescence', predispose older adults to a diminished response to infections with novel pathogens, to autoimmunity, as well as to chronic non-immune disorders including cardiovascular and neurodegenerative diseases, cancers and type-2 diabetes (Fulop et al., 2016; Simon et al., 2015). Immunosenescence is a combination

of immune system-wide changes over the life time of an individual, which are much better studied (examples can be found in the next paragraph on T and B cells), and cellular changes in individual immune cells, which we will term immune cell senescence. To be able to establish to which degree immune cell senescence contributes to immunosenescence, it will be important to first establish which immune cells undergo cellular senescence with age, as it is much less studied. This review will focus on techniques to detect the cellular changes leading to immune cell senescence.

A recent landmark study by Alpert et al. (2019) which has used 'multi-omics' technologies to uncover the cell subsets linked with age, provides longitudinal metrics of immune age. In addition, several recent reviews on the topic of immune system-wide changes give extensive overviews of surface markers and cell populations that emerge during ageing (Alpert et al., 2019; Drew et al., 2018; Frasca, 2018; Judge et al., 2020; Xu & Larbi, 2017; Yarbro et al., 2020). Thus, here we will only briefly summarise some of the most important immune system-wide changes and focus on the available strategies that can be used to detect them.

T and B lymphocytes are major players of the adaptive immune system (Lanier, 2013). Their diverse antigen-recognition repertoire enables naïve T and B cells to respond specifically to a variety of foreign antigens. Their antigen-experienced memory population provides long-lasting protection by responding to previously encountered antigens in a more rapid and robust manner. However, during ageing, the production of naïve lymphocytes declines, shrinking the repertoire of antigen-specific cells able to generate new memory cells (Salam et al., 2013). This compromises the ability of an aged immune system to fight against new pathogens and establish memory responses to these *de novo* antigens. Meanwhile, antigen-experienced cells accumulate and undergo oligoclonal expansion in the aged individuals, reflecting lymphopenia-driven homeostatic proliferation, the adaptive response from a reduced naïve pool and the effect of past and persistent infections. Some subsets of lymphocytes, characterised by reduced antigen-receptor signalling and innate-like phenotypes, are significantly increased in frequency during ageing. Due to their altered function and low proliferation rates, many of these highly inflammatory cells were the first to be termed senescent, including effector memory T cells re-expressing CD45RA (T_{EMRA}), and late/exhausted memory B cells (LM B cells) (Callender et al., 2018; Colonna-Romano et al., 2009; Di Mitri et al., 2011; Frasca et al., 2017; Lanna et al., 2014). However, whether they are truly 'senescent' is still putative, since it has been reported that they, or their subpopulation, are able to proliferate under specific conditions (Di Mitri, 2011; Hao et al., 2011; Verma et al., 2017). Age-associated changes also correlate with the expression of certain surface molecules, which can be detected using antibody staining via flow cytometry. One example is the increased expression of CD57 in T cells (and natural killer cells, which belong to the innate immune system) during ageing, which has been linked with senescent-like phenotypes (Alpert et al., 2019; Brenchley et al., 2003; Lopez-Verges et al., 2010). Downregulation of CD27 and CD28, and upregulation of KLRG1 are also linked with functionally

deficient T cells (Henson & Akbar, 2009; Plunkett et al., 2007). In Table 1, we summarise these T and B cells that accumulate with age and their surface markers.

The deleterious effect of ageing on the adaptive immune system has been extensively investigated (Pawelec et al., 1999). However, there is accumulating evidence suggesting that innate immune systems are also affected, contributing to the emergence of age-related phenotypes in the elderly (Ray & Yung, 2018). Monocytes/macrophages, dendritic cells and natural killer cells have all been reported to undergo changes in number and phenotype during ageing (Almeida-Oliveira et al., 2011; Bhushan et al., 2002; Hearps et al., 2012; Nyugen et al., 2010; Seidler et al., 2010). The age-related myeloid bias during haematopoiesis can explain the increased numbers of macrophages and dendritic cells to a certain extent. Some explanations for the myeloid bias have been put forward, including: (1) a cell-intrinsic issue with the aged hematopoietic stem cell (Pang et al., 2011), (2) the aged microenvironment (Ergen et al., 2012), and (3) increased number of senescent cells that leads to an increase in macrophages and NK cells for their removal (Hall et al., 2016; Sagiv et al., 2016).

Many of their functions are also affected, and these are more likely to be due to immune cell senescence. For example, monocytes and neutrophils from aged humans (or mice) display decreased phagocytosis and ROS production, which impairs their antimicrobial capacity (Butcher et a., 2001; Hearps et al., 2012; McLachlan et al., 1995; Wenisch et al., 2000). The antigen-presenting ability of dendritic cells relies on their ability to phagocytose, which declines with age and may further affect the establishment of adaptive immunity (Agrawal et al., 2007). Another feature of an aged innate immune cell is the dysregulated cytokine/chemokine secretion, mainly studied in macrophages (and recently in T cells), which contributes to fuel the pro-inflammatory environment observed during ageing, termed 'inflamm-aging' (Desdin-Mico et al., 2020; Franceschi et al., 2017; Hearps et al., 2012; Qian et al., 2012).

Inflamm-aging refers to chronic, low-grade inflammation and is possibly caused by prolonged infection, dysregulated metabolism and accumulation of senescent cells (Franceschi et al., 2000, 2018). Some inflammatory molecules in blood, including pro-inflammatory cytokines, chemokines and acute-phase proteins, are widely used as readouts to assess inflamm-aging (Franceschi et al., 2017). From an evolutionary perspective, inflammation plays an important role in the short-term clearance of pathogens in early life (Franceschi et al., 2000). However, the chronic inflammation with age results in accelerated systemic ageing, leading to negative physiological outcomes that are not limited to immunity, which reinforces the pleiotropy theory of ageing.

