

Overcoming immunosenescence: Strategies to modulate DNA damage and senescence in immune cells and fibroblasts



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Abstract

Growing evidence suggests that DNA damage and cellular senescence govern age-related immune dysfunction, termed immunosenescence. Interventions that prevent the harmful consequences of DNA damage (genoprotectors) are therefore an attractive target to improve older people's immunity. Low-dose mTOR inhibition is a potent anti-ageing strategy and improves adaptive immune responses in older people, though how this intervention affects DNA damage and senescence in stromal and immune cells is unclear. In this thesis, I first optimised and validated DNA damage-induced senescence (DDIS) in primary human fibroblasts and observed that these express high levels of *WRN*, a DNA repair factor. Using phage display, I identified several WRN-interacting sequences that resemble human proteins involved in mTOR signalling and protein synthesis. Accordingly, both DDIS fibroblasts and age-associated peripheral immune subsets displayed high levels of mTOR activity and p53/p21-dependent DDR signalling. Continuous treatment with a pan-mTOR inhibitor, AZD8055, rescued a wide range of senescence phenotypes in DDIS fibroblasts, including the presence of DNA damage foci, suggesting that mTOR inhibition is genoprotective. To test whether this also occurred in immune cells, I developed a DNA damage assay in primary human T cells and found that both rapamycin and AZD8055 prevented the accumulation of DNA lesions, reduced DDR signalling, and increased cell survival after genotoxic treatment. Using an optimised autophagy and DNA damage assay, I observed that mTOR inhibition may elicit these genoprotective effects by inducing autophagy. Finally, I report the results from the UK's first single-blind, placebo-controlled trial of the impact of low-dose rapamycin in older adults on features of immunosenescence, using 27-colour spectral flow cytometry. Here, rapamycin treatment decreased the expression of immune exhaustion markers and p21 across immune subsets. These data support the notion that mTOR inhibition is a genoprotective strategy and provide a mechanism for its gero- and immunoprotective effects in older people.

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Several parts of this thesis are reproduced from a review that I published (Kell et al., 2023), primarily in the main introduction and discussion (chapters 1 and 6), and also in the equivalent sections across the three results chapters 3–5. Additionally, the latter part of chapter 6 is based on an essay I submitted for a competition, discussing sexual dimorphisms in ageing.

Phage display (section 3.2.6) was performed using purified WRN helicase gifted by Professor Opher Gileadi and Dr Joseph Newman; these and the methylation-specific PCR experiments were performed alongside Dr Ivan Boubriak (sections 3.2.5–3.2.6). Experiments investigating zeocin-induced fibroblast senescence (section 3.2.7) were performed by Matthew Viner under my supervision. PBMCs from older patients with osteoarthritis (section 5.2.2) were provided by Professor Janet Lord. All PBMCs from the Rapamune trial (led by Professor Philip Atherton) were isolated by Dr Nima Gharahdaghi and Professor Daniel Wilkinson following my training.

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List of abbreviations

53BP1	p53-binding protein 1
ADCC	antibody-dependent cellular cytotoxicity
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
ATM	ataxia-telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related
BafA ₁	bafilomycin A ₁
BCR	B cell receptor
BER	base excision repair
BMI	body mass index
BMT	bone marrow transplant
CAR	chimeric antigen receptor
CCF	cytoplasmic chromatin fragment
ChIP	chromatin immunoprecipitation
CHX	cycloheximide
COVID-19	coronavirus disease 2019
CPD	cumulative population doubling
CTRL	control
DAMP	damage-associated molecular pattern
D+Q	dasatinib + quercetin
DC	dendritic cell
DDIS	DNA damage-induced senescence
DDR	DNA damage response
DES	diethylstilbestrol
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DN	double negative (e.g. DN B cell)
DNA-SCARS	DNA segments with chromatin alterations reinforcing senescence
dNTP	deoxynucleotide triphosphate
DP	double positive (e.g. DP NK cell)
DREAM	Dp/Retinoblastoma(Rb)-like/E2F and MuvB
DSB	double-strand break
dsRNA	double-stranded ribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
FACS	fluorescence-activated cell sorting
FBS	foetal bovine serum
FcR	Fc receptor
FKBP12	FK506 binding protein 12
FMO	fluorescence minus one

gMFI	geometric mean fluorescence intensity
HIC	human immune challenge
HR	homologous recombination
HSC	haematopoietic stem cell
ICL	interstrand crosslink
KLH	keyhole limpet haemocyanin
MAPK	mitogen-activated protein kinase
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MMC	mitomycin C
MMP	mitochondrial membrane potential
MMR	mismatch repair
MMS	methyl methane-sulfonate
MSP	methylation-specific polymerase chain reaction
mTOR	mechanistic target of rapamycin
mTORC1/2	mechanistic target of rapamycin complex 1/2
mTORi	mechanistic target of rapamycin inhibition
NAC	N-acetyl-L-cysteine
NER	nucleotide excision repair
NHEJ	non-homologous end joining
NHS-BT	National Health Service blood transfusion
NK	natural killer
NKR	natural killer receptor
NR	nicotinamide riboside
OA	osteoarthritis
OIS	oncogene-induced senescence
OPP	O-propargyl puromycin
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD	population doubling
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RAPA	rapamycin
REC	research ethics committee
ROS	reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute 1640
RS	replicative senescence
RT	room temperature
SAHF	senescence-associated heterochromatin foci
SASP	senescence-associated secretory phenotype
SA β G	senescence-associated β -galactosidase
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SCAP	senescent cell anti-apoptotic pathway

scRNA-seq	single cell RNA sequencing
SEM	standard error of the mean
SIPS	stress-induced premature senescence
SSB	single-strand break
TCM	central memory T cell
TCR	T cell receptor
TEM	effector memory T cell
TEMRA	effector memory T cell re-expressing CD45RA
TFEB	transcription factor EB
TN	naïve T cell
T _{reg}	regulatory T cell
VIS	virus-induced senescence
VSV	vesicular stomatitis virus
VZV	varicella zoster virus
WRN	Werner protein
WS	Werner syndrome
XCI	X chromosome inactivation
γH2AX	histone H2AX phosphorylated at ser ¹³⁹

1

Introduction

The central role of DNA damage in
immunosenescence

1.1.1 Ageing and immunosenescence

The past century has seen a marked increase in overall human lifespan (Dong et al., 2016, Oeppen and Vaupel, 2002), largely arising from improvements in diet, lifestyle, sanitation, and healthcare. However, this increase in life expectancy has not been met by an increase in healthy life expectancy, termed healthspan, leading to an increasingly long end-of-life period spent in disability and ill health (Salomon et al., 2012). Thus, ageing is the biggest risk factor for developing chronic diseases and multimorbidity (Barnett et al., 2012). In relation to this, older people experience an overall decline in their responses to immune challenges such as vaccination (Wagner et al., 2018, Lord, 2013), resilience against novel pathogens (e.g. SARS-CoV-2 (O'Driscoll et al., 2021, Hou et al., 2022)), and diminished antigen-specific memory responses (Vukmanovic-Stejcic et al., 2018), together with reduced immunosurveillance for cancer neoantigens (Ovadya et al., 2018, Park et al., 2024). Age-related immune dysfunction is also associated with an increase in low-grade, sterile inflammation, termed inflammageing (Franceschi et al., 2000). These maladaptive changes in immunity are broadly termed immunosenescence (Teissier et al., 2022). Despite major advances in the field, there are still significant gaps in our understanding of the causes of immunosenescence and of how immunosenescence underpins the age-related increase in infection susceptibility and severity. As the older population is especially vulnerable to novel global health threats, as highlighted by the recent COVID-19 pandemic, new therapies to alleviate immunosenescence are urgently needed. Our increasing understanding of ageing biology may provide such therapies (Cox et al., 2020).

1.1.2 DNA damage as a driver of ageing and immunosenescence

Biological underpinnings of the ageing process have been encapsulated in the hallmarks of ageing (Lopez-Otin et al., 2013, Lopez-Otin et al., 2023, Schmauck-Medina et al., 2022). Immunosenescence is associated with many of these ageing hallmarks, including genome

instability, while stromal cellular senescence and altered intercellular communication contribute to inflammageing (Aiello et al., 2019). However, though many ageing hallmarks overlap with immunosenescence, it is still unclear whether the hallmarks causally drive age-related immune decline and whether they act in a cell-autonomous manner. For example, the proportion of cells that have undergone proliferative arrest and become senescent increases in both haematopoietic (immune) and non-haematopoietic compartments during ageing (Lopez-Otin et al., 2013, Mittelbrunn and Kroemer, 2021, Tuttle et al., 2020). Although many triggers for cellular senescence have been identified (van Deursen, 2014), there is accumulating evidence that these converge upon, and are further driven by, the ageing hallmark of genome instability and persistent DNA damage (Yousefzadeh et al., 2021a, Schumacher et al., 2021). Accordingly, markers of DNA damage are observed to increase in the immune system during ageing, but until recently it was unknown whether this was either causal, or secondary to other age-related organismal changes. In support of a causative role, recent studies have demonstrated that inducing persistent DNA damage in immune cells alone is sufficient to recapitulate ageing-induced immunosenescence and organismal ageing (Yousefzadeh et al., 2021b, Desdin-Mico et al., 2020). While it is not known whether senescence of other cell types would have had this effect, these recent findings suggest that DNA damage in the immune system is sufficient to precipitate immunosenescence and accelerate whole-body ageing. The evidence behind this hypothesis will be introduced and discussed in this chapter.

1.2 DNA damage and cellular senescence

1.2.1 DNA lesions and repair pathways

Endogenous and exogenous factors can lead to a variety of different types of genome lesions on one or both strands of the DNA, and occur at scales ranging from individual

bases to much larger tracts of the genome (Huang and Zhou, 2021). Endogenous sources of DNA damage include reactive oxygen species (ROS), normal by-products of oxidative metabolism which are generated at abnormally high levels by dysfunctional mitochondria (an additional hallmark of ageing (Lopez-Otin et al., 2013)); ROS can induce oxidative DNA damage and lead to DNA replication errors that require DNA excision repair pathways to correct. Exogenous UV light can induce lesions including (6-4) photoproducts and cyclobutane pyrimidine dimers (i.e. covalently linked adjacent cytosine or thymidine bases on one DNA strand), while interstrand crosslinks can be formed by crosslinking agents such as mitomycin C (McKenna et al., 2012). Other exogenous sources of DNA damage, such as ionising radiation, or radiomimetic genotoxins such as etoposide and bleomycin, can induce single- or double-strand breaks (SSBs or DSBs) i.e., breaks in one or both of the DNA strands of a duplex (Mason and Cox, 2012).

Distinct pathways have evolved to repair the different types of DNA lesions (reviewed in (Huang and Zhou, 2021)). These include i) direct reversal of the damage, ii) base excision repair (BER), iii) nucleotide excision repair (NER) for bulkier adducts, iv) mismatch repair (MMR), v) interstrand crosslink (ICL) repair, vi) single-strand break repair and vii) double-strand break repair, chiefly by either homologous recombination (HR) or non-homologous end joining (NHEJ). Damage to the DNA triggers the DNA damage response (DDR), which involves a tightly coordinated recruitment of damage sensors, transducers, and effectors that transiently arrest the cell cycle and drive cell fate decisions to either DNA repair, apoptosis, or senescence. Figure 1.1 provides a summary of DNA lesion types and their canonical repair mechanisms.