Although many of the mentioned alterations have been associated with immune ageing, it remains largely unknown whether they are signs of maladaptation, or the outcome of an adaptation to compensate for a dysregulated immune system. To address this, a detailed profiling of cellular and molecular changes over the lifetime of an individual is essential. Do all changes happen to each cell at once, or just to some and in subsequent steps and does that correlate with

TABLE 1 Subtypes of T and B cells display senescent-like phenotypes and accumulate during ageing

	Senescent-like population	Surface marker	Tissue	Species (Human/ Murine)	Percentage of total T or B cells		Characteristics related to cellular senescence
					Young	Old	
T cell	Senescent-like CD8$^+$ T cells (Henson, 2009)	CD8$^+$ CD27$^-$ CD28$^-$	PBMC	Human	0.5%	50%	↓telomere length (Plunkett, 2007); ↓telomerase activity; ↓proliferation capacity
	Terminally differentiated CD8$^+$ T cell (T$_{EMRA}$) (Le Page et al., 2018)	CD8$^+$ CD45RA$^+$ CCR7$^-$	PBMC	Human	3%	14%	↓proliferation capacity (Callender et al., 2018) ↑pro-inflammatory cytokine
	CD57$^+$ KLRG1$^+$ CD8$^+$ T cells (Onyema, 2012)	CD8$^+$ CD57$^+$ KLRG1$^+$	PBMC	Human	7.9%	32.2%	↓proliferation capacity (Brenchley et al., 2003)
	Virtual memory CD8$^+$ T cells (Tvm)	CD45RA$^+$ PanKIR$^+$ and/ or NKG2A$^+$ (Quinn, 2018)	PBMC	Human	4%	12%	↓proliferative capacity; ↑γH2AX
		CD44^{hi} CD49d^{lo} (Quinn, 2018)	Spleen	Murine	10%	30%	
B cell	Age-associated B cells	CD21/35$^-$ CD23$^-$ CD43$^-$ CD93$^-$ (Hao et al., 2011)	Spleen	Murine, female	3%	12%	↓proliferation capacity ↑pro-inflammatory cytokine
	Age-associated B cell-like cells	CD11c$^+$ CD11b$^+$ CD21$^-$ (Rubtsov, 2011)	Spleen	Murine, female	0.25%	6.64%	NA

function? The good news is that the emerging field of geroscience may be of considerable help. Geroscience assumes that mechanisms underlying ageing and age-related diseases are largely overlapping and shared across cell types (Franceschi et al., 2018). Nowadays, several interconnected 'pillars' promoting ageing have been identified, including mitochondrial health, DNA damage, telomere shortening, chromosome reorganisation, cell cycle arrest, excessive cytokine release, proteostasis and lysosomal health (Kennedy et al., 2014). Applying the wide range of methods that have been developed and tested in multiple tissues and organisms to the immune system will provide important insights into the molecular basis of immunosenescence.

The current COVID-19 pandemic affects mostly the elderly and as such is closely linked to immunosenescence. A case report profiled the kinetics of immune responses in response to SARS-CoV-2 infection. Both innate and adaptive immunological changes were detected before the full resolution of symptoms (Thevarajan et al., 2020). The remodelling of the immune systems during ageing may explain why older adults are more at risk to develop severe COVID-19. Two hallmarks of ageing, which are, shorter telomere length and aberrant mitochondria function, have been hypothesised to indicate a worse prognosis in COVID-19 (Aviv et al., 2020; Kloc et al., 2020). Systematically screening the hallmarks of both immune cell senescence and immune system-wide changes may unravel their relationship with the progression of the disease. Vaccines against SARS-CoV-2 are commonly considered as the best way of protecting the elderly and putting the pandemic to an end (Lurie et al., 2020; Zhang et al., 2020). However, the successful vaccination in senior people is a big challenge, since vaccines are much less immunogenic and effective in this cohort (Weinberger

et al., 2018). Therefore, understanding the molecular mechanisms that underpin immune cell senescence may help to boost the effectiveness of SARS-CoV-2 vaccines and protect the vulnerable older adults. Furthermore, the predisposition of aged people to inflamm-aging may lead to immune pathology. In severe cases, patients suffer from excessive pro-inflammatory cytokines, the so-called 'cytokine storm', possibly promoted by inflamm-aging (Meftahi et al., 2020). Indeed, dexamethasone, an anti-inflammatory glucocorticoid, reduces the 28-day mortality rate in a randomised clinical trial (Group et al., 2020). We believe that understanding the cellular changes that occur with age in immune cells, which may lead to aberrant responses, including inflamm-aging, can help to identify drug targets for COVID-19 treatment (Channappanavar & Perlman, 2020).

In this review, we will compile the most widely used and accepted age-related cellular changes, and highlight the most recent techniques that can be applied to different immune cell types in a high-throughput manner (overviewed in Figure 1 and summarised in Table 2). These techniques, particularly the use of flow cytometry and omics at single-cell resolution, will help to correlate immune cell senescence with immune dysfunction. Furthermore, application of these techniques will potentially help to unveil druggable targets, and help to design drugs to rejuvenate aged immune cells and prevent age-related diseases. Because certain age-associated cell changes are not necessarily characteristics of senescence, and rather are hallmarks of cellular ageing, the next chapter will be divided into hallmarks of cellular senescence and hallmarks of cellular ageing (Gorgoulis et al., 2019; Lopez-Otin et al., 2013). The final part of this review aims to give our readers a perspective of the future directions of ageing research.
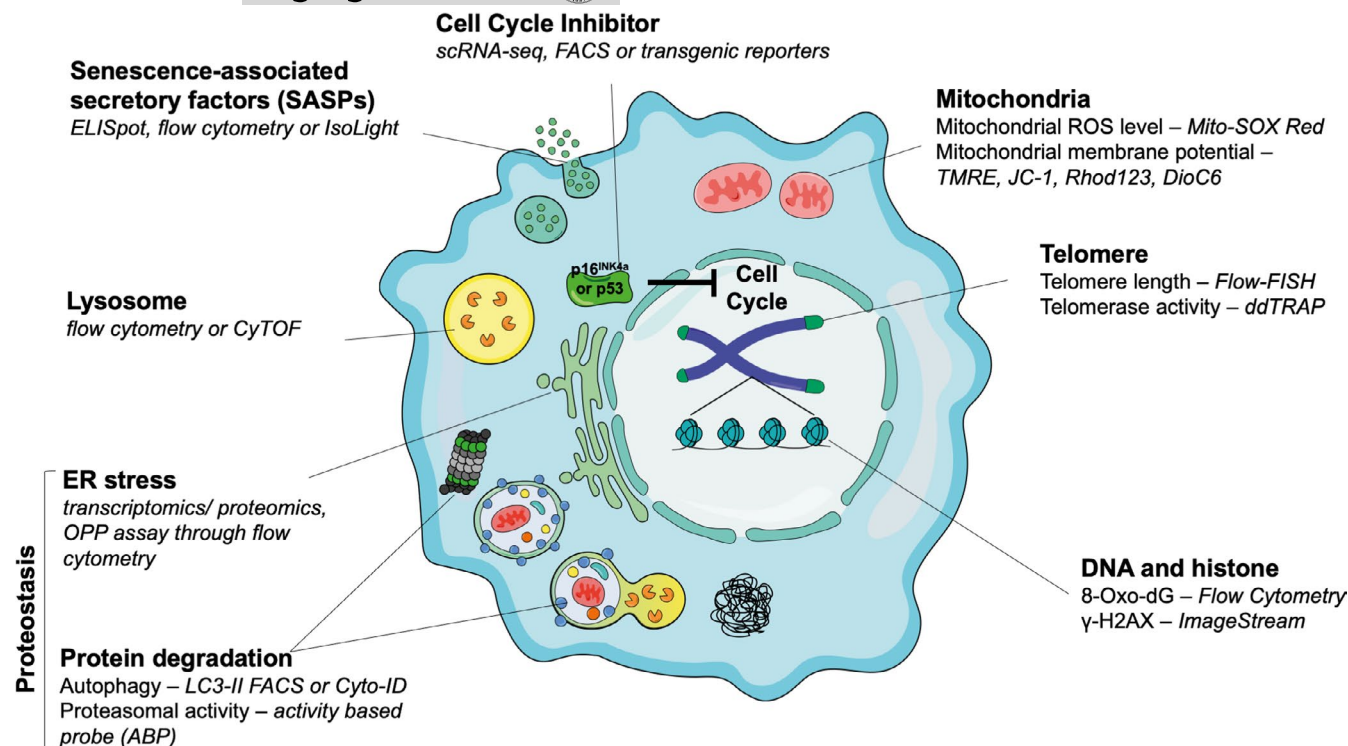
FIGURE 1 Senescence biomarkers and detection techniques. Senescent cells display excessive oxidative stress in mitochondria, which further induces DNA and protein damage and leads to disrupted proteostasis and cell cycle arrest. The lysosomal accumulation of lipofuscin and SA-β-Gal serves as common hallmarks for the identification of senescent cells. The dramatic change in the pro-inflammatory secretome of senescent cells, termed senescent-associated secretory phenotype, might contribute to inflamm-aging, the chronic, low-degree inflammation during ageing