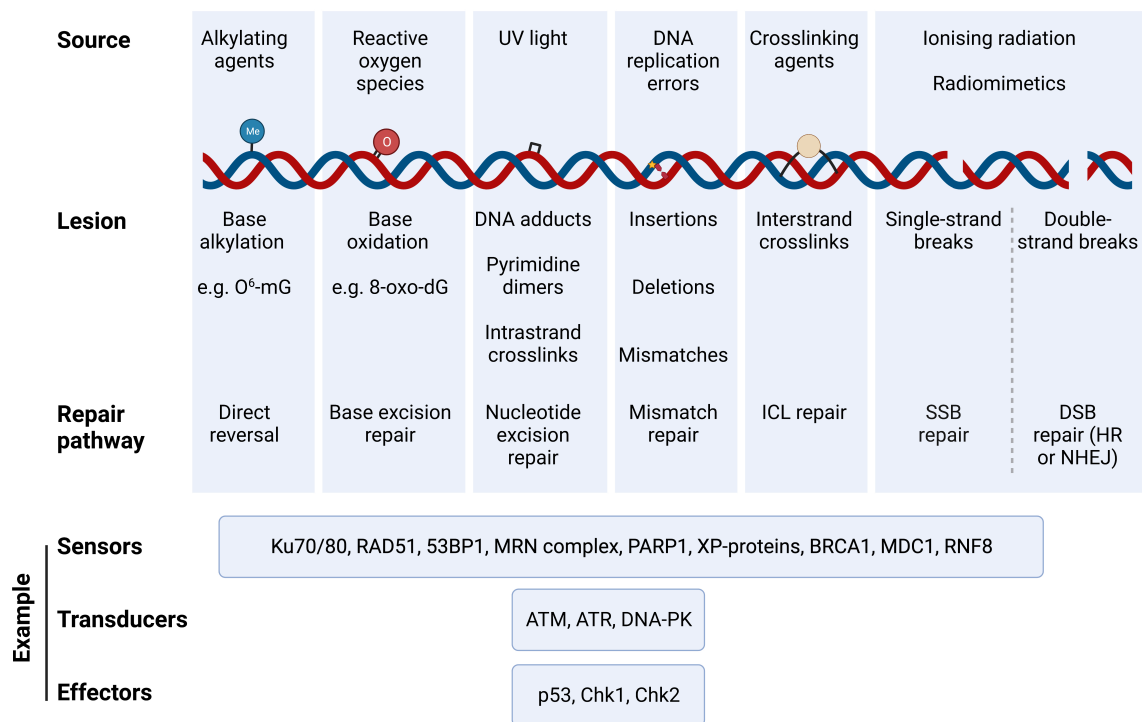


Figure 1.1 Types of DNA damaging agents, resulting lesions, and canonical repair pathways that correct them

DNA experiences a variety of genotoxic insults from both endogenous and exogenous sources. These result in lesions that can be repaired via specific DNA repair pathways triggered through the DNA damage response (DDR): damaged DNA is sensed, signalled, and repaired through a cascade of signalling mechanisms elicited by DNA sensors, transducers, and effectors, which are specific to the DNA lesion type and repair pathway. Examples of eukaryotic DDR proteins are shown.

1.2.2 The DNA damage response

1.2.2.1 DDR and cell cycle arrest

The decision for a cell to divide depends on the integration of a wide variety of signals that ultimately converge on the hyperphosphorylation of Rb, which causes the release of E2F to promote transcription of cell cycle genes (Figure 1.2). DSBs and SSBs trigger the phosphorylation of the DDR master regulators ATM (at Ser¹⁹⁸¹) and ATR (at Thr¹⁹⁸⁹), and their downstream targets, Chk2 (at Thr⁶⁸) and Chk1 (at Ser³⁴⁵) respectively. This leads to the phosphorylation (at Ser¹⁵ and Ser²⁰) and stabilisation of the tumour suppressor, p53,

which drives expression of p21^{Cip1/Waf1} (hereafter p21). Both p21 and p16^{Ink4a} ('p16'), are cyclin-dependent kinase inhibitors (CDKIs) whose expression increases during DNA and other cellular damage. They act to suppress the kinase activities of CDK2 and CDK4/6 respectively, leading to the loss of phosphorylation of Rb, increasing Rb binding to E2F, and downregulating cell cycle gene expression (Petsalaki and Zachos, 2020). Thus DNA damage promotes cell cycle arrest, which is maintained until the DNA has been repaired and DDR signalling has sufficiently declined. Whereas p21 protein is encoded by the *CDKN1A* locus, p16 is encoded at the *CDKN2A* locus, which additionally produces p14^{ARF} from an alternate reading frame (ARF). p14^{ARF} inhibits MDM2-mediated degradation of p53, thus further establishing cell cycle arrest during cellular damage (Lowe and Sherr, 2003).