2 | HALLMARKS OF CELLULAR SENESCENCE

2.1 | Cell Cycle

Common inducers of senescence include telomere erosion, DNA damage, oxidative stress, oncogene activation and chronic mitogen signalling. The downstream signalling cascades induced by these stressors ultimately converge to the p53/p21^{CIP} and/or the p16^{INK4a}/pRB pathways, which directly act on cyclin-dependent kinases to inhibit the cell cycle (Bruce et al., 2000). Both pathways contribute to the initial growth arrest during cellular senescence (Prieur et al., 2011). However, studies show that the type of stress signal determines which pathway takes the leading role. For instance, DNA damage upregulates p21^{CIP} levels in a p53-dependent manner (Mlynarczyk & Fåhraeus, 2014), while p38-MAPK-mediated increase in mitochondrial ROS levels stimulates p16^{INK4a} expression (Luo et al., 2011).

2.1.1 | p16^{Ink4a}

Quantitative polymerase chain reaction (qPCR) has long been the benchmark technique for measuring mRNA levels. Some studies show an increase of p16^{INK4a} mRNA in human peripheral T cells (Liu et al., 2009), as well as mouse B cells during ageing (Liu et al., 2011). Due to

the heterogeneity of senescence within the same cell population, it is necessary not only to measure the average change of p16^{INK4a} mRNA level over many cells, but at single-cell resolution. At present, single-cell RNA sequencing (scRNA-seq) allows us to identify p16^{INK4a}-expressing cells in a high-throughput manner, while also revealing other differentially expressed genes (Enge et al., 2017). Applying the technique to immune cells may help to identify whether they are senescent. Transgenic p16^{INK4a}-reporter mice also shed some light on the issue, and several mouse lines generated with different reporter-constructs are available (Demaria et al., 2014; Liu et al., 2019). A recent article demonstrates that less than 1% of T or B cells (among their total respective peripheral populations) are p16^{INK4a} positive in aged mice. This is a much lower frequency than the one found in other cell populations, such as cartilage and fat progenitor cells, among which around 6% of the cells express p16^{INK4a}. Thus, in comparison with other tissues, it remains to be elucidated whether p16^{INK4a} is a reliable biomarker for senescence in immune cells by looking at other cellular changes and correlating them with immune function (Liu et al., 2019). One explanation is that the p16^{INK4a} promoter activation does not reflect mRNA abundance. The senescent lymphocytes accumulate high levels of the p16^{INK4a} transcript with marked stability, but due to technical difficulties p16^{INK4a} protein levels are not easy to detect with this reporter line (Liu et al., 2019).

To measure p16^{INK4a} protein expression, flow cytometry or its multiparameter derivative CyTOF (time-of-flight mass cytometry),

TABLE 2 The detection techniques of senescent biomarkers and their applications in immune cells

Category	Phenotype during ageing	Detection techniques	Phenotype in aged immune cells	References
Mitochondrial damage and oxidative stress	cellular ROS ↑	H_2-DCFDA or CellROX staining for flow cytometry	Increase in bone marrow-derived and tissue-resident macrophages	Sebastian et al. (2009) Vida et al. (2017)
	mitochondrial ROS ↑	Mito-SOX Red staining for flow cytometry	Increase in naïve and central memory $CD8^+$ T and antibody-secreting cells	Sanderson and Simon (2017); Kurupati et al. (2019)
	mitochondrial membrane potential ↓	TMRE, JC-1, Rhodamine 123 or DioC6 staining for flow cytometry	No change in $CD8^+$ T nor antibody-secreting cells	Sanderson and Simon (2017); Kurupati et al. (2019)
p16^{INK4a} (similar to p53)	↑	Single-cell RNA sequence	Increase in human peripheral T cells and mouse B cells	Liu et al. (2011) Liu et al. (2009)
		Antibody staining for flow cytometry or transgenic reporter mouse line	Minor increase in p16^{INK4a+} T and B cells	Liu et al. (2019)
Senescence-associated secretory phenotype	↑	qPCR single-cell RNA sequence	Increase in mouse senescent-associated T cells, human T_{EMRA} cells, late/exhausted memory B cells, mouse bone marrow myeloid cells and neutrophils	Fukushima et al. (2018) Callender et al. (2018) Frasca et al. (2017) Farr et al. (2016) Uhl et al. (2016)
		ELISpot	IL–6, TNF-α, MMPs	
		Luminex IsoLight	NA	NA
Senescence-associated beta-galactosidase	↑	C_{12}FDG or its analogue for flow cytometry or CyTOF	Increase in memory-phenotype (CD44^{high}) T cells	Liu et al. (2019)
Lipofuscin	↑	biotin-conjugated GL13 staining for flow cytometry	Increase in peritoneal macrophages, microglia, and T cells	Vida et al. (2017) Kushwaha et al. (2018)
DNA damage	8-Oxoguanine↑	flow cytometry	NA	Sampson et al. (2006)
	γH2AX↑	ImageStream	Increase in virtual memory T cells, human HSCs and progenitor cells	Quinn et al. (2018) Durdik et al. (2015) Phadwal et al. (2012)
Telomere	average telomere length↓	Flow-FISH	Shortened in human peripheral T cells	Sanderson and Simon (2017) Fali et al. (2019)
	telomerase activity↓	droplet digital TRAP (ddTRAP)	Declines in resting T and B cells	Lin et al. (2015)
Ubiquitin-proteasome system	proteasome activity↓	activity-based probe (ABP)	Decrease in lymphocytes and T cells	Carrard et al. (2003) Ponnappan et al. (1999) Arata et al. (2019)