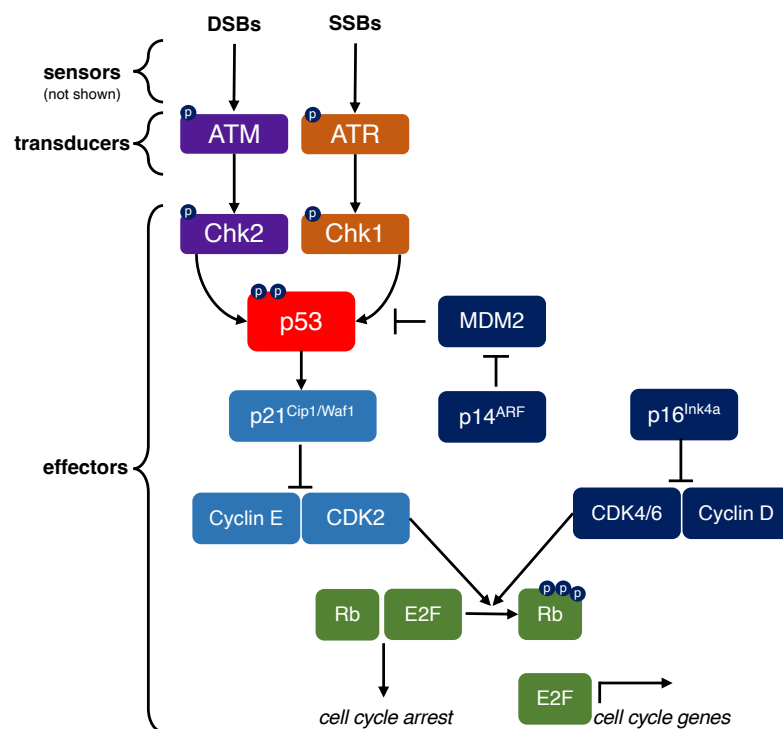


Figure 1.2 Simplified schematic of the DNA damage response and cell cycle arrest

Overview of the major pathways in which cellular and DNA damage trigger inhibition of cyclin-dependent kinases, and induction of Rb-mediated inhibition of E2F and cell cycle gene expression.

1.2.2.2 Detecting DNA damage

The presence of DNA damage can be probed by measuring the activation and/or localisation of proteins involved in the DDR pathways, such as γ H2AX (histone variant H2AX phosphorylated at Ser¹³⁹), p53-binding protein 1 (53BP1) (Huang and Zhou, 2021), and the phosphorylation of key DDR master regulators, ATM (at Ser¹⁹⁸¹) and ATR (at Thr¹⁹⁸⁹). It is possible to probe for specific DNA lesions using antibodies (e.g. 8-oxo-dG (Sanderson and Simon, 2017)), while DNA fragmentation due to SSBs and DSBs can be directly measured using single cell gel electrophoresis (the comet assay); inverse comet assays can instead provide a readout of extent of interstrand crosslinking (Swift et al., 2020). Overall, cells have evolved a plethora of mechanisms to repair DNA damage; whether these are implemented successfully plays a crucial role in determining cell fate. In the case that there is insufficient repair of DNA damage, cells either undergo apoptosis, or permanently exit the cell cycle and enter senescence.

1.2.3 Cellular senescence

Cellular senescence is a state of essentially permanent proliferative arrest accompanied by a host of morphological, metabolic, and functional changes, and was originally discovered during long-term *in vitro* culture of human diploid cells (Hayflick and Moorhead, 1961). Senescent cells play roles in normal physiology but accumulate in tissues during normal ageing, and especially in tissues impacted by age-related diseases (van Deursen, 2014, Tuttle et al., 2020). Correspondingly, an increased senescent cell burden is a hallmark of ageing (Lopez-Otin et al., 2023). This section will discuss the roles of senescent cells in physiological homeostasis and in ageing, as well as their characteristic features (Figure 1.3).

1.2.3.1 Cellular senescence in normal physiology

Senescent cells are important sentinels of tissue damage and are intricately involved in pro-regenerative processes. For example, senescent cells are induced at sites of cutaneous wounds in mice, and the genetic deletion of p16-expressing senescent cells impairs wound closure (Demaria et al., 2014). The importance of senescent cells spans the animal kingdom; for example, in the newt regenerating limb, the transient induction of senescent cells drives the dedifferentiation of muscle cells into stem-like progenitor cells (Walters et al., 2023). Similarly, senescent fibro-adipogenic progenitor cells support muscle regeneration in mice (Chen et al., 2023). Furthermore, removal of p16^{high} senescent cells in the mouse liver causes fibrosis due to the permanent loss of liver sinusoidal endothelial cells, which have important detoxifying and structural functions (Grosse et al., 2020). Senescent cells also have roles in embryonic development and pregnancy; for example, senescent cells are induced at the apical ectodermal ridge of the developing limb bud (Storer et al., 2013, Munoz-Espin et al., 2013). Likewise, the fusion of cytotrophoblasts to form the multinucleate, non-dividing syncytio-trophoblast of the placenta also involves senescence induction; insufficiency of this process is associated with placental syndromes, such as pre-eclampsia (Cox and Redman, 2017, Chen et al., 2006).

One of the main functions of cellular senescence is its cell-intrinsic and -extrinsic abilities in preventing cancerous transformation (Campisi, 2005). Cellular senescence is characterised by cell cycle arrest in response to internal damage, thus limiting the effects of oncogenic DNA mutations, or restricting the pro-proliferative signals that may arise from Ras, BRAF, Myc, or other oncogene hyperactivation. Furthermore, the pro-secretory phenotype of senescent cells induces paracrine senescence of bystander cells (Nelson et al., 2012), thus preventing the effects of pro-carcinogenic signals in the local environment (Bousset and Gil, 2022). However, senescence plays divergent roles in tumour progression: whereas early senescence generally limits tumour growth, senescent cells in the tumour

microenvironment (TME) at later time points promote cancer progression, primarily through their secretion of pro-inflammatory and tissue-remodelling cytokines (Davalos et al., 2010). For example, co-injecting tumour cells (either of epithelial or breast cancer origins) with senescent fibroblasts significantly enhanced tumour growth in mice, compared to co-injection of control non-senescent fibroblasts, demonstrating the pro-tumorigenic effects of stromal cell senescence in the TME (Krtolica et al., 2001).

Interestingly, pro-senescence treatments show promise for cancer therapies. Here, tumours are treated with senescence-inducing agents, such as cyclin kinase inhibitors (e.g. palbociclib, a CDK4/6 inhibitor), and then subsequently removed using senolytics, in so-called 'one-two punch' therapies (Bousset and Gil, 2022). In summary, cellular senescence plays physiological roles in supporting pro-regenerative environments and development, and pro-senescence therapies can be leveraged to render cancer cells sensitive to senolytic treatments.