TABLE 2 (Continued)

Category	Phenotype during ageing	Detection techniques	Phenotype in aged immune cells	References
Autophagy	macroautophagy flux↓	LC3-II flow cytometry	Decrease in macrophages, T and B cells	Puleston et al. (2014), Stranks et al. (2015), Bharath et al. (2020), Zhang et al (2019)
	Chaperon-mediated autophagy↓	KFERQ-tagged protein reporters; Co-stain with HSC70 and LAMP2A	Decrease in T cells	Valdor et al. (2014)
ER stress	BiP ↓ UPR ↑ Translation ↓	Western Blot or qPCR for BiP; Flow cytometry or in vivo imaging for protein translation	Decrease of BiP expression and protein translation in several murine tissues	Naidoo et al. (2008), Zhang et al. (2019), Signer et al. (2014), Liu, Xu et al (2012)

which both rely highly on the specificity of the antibodies, are alternatives (Cheung & Utz, 2011). CyTOF currently can detect more than 50 features in a single cell simultaneously (Olsen et al., 2019). The high-dimensional data generated enable more detailed characterisation of p16[INK4a+] immune populations and identification of surface markers and ageing biomarkers correlating it with p16 expression. One hurdle is that p16[INK4a] antibodies have not yet been validated for either method. However, other methods independent of highly specific antibodies, such as single-cell mass spectrometry-based proteomics, are emerging, which will help to answer whether and how p16[INK4a] expression contributes to immune cell senescence (Dou et al., 2019).

2.1.2 | p53-p21[CIP] signalling pathway

p53 is a tumour suppressor gene, which is frequently mutated in human cancer (Yue et al., 2017). Upon DNA damage responses, p53 protein undergoes post-translational modification and induces cell cycle arrest and/or apoptosis through its transcription factor activity. Thus, it is crucial to the maintenance of genome stability. Similar to p16[INK4a], p53 also participates in cellular senescence. One of its downstream genes, *p21[CIP]*, is a cyclin-dependent kinase inhibitor, which halts the progress of the cell cycle through interacting with multiple types of cyclin-dependent kinases (He et al., 2005). The activation of p53-p21[CIP] pathway has been widely observed in senescent cells, though only at the early stage, which suggest to its role in induction of cellular senescence (Stein et al., 1999). The p53 pathway has complex roles in ageing, depending on the persistence of the stimuli, which activate p53 expression (Liu & Sharpless, 2009). Transient stimuli-triggered p53 activity contributes to genome stability through temporarily pausing cell cycle for DNA repair. However, continuous p53 activation caused by prolonged stress leads to senescence or cell death (Liu & Sharpless, 2009). Aged splenocytes and thymocytes display elevated p53 levels, suggesting the accumulation of DNA damage (Kapasi & Singhal, 1999). Different p53 isoforms can have a different impact on ageing. The age-associated human peripheral CD8^{+} T cells (CD28^{-} CD57^{+}) show decreased Δ133p53 and increased p53β levels (Mondal et al., 2013). Reconstituting Δ133p53 expression recovers the proliferation of these age-associated T cells and rescues their senescent phenotypes, suggesting a potential therapeutic strategy for treating immune cell senescence.

The methods to detect p16[INK4a] and p53 are similar. Note that p53 activity is determined by its various post-translational modifications (PTM) and isoforms (Liu et al., 2019). A systematic analysis with PTM- and isoform-specific antibodies will fully uncover p53's role in immune ageing.

2.2 | Telomere attrition

Telomeres are special structures at the end of linear chromosomes, which, in mammalian cells, consist of typical telomeric DNA repeats (TTAGGG) and associated proteins (Shay & Wright,

2019). The protein complex prevents telomeres from being recognised as damaged DNA and represses the DNA damage response. During DNA replication, the replicate machinery cannot fully copy the telomeric DNA, leading to the shortening of telomere each time, termed 'end-replication problem' (Shay & Wright, 2019). Once the telomeric DNA shortens below a threshold, it may cause DNA breaks and replicative senescence (d'Adda di Fagagna, 2003). Thus, telomere shortening, which is a cause of telomere dysfunction, can be used as a readout to detect cellular senescence (Bernadotte et al., 2016).

In the immune system, the average telomere length decreases both in lymphocytes and granulocytes with ageing, with lymphocytes experiencing more pronounced telomere shortening (Aubert et al., 2012). The telomere length also varies between different subtypes of human $CD8^+$ T lymphocytes. Naïve populations usually display longer telomeres than the highly differentiated ones, leading to higher proliferative potential (Sanderson & Simon, 2017). Thus, both telomere attrition and population shift from naïve to memory and effector cells lead to the overall shortening of telomeres in T cells (Fali et al., 2019). Flow-FISH (fluorescence in situ hybridisation) has been widely used to measure average telomere length in hematopoietic lineages (Baerlocher et al., 2006). It combines flow cytometry with the hybridisation of telomeric PNA probes to cells in suspension. Telomere length by Flow-FISH correlates with mitochondrial stress in T cells (Sanderson & Simon, 2017) and lymphocyte function, predicting immune responses to vaccination in the elderly (Najarro et al., 2015).

Telomerase is a reverse transcriptase that maintains telomere length (Pfeiffer & Lingner, 2013). Due to their high telomerase activity, immortal cell lines, germline cells, stem cells or cells at early stage of fetal development may escape replicative senescence caused by telomere dysfunction (Liu et al., 2007; Montalto et al., 1999). While in resting lymphocytes, telomerase activity is usually low, mitogen stimulation can transiently upregulate telomerase activity (Hiyama et al., 1995). One study showed that with age telomerase activity declines in resting T and B cells (Lin et al., 2015). To measure telomerase enzyme activity at single-cell level, traditional telomere repeat amplification protocol (TRAP) was adapted to a digital format, called 'droplet digital TRAP' (ddTRAP) (Ludlow et al., 2014). This method allows the quantification of telomerase-extended products in a single cell with high accuracy and reproducibility. Several studies managed to monitor telomerase activity in single T cells with ddTRAP (Huang et al., 2017; Tedone et al., 2019).