1.2.3.2 Cellular senescence regulates health- and lifespan

The accumulation of senescent cells with ageing is intimately linked with immunosenescence (discussed later, section 1.3.4.1). Senescent cell accumulation is a causative factor in ageing, contributing to age-related morbidity and mortality – for instance, transplanting senescent cells into mice accelerates physical frailty and shortens lifespan in a dose-dependent manner (Xu et al., 2018). Moreover, deletion of senescent cells via genetic (Baker et al., 2016) or pharmacological means (e.g. fisetin (Yousefzadeh et al., 2018), or dasatinib + quercetin, 'D+Q' (Xu et al., 2018)) increases median lifespan and ameliorates a range of age-related conditions in mice, thus extending healthspan. Importantly, such senolytic treatment also improved survival in older mice following infections with coronavirus (Camell et al., 2021, Cox and Lord, 2021) and influenza

(Lorenzo et al., 2022), potentially through reducing inflammatory signalling. Cellular senescence therefore drives ageing and inflammation.

1.2.4 Features of cellular senescence

Although no unique biomarkers for senescence have yet been described, senescent cells can be detected by their presentation of a panel of senescence-associated features. Senescence markers in the immune system are like those in other cell types, and a variety of tools, including flow cytometry, have proven useful in identifying senescent immune cells (Zhou et al., 2021). In this section, the major features of senescent cells are discussed and are summarised in Figure 1.3.

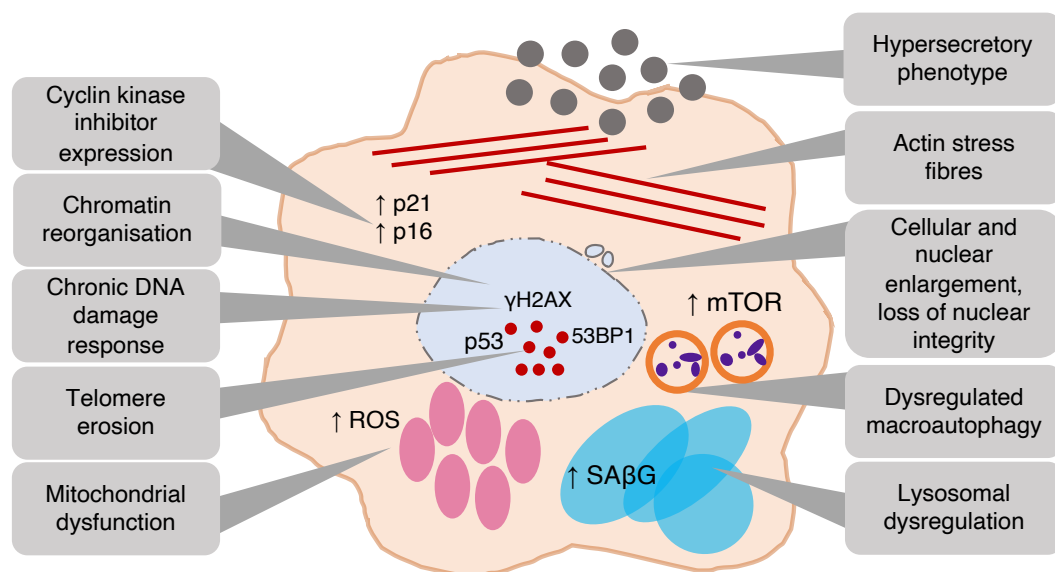


Figure 1.3 Features of cellular senescence

Senescent cells are essentially non-proliferative and display high levels of cyclin kinase inhibitors, p21 and p16. Breakdown of nuclear structure and persistent DNA damage response signalling are exacerbated by reactive oxygen species (ROS) accumulating from dysfunctional mitochondria that are not removed by autophagy (mitophagy). Lysosomal mass increases leading to high senescence-associated β -galactosidase (SA β G) activity, and mechanistic target of rapamycin (mTOR) is hyperactive. Senescent cells also display a highly active secretome, termed the senescence-associated secretory phenotype (SASP). Generally, senescent cells are enlarged and display actin stress fibres.

1.2.4.1 Upregulation of cyclin kinase inhibitors

Senescence is associated with high expression of the tumour suppressor p53 (and also at times Rb) and the cyclin-dependent kinase inhibitors, p21 and p16, which establish and maintain proliferative arrest (Figure 1.2) (van Deursen, 2014). p21 and p16 show differing expression dynamics during the induction of senescence in some cells: whereas p21 expression increases at an early stage of senescence induction, p16 expression increases later, at the point of p21 decline (Stein et al., 1999). In this way, p21 positivity may be a marker of the establishment of senescence, whereas p16 positivity is a feature of senescence maintenance and the onset of other senescence features, such as SA β G activity (Sturmlechner et al., 2021). p21⁺ and p16⁺ senescent cells produce differing secretomes, with the p21⁺ senescent cell secretome being particularly immunogenic (Sturmlechner et al., 2021). Finally, p16⁺ or p21⁺ senescent cells drive different biological outcomes, as evidenced by a study where ionising radiation-induced osteoporosis in mice could be alleviated by the deletion of p21⁺ but not p16⁺ senescent cells (Chandra et al., 2022).

1.2.4.2 Morphological changes and actin reorganisation

In *in vitro* 2D culture, senescent cells adopt a large, wide, flat shape (Walters et al., 2016). It is not clear whether this occurs in the senescence of non-adherent immune cells, such as T cells. Senescent cells display actin reorganisation, manifesting as the presence of large, stabilised, filamentous actin stress fibres that can be detected using a fluorescent phalloidin probe (Walters et al., 2016, Chen et al., 2000). Cell enlargement and the presence of stress fibres are attributed to the upregulation and phosphorylation of cofilin-1, an actin depolymerising factor (Tsai et al., 2021). While fibroblasts with constitutively unphosphorylated mutant forms of cofilin-1 do not increase in size with replicative senescence, they nevertheless enter proliferative arrest, suggesting that these

morphological changes are a consequence, and not a cause, of senescence (Tsai et al., 2021). Enlargement of senescent cells may plausibly enhance their ability to adhere and spread across their environment to exert their tumour-suppressive properties. Additionally, these morphological features may promote intercellular communication and delivery of dysfunctional mitochondria from senescent to non-senescent cells via tunnelling nanotubes (Walters and Cox, 2021).

1.2.4.3 Senescence-associated β -galactosidase activity

Cellular senescence is accompanied by an increase in both lysosomal number and mass (Robbins et al., 1970). The high lysosomal load of senescent cells consequently leads to high levels of the lysosomal enzyme, β -galactosidase; thus, even at suboptimal pH 6.0, senescent cells display high β -galactosidase activity, termed senescence-associated β -galactosidase (SA β G) (Dimri et al., 1995, Kurz et al., 2000). SA β G activity can be assessed with the chromogenic substrate, X-gal, or fluorogenic substrate, C₁₂FDG (Martinez-Zamudio et al., 2021). Interestingly, though SA β G activity is an established feature of senescent cells, it is not required for senescence: fibroblasts from patients with G_{M1}-gangliosidosis, which experience total loss of function of lysosomal β -galactosidase, maintain the ability to enter senescence (Lee et al., 2006). Furthermore, normal phagocytes such as macrophages display high lysosomal content and stain positively for SA β G, even when they are not senescent (Bursuker et al., 1982, Kopp et al., 2007). More generally, as macrophages are inflammatory, secretory cells with a low proliferation rate, they share many similarities with senescent cells (Behmoaras and Gil, 2021).

Senescent cells can be identified *in vivo* using nanoprobe containing indocyanine green J-aggregates, called NanoJaggs, which accumulate in lysosomes; these NanoJaggs can then be detected using photoacoustic tomography (Baker et al., 2024). Conjugating the

NanoJaggs with senolytic drugs may allow for more targeted senolytic treatments at lower doses, though the effects on cell types with naturally high lysosomal contents, such as phagocytes, must be monitored.

1.2.4.4 Senescence-associated secretory phenotype

Senescent cells possess a unique secretome consisting of inflammatory cytokines, matrix-remodelling proteins, growth factors, and extracellular vesicles, collectively termed the senescence-associated secretory phenotype (SASP) (Coppe et al., 2008, Rodier et al., 2009). Though the SASP is a feature of senescence, the exact components of the senescent cell secretome are dependent on the cell type, mode of senescence induction (e.g. replicative, DNA damage, oncogene-induced), and stage of senescence (e.g. early, middle, or late) (Oguma et al., 2023). The SASP is highly immunogenic (containing, for example, IL-6, IL-8, and TNF- α); thus, senescent cells attract immune cells, such as CD8⁺ T cells and NK cells, to coordinate their removal (Ovadya et al., 2018, Pereira et al., 2019). Hence, senescence is a physiological state that evolved to aid tumour cell removal and tissue remodelling. Additionally, the SASP drives paracrine senescence of local cells, which may provide another tumour-suppressive mechanism that restricts cancerous transformation. Interestingly, the SASP is dependent on the presence of mitochondria, as selective removal of these organelles prevented the induction of IL-6, a SASP component, after radiation-induced senescence (Correia-Melo et al., 2016). Extending these findings in a recent study, the SASP was found to be primarily driven by the mitochondrial release of dsRNA into the cytoplasm, which triggers innate immune signalling and pro-inflammatory cell secretions (Lopez-Polo et al., 2024). Hence, inhibition of mitochondrial RNA polymerase with IMT1 limited the expression of multiple SASP components (Lopez-Polo et al., 2024). However, SASP inhibition did not modulate other senescence phenotypes, such as *CDKN1A* (p21) expression, suggesting that the SASP is a feature, but not a central

driver, of cell-intrinsic senescence, though the SASP can drive cell-extrinsic bystander senescence (Lopez-Polo et al., 2024, Nelson et al., 2012).

The SASP can drive maladaptive outcomes during ageing. Age-related accumulation of senescent cells probably contributes to the sterile, low-grade inflammatory tissue environment, termed inflammageing (Franceschi et al., 2000). Furthermore, the SASP contributes to the tissue destruction associated with certain age-related diseases, such as osteoarthritis, mediated by matrix-degrading cytokines (Jeon et al., 2017). Likewise, the chemoattraction by senescent cells of certain immune cells, such as neutrophils, promotes oxidative damage and tissue destruction in the liver (Lagnado et al., 2021). Finally, the SASP can inhibit adequate adaptive immune responses, particularly by modulating T cell differentiation and function, thus driving immunosenescence (discussed later in section 1.3.4.2) (Chambers et al., 2021a, Lorenzo et al., 2022).

1.2.4.5 Dysregulated macroautophagy

Macroautophagy, hereafter referred to as autophagy, is one of the major cellular degradation pathways, in addition to degradation by the proteasome. Autophagy targets the degradation of both macromolecules (such as proteins, lipids, and cytoplasmic DNA) and larger organelles, such as the mitochondria (mitophagy), endoplasmic reticulum (ER-phagy), and peroxisomes (pexophagy) (Lopez-Otin et al., 2023). Autophagy involves the enclosure of targets inside a double-membrane-bound structure called an autophagosome. Upon fusion with the lysosome, autophagosomal luminal contents are exposed to the lysosome's low pH and degradative enzymes, causing breakdown into their constituent components. These components can be used by the cell for biosynthesis of new macromolecules during nutrient scarcity and also for external provision of nutrients and signalling molecules to support cellular crosstalk (Piletic et al., 2023).