2.3 | Senescence-associated secretory phenotype

The senescence-associated secretory phenotype is another typical feature of senescent cells. Senescent cells secrete a spectrum of soluble factors including pro-inflammatory cytokines, chemokines, growth factors and proteases. In a recent article, Nathan et al. introduces the 'SASP atlas', which profiles SASP factors in human lung

fibroblasts and renal cortical epithelial cells (Basisty et al., 2020). In young individuals, the SASP contributes to tissue homeostasis by facilitating myofibroblast differentiation and wound healing (Demaria et al., 2014). However, as senescent cells accumulate with age, chronic SASP promotes degenerative changes in neighbouring cells and age-related pathologies, and possibly inflamm-ageing. This is supported by studies using senolytics that specifically eliminate senescent cells through pharmacological or genetic methods (definitive evidence revealing that senescent cells accelerate age-related phenotypes systematically) (Baker et al., 2011; Zhu et al., 2015). Therefore, inhibiting SASP production may become a target of anti-ageing therapies (Laberge et al., 2015).

SASP factors can potentially create a pro-inflammatory environment and recruit immune cells (Prata et al., 2018). The immune cells may further produce more pro-inflammatory cytokines and aggravate age-related pathologies. Some age-associated adaptive immune populations are able to secrete factors belonging to SASP. The terminally differentiated T_{EMRA} cells in humans, and the T_{EMRA}-like virtual memory $CD8^+$ T cells in mice, which accumulate during ageing, exhibit a uniquely high inflammatory secretory profile characteristic of SASP, which is regulated by p38-MAPK signalling pathway (Callender et al., 2018; Quinn et al., 2018). However, it is still elusive whether increased SASP serves as a homeostatic, adaptive response to the age-associated decline in antigen-specific immunity. The function of $SASP^+$ immune cells needs to be carefully determined in vivo.

A large proportion of SASP factors may originate from the innate immune system. Aged $CD14^+$ myeloid cells isolated from mouse bone marrow show increased expression of 36 established SASP-related mRNA (Farr et al., 2016). Similarly, synthesis of pro-inflammatory (IL-1α, IL-1β, IL-6, TNFα and IFNγ) cytokines was significantly increased in aged neutrophils (Uhl et al., 2016) and surprisingly T cells contribute to age-related pro-inflammatory cytokine production (Desdin-Mico et al., 2020). Although the secretome of aged immune cells overlaps with SASP, whether and how it is associated with cellular senescence still needs to be ascertained. Detailed profiling of SASP components, other hallmarks of senescence as described herein and the upstream signalling inducing such phenotype will solve this question.

To measure cytokine secretion at the single-cell level, traditional methods involve enzyme-linked immune absorbent spot (ELISpot) and flow cytometry-based assays. To detect all relevant cytokines simultaneously, several techniques offer a solution (Xu et al., 2019). Cell culture supernatants containing SASPs are added to a mixture of beads (Luminex) pre-coated with analyte-specific capture antibodies, followed by detection with fluorescent dyes and flow-based analysis. Though enabling multi-analyte profiling, the method is not easily applicable to single cells. However, another technique, called IsoLight, combines sandwich ELISA and fluorescence signal detection (Liu et al., 2020) and is based on a single-cell, highly multiplexed chip consisting of an antibody barcode array. It enables the quantification of 40+ key secreted proteins from up to ~10,000 live single cells. For surface staining, antibodies that do not affect intracellular

signalling pathways are required to avoid changes in the cell secretome. Such platforms can further be adapted to include common SASP factors for ageing biomarker development.

2.4 | Senescence-associated beta-galactosidase

Senescence-associated beta-galactosidase (SA-β-gal) activity, together with p16[INK4a] expression, serves as the most widely used biomarker for detecting cellular senescence (Lee et al., 2006). Normally, the lysosomal beta-galactosidase displays peak activity of hydrolysing the beta-galactosides between pH 4.0 and 4.5. In senescent cells, due to the accumulation of the enzyme, such activity is detectable at pH 6.0 (Dimri et al., 1995). This biomarker was first described in senescent human cells in vitro and in ageing skin in vivo through histochemical staining. Despite being used in many senescence studies, SA-β-gal activity cannot be used alone, since its activity at pH 6.0 also occurs in immortal cell lines or quiescent primary cells upon high confluency or serum starvation (Cho & Hwang, 2012). As mentioned before, multiple different measurements are needed to establish the senescent status of a cell.

Enhanced SA-β-gal activity has been observed in immune cells under different physiological conditions. In murine secondary lymphoid organs, memory-phenotype (CD44^{high}) T cells show increased SA-β-gal activity in aged mice when compared to their naïve counterparts (Shimatani et al., 2009). Injection of senescent fibroblasts into mouse peritoneal cavity leads to p16[INK4a+] macrophages recruitment with high SA-β-gal expression (Liu et al., 2019). Detection of SA-β-gal activity by chemogenic and fluorogenic substrates is either performed by histology on fixed tissues or by flow cytometry, allowing its measurement at both population and single-cell level. C_{12}FDG (5-dodecanoylaminofluorescein di-β-D-galactopyranoside) is a galactosidase substrate covalently modified with a 12-carbon lipophilic moiety (Debacq-Chainiaux et al., 2009). Once inside the cell and cleaved, the fluorescent product is incorporated into the membrane structure of the cell, where it is retained. Since the SA-β-gal activity needs to be detected at pH 6.0, the cells are pre-treated with lysosomal alkalisation reagents. Most importantly, cells are still alive after the detection, enabling further characterisation of SA-β-gal[+] populations through in vivo transfer. Another galactosidase probe, suitable for mass spectrometry detection through adding a tellurophene reporter group, is based on a similar mechanism (Lumba et al., 2017). One caveat of both methods is the potential physiological alterations of elevated lysosomal pH or the in vitro culture when treated with alkalisation reagents. Therefore, it might be necessary to fix cells prior to staining in certain sensitive cell types.

2.5 | Lipofuscin

Lipofuscin is a type of autofluorescent lipopigment enriched in aged neurons, muscle and skin (Moreno-Garcia et al., 2018).

While the nature and structure of lipofuscin shows tissue specificity and temporal heterogeneity, it is universally composed of lipids, metals and misfolded proteins (Hohn et al., 2010; Terman & Brunk, 2004; Zglinicki et al., 1995). The lipopigment accumulates in lysosomes and cytosol over time which, on one hand, delineates a specific cellular senescence pattern, and on the other hand, aggravates the defects of lysosomal degradation pathways during ageing (Terman et al., 1999). The levels of lipofuscins in both peritoneal macrophages, microglia and T cells from aged mice are significantly higher than those from young (Kushwaha et al., 2018; Singh Kushwaha et al., 2018; Vida et al., 2017). To customise lipofuscin detection to immune cell populations, a novel method involving an analogue of Sudan Black B (SBB), called GL13, has been developed that stains of a variety of lipids, which together make up 20%–50% of lipofuscin aggregate. With enhanced sensitivity through coupling with biotin, it enables its detection using anti-biotin antibodies conjugated with fluorescent dyes. This allows the lipofuscins to be identified in cells using both microscopy and flow cytometry.

3 | HALLMARKS OF CELLULAR AGEING

3.1 | Cell stress and damage

Cells face continuous cellular damage throughout their lifetime. To guarantee homeostasis, cells evolve multiple maintenance mechanisms, which respond timely to various forms of stress and activate pathways to repair damage. However, the age-related decline of repair mechanisms causes accumulation of damage, acting as a main driving force of cellular ageing (Harman et al., 1983; Schieber & Chandel, 2014). While it is accepted that naïve and memory T cells and tissue-resident macrophages are generally considered to be long-lived, and have a high chance to accumulate damage over lifetime, it is less clear whether long-lived immune cells escape senescence or whether all senescent immune cells undergo apoptosis. However, damage can also be found in short-lived cells, such as neutrophils and monocytes that may only survive for 1–2 days. The mechanisms that culminate in a short-lived cell exhibiting an aged phenotype are yet to be elucidated, but they might involve inheritance of damage from their longer-lived progenitors, which can undergo asymmetric cell division. Thus, in this section the term cellular ageing will be used to refer to the functional status of the cells that exhibit inefficient repair mechanisms, as a result of accumulated damage and stress, which is not necessarily a result of longer lifespan.

3.1.1 | Oxidative stress and mitochondrial dysfunction

Reactive oxygen species (ROS) refer to a class of radical and non-radical oxygen species. The majority of cellular ROS (cROS) are generated endogenously through mitochondrial aerobic respiration

(Schieber & Chandel, 2014). Meanwhile, other environmental stimuli, such as growth factors, inflammatory cytokines and ionising radiation, can also induce cROS, some of which are needed for signalling. Excessive levels of these chemically-reactive species cause oxidative damage to various macromolecules, including DNA, proteins, and lipids. It is crucial for the cell to tightly regulate cROS levels and maintain the redox homeostasis through several antioxidant systems. However, during ageing, cellular oxidant/antioxidant ratio becomes unbalanced, usually in favour of oxidants and excessive ROS, which impairs cell homeostasis and function (Harman et al., 1983).

As mitochondrial ATP production in the electron transport chain is the main producer of cROS, mitochondrial DNA (mtDNA) and proteins are especially vulnerable to ROS (Sandalio et al., 2013). Indeed, age-associated increase of mtDNA mutations has been found in multiple tissues (Sun et al., 2016). It impairs the normal function and integrity of mitochondria, inducing more ROS production and their leakage into the cytosol. The positive feedback loop of oxidative stress leads to cellular ageing over time. To systematically describe the mitochondrial changes occurring during ageing, mROS and mitochondrial membrane potential (MMP) are usually tested.

T-cell activation and differentiation involves metabolic reprogramming. It is necessary to distinguish among T-cell subsets to accurately compare oxidative stress and mitochondrial quality between old and young. Our group previously demonstrated that mROS levels significantly correlate with ageing in human naïve and central memory CD8^{+} T cells (Sanderson & Simon, 2017). In line with this, antibody-secreting cells from old donors display an increase in mROS (Kurupati et al., 2019). Among innate immune cells, the classical monocytes isolated from human peripheral blood show no changes in mROS or MMP during ageing (Pence & Yarbro, 2018). Similarly, mROS production in aged murine neutrophils does not differ from their young counterparts (Uhl et al., 2016). However, aged bone marrow-derived and tissue-resident macrophages are susceptible to the accumulation of intracellular ROS (Sebastian et al., 2009). In summary, aged immune cells do not necessarily display the same mitochondrial phenotypes, requiring its detection in a cell type-specific manner.

Oxidative stress and mitochondrial dysfunction can be assessed by fluorescent probes compatible with flow cytometry. To detect ROS, several probes are commercially available, including the H_2-DCFDA and CellROX reagents (Zhang et al., 2018). After passively diffusing into the cell, H_2-DCFDA, with its acetate groups cleaved by intracellular enzymes, undergoes oxidation by ROS and turns into a fluorescent compound (Zhu et al., 1994). One caveat of the dye is its conversion to fluorescent product accelerates in the presence of serum, heme and metalloporphyrins (Korystov et al., 2007; Ohashi et al., 2002). CellROX is available in a series of commercial fluorogenic dyes with different emission spectra. However, due to its unclear structure, reaction chemistry and kinetics, CellROX reagents may not be easily applicable to detect oxidative stress (Cheng et al., 2018).

Mito-SOX Red has routinely been used to determine mROS levels (Zielonka et al., 2008). It can be oxidised by mitochondrial $O_2^{\cdot -}$ and yields a fluorescent oxidation product. However, high concentration of Mito-SOX can impair mitochondrial function, also leading to the cytosolic localisation of the probe, undermining its mitochondrial specificity (Robinson et al., 2006). Thus, it is necessary to titrate its working concentration before use. Moreover, the dye is light-sensitive and prone to auto-oxidation, which may compromise the accuracy of superoxide detection by flow cytometry or confocal microscopy (Zielonka et al., 2008).

MMP is the driving force for mitochondrial ATP synthesis. It reflects the quality of mitochondria since depolarisation impairs mitochondrial function. The use of several common fluorescent dyes, including tetramethylrhodamine ethyl ester (TMRE), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide (JC-1), Rhodamine 123 and 3,3′-dihexyloxacarbocyanine iodide (DioC6), facilitates the monitoring of MMP (Perry et al., 2011). These dyes are all lipophilic cationic and accumulate within mitochondria in inverse proportion to MMP. The comparison of their advantages and pitfalls has been previously summarised (Perry et al., 2011). Note that both mROS and MMP level need to be normalised with MitoTracker Green (MTG), a fluorescent probe to measure total mitochondrial mass (Kurupati et al., 2019).