Disabled autophagy is a hallmark of ageing. However, the role of autophagy in cellular senescence is somewhat less clear. On the one hand, autophagy is observed to be upregulated within the early stages of oncogene-induced senescence, potentially to sustain the high energy requirements for the hypersecretory senescence phenotype (see section 1.2.4.4) (Narita et al., 2011). On the other hand, knockdown of key autophagic components, such as *ATG7*, *ATG12*, and *LAMP2*, drives primary human dermal fibroblasts into premature replicative senescence, suggesting that autophagy is geroprotective (Kang et al., 2011). Autophagy-deficient fibroblasts displayed high p53, p21, high SA β G activity, and lysosomal hypertrophy, in addition to high mitochondrial mass and elevated ROS levels (Kang et al., 2011). The accumulation of senescence features in replicatively-senescent and autophagy-deficient cells was linked to high levels of ROS, as these senescence features, including γ H2AX⁺ DNA damage, were prevented by continuous culture with *N*-acetyl-L-cysteine (NAC), a ROS scavenger (Kang et al., 2011). The contrasting roles of autophagy in cellular senescence reported by the above studies may be attributed to the different types of senescence models utilised: while Narita et al. (2011) utilised Ras-driven oncogene-induced senescence, Kang et al. (2011) assessed senescence induction in an unperturbed state as cells entered replicative senescence (Narita et al., 2011, Kang et al., 2011). Autophagy is decreased in immune cells, such as B cells and T cells, from older donors and, importantly, pharmacological enhancement of autophagy improved immune responses in an aged context (Zhang et al., 2019, Alsaleh et al., 2020). It is unclear why autophagy declines with ageing. The increase in activity of its negative regulator, mTOR, during cellular senescence and ageing of human tissues may provide one explanation (Walters et al., 2016, Markofski et al., 2015). Additionally, TFEB, a master regulator of autophagy and lysosomal biogenesis, is decreased in aged cells, such as immune cells (Alsaleh et al., 2020). Overall, despite the conflicting evidence about the role of autophagy

in cellular senescence, loss of autophagy in the context of immunosenescence is definitively maladaptive.

1.2.4.6 Mitochondrial dysfunction

Mitochondria are organelles governing cellular metabolism and survival, with a primary role in energy generation in the form of ATP. Cellular senescence is associated with an increase in mitochondrial mass (Passos et al., 2007), possibly due to defective mitophagy (see above) and excessive ROS release, due to a lower mitochondrial membrane potential (MMP) that results in loss of the proton motive force and subsequently defective oxidative phosphorylation (Miwa et al., 2022). Excessive ROS release can cause oxidative damage of both genomic and mitochondrial DNA (Henson et al., 2014). This leads to a vicious cycle: since the mitochondrial genome encodes key components of the electron transport chain (ETC), oxidative damage, combined with low levels of mitochondrial DNA repair, leads to further disruption in oxidative phosphorylation and hence higher ROS levels (Rong et al., 2021). In this way, mitochondria participate in several positive feedback loops that act to deepen senescence via DNA damage induction (discussed in section 1.2.5.1).

Dysfunctional mitochondria govern senescence phenotypes. As mentioned earlier, the presence of mitochondria is necessary for the pro-inflammatory SASP of senescent cells (Correia-Melo et al., 2016). This may be due to their release of a wide range of damage-associated molecular patterns (DAMPs), such as cytoplasmic mtDNA, which stimulates the cGAS-STING pathway and inflammasome activation, driving the cleavage and release of pro-inflammatory molecules such as IL-8 and IL-1 β (Miwa et al., 2022). Overall, dysfunctional mitochondria play a central role in cellular senescence by linking autophagy, DNA damage, and cell cycle arrest. Excitingly, a recent study has leveraged the low mitochondrial membrane potential of senescent cells by co-administering mitochondrial

uncoupling agents, to which senescent cells are uniquely sensitive, to potentiate lower doses of senolytic drugs *in vivo* (Fielder et al., 2024).

1.2.4.7 Nuclear and DNA damage

Some of the most salient features of senescent cells concern nuclear alterations. Senescent cells exhibit enlarged nucleoli (Buchwalter and Hetzer, 2017), chromatin rearrangements (e.g. senescence-associated heterochromatin foci, or SAHFs (Narita et al., 2003)), and nuclear lamina breakdown leading to cytoplasmic chromatin fragments (CCFs (Ivanov et al., 2013)). Indeed, loss of lamin B1 from the nuclear envelope is one of the classical biomarkers of senescence (Ogrodnik et al., 2024). More generally, assessing senescence-associated changes in nuclear size and morphology alone is sufficient to identify senescent cells using machine learning techniques (Duran et al., 2024).

Persistent DNA damage is a hallmark of senescence (Passos et al., 2007). After the induction of the original DNA damage and entry of cells into senescence, senescent cells maintain persistent expression of DNA damage markers such as γ H2AX and 53BP1, which associate with nuclear PML bodies and drive other features of senescence, such as the SASP (Rodier et al., 2009). These persistent DNA damage foci present in senescent cells are distinct from acute DNA lesions and are termed DNA-SCARS (DNA segments with chromatin alterations reinforcing senescence) (Rodier et al., 2011). Failure of DNA repair pathways with ageing may explain the persistence of DNA damage and chronic DDR signalling, leading to the accumulation of deleterious senescent cells which drive age-related diseases and immunosenescence.

1.2.5 DNA damage drives cellular senescence and accelerated ageing

A variety of senescence inducers have been characterised, including oncogene activation leading to oncogene-induced senescence (OIS). Additionally, some viruses, (e.g. SARS-CoV-2) and fusogenic viruses (e.g. the measles virus) lead to virus-induced senescence (VIS) (Lin et al., 1998, Lee et al., 2021, Chuprin et al., 2013). DNA damage can trigger senescence, while persistent DNA damage is often a feature of senescence arising from other inducers (Fumagalli et al., 2014). Generally, an imbalance between DNA damage and repair is a primary driver of cell cycle proliferative arrest resulting in one of three key cell fates: persistent arrest, cell death, or cell senescence. How this imbalance arises depends on the type of damage signal involved.