3.1.2 | DNA damage

Cells are constantly exposed to DNA damaging agents, including chemical mutagens, irradiation and oxidative stress (Jackson & Bartek, 2009). To maintain genome stability, an evolutionary conserved pathway known as DNA damage response (DDR) can sense DNA damage and recruit the repair machineries to fix the DNA lesion. DDR mediates transient checkpoint through activating the p53-p21[CIP] signalling pathway before the removal of DNA damage (Ou & Schumacher, 2018). However, if DNA damage is persistent, cells may undergo apoptosis or enter cellular senescence. It can be inferred that some senescent cells may contain more DNA lesions and higher DDR level than non-senescent ones.

8-Oxoguanine (8-oxoG) is one of the most common DNA lesions resulting from guanine being modified by ROS. During ageing, 8-oxoG accumulates in various mammalian tissues (Nie et al., 2013). As mitochondrial DNA is more vulnerable to oxidative stress due to its structure and location, it displays increased 8-oxoG both in vivo and in vitro (Barreau et al., 1996; Mecocci et al., 1993). Mice deficient in 8-oxoG-repair enzymes show high level of 8-oxoG in mitochondrial DNA, which is linked with the onset of neurodegenerative diseases (Leon et al., 2016). Quantitation of cellular 8-oxoG level can be achieved through antibody staining and followed by flow cytometry.

DNA damage also gives rise to epigenetic changes. H2AX is a minor histone H2A variant and detection of H2AX foci can serve as a readout for DNA double-strand breaks, as well as genomic instability and telomere dysfunction (Mah et al., 2010; Nagelkerke & Span, 2016). Several kinases mediate the phosphorylation of its Ser-139 residue and the formation of γH2AX foci at the DNA break site. γH2AX modifications can increase DNA accessibility, further enhancing the recruitment and accumulation of certain DDR proteins. Since each DNA break corresponds to one γH2AX focus

(Sedelnikova et al., 2002), and, unlike other repair proteins, γH2AX is formed *de novo* upon DNA damage (Ayoub et al., 2008), it can serve as a robust biomarker reflecting the increased level of DNA damage during ageing. The number of γH2AX foci increases in human hematopoietic stem cells and progenitor cells with ageing (Quinn et al., 2018). Mouse virtual memory T cells, which are considered to be the senescent population, also show higher γH2AX level than other populations (Phadwal et al., 2012). To detect cellular γH2AX levels with high sensitivity and reliability in immune cells, we adapted the traditional immunofluorescence-based assay to imaging flow cytometry, a technique that combines the speed of a traditional flow cytometer with the resolution and imaging of a microscope (Phadwal et al., 2012). It enables the high-throughput analysis of γH2AX signal as well as the visualisation of its foci, which guarantees the staining specificity, for example, in human T cells (Phadwal et al., 2012).

4 | PROTEOSTASIS

4.1 | ER stress

The endoplasmic reticulum hosts the unfolded protein response (UPR), an important stress triggered pathway that enables the suppression of protein aggregation (Walter & Ron, 2011), and promotes cell homeostasis (Hetz et al., 2015). The orchestrated activation of three sensors is key to the UPR: the dual kinase/endoribonuclease inositol-requiring enzyme 1α (IRE1α), the kinase PKR-like ER-resident kinase (PERK), and activating transcription factor 6α (ATF6α). Their activation follows the detachment of the binding immunoglobulin protein (BiP, GRP-78), a heat shock protein 70 chaperone, and synergistically culminates in increased chaperone activity, translation inhibition and improved proteostasis (Bertolotti et al., 2000; Ye, 2000). Proteostasis relies on the activation of degradation and recycling mechanisms downstream of the UPR, such as the ubiquitin-proteasome system and autophagy, which determines cell death or survival (Hetz et al., 2012, 2015).

Ageing is accompanied by a dysregulation of the UPR, which contributes to loss of proteostasis (Martinez et al., 2017). It has been observed in rodent tissue that the expression and activity of key chaperones, such as BiP, declines during ageing, which facilitates the accumulation of improperly folded proteins, and the formation of protein aggregates (Paz Gavilan et al., 2006). Thus, detection of low BiP levels (both mRNA or protein) may be an important tool to molecularly identify immune cell senescence. Lower levels of BiP also elicit UPR activation, which culminates in impaired protein translation. Hence, ER stress can be assessed by measuring nascent proteins with O-propargyl-puromycin (OPP) assays by flow cytometry (Zhang et al., 2019).

4.1.1 | Ubiquitin-proteasome system

The ubiquitin-proteasome system (UPS) acts as a major component of the intracellular protein catabolic pathway. Through the removal of misfolded or damaged proteins, it helps to maintain protein homeostasis under various forms of cellular stress (Chen et al., 2011). UPS-mediated degradation involves two stages – the ubiquitination and the proteasome-mediated degradation. Ubiquitin tags protein substrates to deliver them to the proteasome for degradation. Proteasomes are barrel-shaped proteolytic machineries, containing 20S 'core' catalytic chambers, and 19S 'cap' regulatory particles, which bind the polyubiquitinated substrates and translocate them into the central chamber (Kimura et al., 2015). The most frequent form of the 20S core is the constitutive proteasome. In immune cells, a special form of proteasome, called the immunoproteasome, plays a crucial role in digesting intracellular proteins of viral origins (Nathan et al., 2013). It helps to produce small peptides for further MHC-I-mediated antigen presentation.

Proteasome activity declines progressively in aged mammalian cell (Carrard et al., 2003). Overexpressing proteasomal subunits has been shown to promote proteostasis and longevity in various organisms (Chondrogianni et al., 2015; Nguyen et al., 2019). Aged human lymphocytes display decreased proteasome function, mainly due to the post-translational modification of the proteasome subunits (glycation, lipid peroxidation product conjugation and ubiquitination) (Carrard et al., 2003). Moreover, TCR-stimulated aged CD4^{+} T cells also fail to induce transcriptional upregulation of proteasome subunit genes (Arata et al., 2019). In T cells from elderly donors, decreased proteasome activity leads to compromised IκBa degradation upon TNFα stimulation, increasing NF-κB signalling and IL2R upregulation (Ponnappan et al., 1999).

Methods to detect the activity of different UPS components, including the proteasome, E3 ubiquitin ligases and deubiquitinating enzymes (DUBs), have been comprehensively summarised previously (Melvin et al., 2013). Here, we focus on the technique suitable for the study of primary immune cells. Fluorescently labelled activity-based probes (ABPs) are a class of chemical compounds with three domains – a reactive group, a tri- or tetrapeptide recognition element and a reporter tag (Hewings et al., 2017). Once entering the proteasomal catalytic chamber, ABPs can be covalently linked to the active-site Thr residues, detectable by flow cytometry or fluorescent microscopy. Existing subunit-selective ABPs can distinguish all six types of constitutive and immunoproteasome subunits, therefore allowing to profile the changes in each subunit during ageing. While this is limited by the fact it inhibits the catalytic function irreversibly, ABP is still a powerful tool to measure the proteasomal activity in intact cells without the necessity of transgene or transfection (Schipper-Krom et al., 2019).

4.1.2 | Autophagy

Autophagy is a conserved lysosome-dependent degradation pathway in eukaryotic cells. There are three types of autophagy – macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). We focus on macroautophagy, the most prevalent form (hereafter referred to as autophagy) (Feng et al., 2014) and CMA,

as both are closely related to ageing. Autophagy is a crucial homeostatic regulator of cell metabolism, differentiation and cell growth, balancing cell survival and death. During ageing, autophagic activity declines across multiple tissues, as is the case for macrophages, T and B lymphocytes (Bharath et al., 2020; Puleston et al., 2014; Stranks et al., 2015; ; Zhang et al., 2019). Several anti-ageing interventions, including behavioural (calorie restriction or exercise) or pharmacological (reducing TOR or insulin/insulin-like growth factor signalling, activating AMPK or sirtuins) converge in the induction of autophagy (Cabo et al., 2014). Possible mechanisms of how autophagy delays ageing involve the removal of protein aggregates or damaged mitochondria, prevention of stem cell attrition and oncogenic transformation, and the reduction of inflammatory responses (Rubinsztein et al., 2011). Therefore, autophagy acts both as a biomarker of ageing, and as a popular anti-ageing target. Similarly, CMA activity declines during ageing. In mouse T cells, CMA is crucial to TCR-mediated activation through degrading inhibitory molecules, whereas its compromised capacity during ageing negatively impact the TCR response (Valdor, 2014).

Autophagy requires core molecular machineries composed of different autophagy-related proteins (Feng et al., 2014). It starts from a double-membrane structure, and as this elongates, it either engulfs bulk cytosolic content or selectively sequesters a large spectrum of cargoes (Gatica et al., 2018). LC3/GABARAP (Atg8) family proteins play a crucial role in the selectivity. LC3-II, the lipid-modified form of LC3, is anchored into the double membranes and recruits autophagy receptors, which carries polyubiquitinated autophagic cargos (Kabeya et al., 2000). The autophagosome together with its cargo is delivered to the lysosome for degradation. The membrane-bound LC3-II is used as a readout for autophagy by microscopy, as it appears punctate, or by Western blot, as it runs at different molecular weight (Phadwal et al., 2012). In immune cells, flow cytometry or imaging is ideal as it can be combined with surface markers. As the fluorophore-conjugated LC3 antibody detects both LC3-I and II, we use saponin to selectively permeabilise plasma membrane and flush away the cytosolic non-membrane bound LC3-I. For LC3-II levels to reflect autophagic flux, it is blocked with lysosomal inhibitors, such as chloroquine or bafilomycin A1, that prevent the fusion between autophagosome and lysosome and therefore accumulates LC3 levels over the time of treatment (Homewood et al., 1972; Mauvezin & Neufeld, 2015).

An alternative way to measure autophagic flux is by cationic amphiphilic tracer dyes. Cyto-ID is an autofluorescent compound displaying a preference for autophagosomal membrane lipids (Niemann et al., 2001). These dyes have been widely applied to detect the autophagic flux in living cells with flow cytometry (Chan et al., 2012). However, some evidence suggests that during ageing autophagosomes fail to fuse with lysosomes and accumulate in the cell (Zhang et al., 2019). Measuring LC3-II should be accompanied by detecting the extent of autophagosome-lysosome fusion.

CMA substrates include proteins with KFERQ-like motif, which enables their binding to heat shock cognate 71 kDa protein (HSC70, a cytosolic chaperone). The substrate-chaperone complex

is recruited to lysosomal surface by lysosome-associated membrane protein type 2A (LAMP2A). LAMP2As are assembled into multimeric translocation complex to internalise the unfolded protein for intralysosomal degradation. To monitor CMA level in vivo, animals with transgenic reporters were developed. The fluorescent protein reporter, tagged with KFERQ sequence, is degraded specifically by CMA (Dong et al., 2020). Cells with high CMA level display lower fluorescent signal, which can be quantified by both microscopy or flow. Another method involves immunostaining of HSC70 and LAMP2A (Kaushik & Cuervo, 2009). Since lysosomes competent for CMA contains both molecules, the abundance of these lysosomes can indirectly reflect CMA level (Cuervo et al., 1997).

5 | CONCLUSION AND PERSPECTIVES

The recent decade has witnessed a progression in the field of immune ageing. We have a comprehensive knowledge of the components of immune system, and how they respond in different physiological contexts. More importantly, immune ageing is at the centre of many age-related phenotypes and diseases (Simon et al., 2015). Understanding its molecular and cellular mechanisms becomes more urgent with global population ageing, which also highlights the immense translational potential of strategies able to modulate and rescue immunosenescent phenotypes. The ongoing COVID-19 pandemic is a vivid example. Old individuals are more susceptible to SARS-CoV-2 viral infection and complications accompanied by a severe cytokine storm (Liu, Li, et al., 2020). Thus, investigating ways to rejuvenate aged immune cells and promote robust and efficient immune responses in the elderly has become an urgent matter, and the reason why we believe that immune ageing research will be particularly incentivised after the pandemic. It will be important to first establish which immune cells increasingly undergo cellular senescence with age, before drawing any conclusions to which degree immune cell senescence contributes to immunosenescence in mammals.

Immune cells are one of the most accessible human samples, thus allowing easier 'bench to bedside' translation. It is especially relevant to advance in complex topics such as ageing. However, immune cells are very heterogeneous in phenotype and function, which requires systematic analysis at high resolution with single-cell omics techniques to dissect certain phenomena (Goldman et al., 2019). Thanks to the recent advances of cloud computing, large amounts of data generated can be stored and processed with unprecedented speed. Artificial intelligence technology can further help to decipher hidden patterns among the large data volume and unravel more robust biomarkers of ageing. It is worth noting that one age-related hallmark does not necessarily co-exist with others. Some of them may appear earlier and drive cells into ageing, forming a hierarchical relation (Lopez-Otin et al., 2013). Adaptation of traditional methods to fit the omics era and measure all hallmarks simultaneously is necessary, forming a 'toolkit' of ageing detection. This toolkit can be further applied for the exploration of other tissues, to reveal the diverse facets of ageing in the whole organism.

CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

D. Z and M. B. wrote the manuscript, and K. S. edited the manuscript.

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