Replicative senescence (RS), the type of cell senescence thought to arise most commonly during normal ageing, is a consequence of telomere attrition (Harley et al., 1990). Loss of telomeric DNA at every round of DNA replication gradually erodes telomeres until they become critically short, which impairs their stabilisation by the protective shelterin complex (Rossiello et al., 2014). Uncapped telomeres are sensed as DSBs, triggering a persistent DDR and permanent exit from the cell cycle. The shortening of telomeres towards this critical length after each mitotic cell cycle therefore fundamentally limits the replicative lifespan of cells (the Hayflick limit), i.e., drives replicative senescence. Maintaining telomere length and capping is therefore important for chromosomal stability and to prevent early onset of senescence. Correspondingly, ectopic expression of telomerase, which extends telomeres, overcomes premature senescence phenotypes in progeroid Werner syndrome cells *in vitro* (Wyllie et al., 2000), while experimental telomerase reactivation promotes tissue rejuvenation in mice (Jaskelioff et al., 2011). Moreover, even where telomere length has not reached the point of shelterin destabilisation, DNA damage – particularly formation of 8-oxo-dG lesions – within the telomeric regions triggers DDR signalling, leading to cell cycle arrest and senescence (von Zglinicki, 2000).

Unlike most somatic cells where telomerase is repressed, adaptive immune T and B cells can upregulate telomerase expression, which prolongs their replicative potential; this is likely to be an evolved response to permit multiple rounds of clonal proliferation on antigenic stimulation without inducing replicative exhaustion (Akbar et al., 2016). Remarkably, T cells from a mouse immunised against vesicular stomatitis virus (VSV) could be transferred and expanded in 51 successive recipient mice, over 10 years, without showing any proliferative dysfunction or telomere shortening (Soerens et al., 2023). Nevertheless, T cells from aged donors do display short telomeres (Sanderson and Simon, 2017, Lin et al., 2015), possibly because their ability to upregulate telomerase is impaired during ageing, especially in the CD27⁺CD28⁺ senescent T cell population (Patrick et al., 2019, Plunkett et al., 2007). Interestingly, a recent study identified a novel mechanism by which human T cells may acquire telomeres delivered in extracellular vesicles from antigen-presenting cells, which increases their replicative capacity and prevents senescence (Lanna et al., 2022). It would be particularly interesting to determine whether this intercellular telomere transfer occurs as efficiently in old as it does in young individuals, as failure with ageing could potentially contribute to telomere attrition in T cells.

Stress-induced premature senescence (SIPS) can occur, for example, in response to oxidising environments and can result in telomere oxidation. Notably, up to half of all DNA damage foci (as measured by a combination of immunofluorescence and ChIP) have been reported to localise at telomeres (Hewitt et al., 2012) suggesting either that telomeres are particularly prone to damage, or that their heterochromatin nature precludes effective repair (Fumagalli et al., 2012, von Zglinicki, 2002). Telomeric vulnerability due to high guanine content and unusual DNA topology, such as G4 quadruplexes, remains the most likely explanation.

As well as oxidative damage, exposure to genotoxic agents can drive DNA damage-induced cellular senescence (DDIS). Following transient cell cycle arrest immediately after

DNA damage, in cases where DNA lesions are successfully repaired (Figure 1.1), the cell may re-enter the proliferative cell cycle. However, in situations where the damaging agent persists, where the lesion burden is high, or where the cell is unable to repair the DNA lesions (e.g. through loss of function of critical DNA repair factors), persistent DDR signalling can result in irreversible cell cycle arrest and DDIS. DDIS is considered to have evolved as a tumour-suppressive strategy to limit cancerous transformation in the presence of a high lesion burden (Campisi, 2005).

DNA damage is therefore well established as a driver of senescence. It is also strongly associated with pathology during ageing (Schumacher et al., 2021). The majority of human premature ageing (progeroid) syndromes (e.g. Werner syndrome, Rothmund-Thomson syndrome, Bloom syndrome, Cockayne's syndrome, Fanconi's anaemia, and Ataxia-Telangiectasia) result from mutations in DNA repair factors (WRN, RECQL4, BLM, CSB, FANC proteins, and ATM, respectively); patients show premature ageing across multiple organs often accompanied by greatly elevated cancer incidence, and patient-derived cells senesce prematurely in culture (Cox and Faragher, 2007). In Hutchinson-Gilford progeria – where the driver mutation occurs in the lamin A/C gene, i.e. not a DNA repair factor – the resulting instability of the nuclear lamina leads to significant genome instability and DNA breakage (Liu et al., 2005). Hence progeroid syndromes provide strong evidence that DNA damage can be a major driver of senescence and ageing (Yousefzadeh et al., 2021a).

1.2.5.1 DNA damage is central to positive feedback loops that deepen senescence

Further to DNA damage being an inducer of senescence, unrepaired DNA lesions propagate further cellular dysfunction and hallmarks of ageing, thereby positioning DNA damage as a major node in senescence and ageing (Schumacher et al., 2021). For example, free DNA ends at sites of damage are recognised by poly-ADP ribose polymerase

1 (PARP1) which uses NAD⁺ as a substrate for sensing the damaged DNA (Yoshino et al., 2018). Continuous and persistent DNA damage therefore leads to increased PARP1 activity and depletion of NAD⁺ stores, resulting in reduced function of other NAD⁺-requiring enzymes such as the sirtuins. These are a family of NAD-dependent deacetylases, some of which are important regulators of chromatin organisation and mitochondrial health and have been associated with longevity (Fang et al., 2014). Maintaining sirtuin activity limits senescence: for example, the age-related loss of SIRT6 activity results in heterochromatin decompaction and expression of retroviral cDNA in the cytoplasm, triggering innate cGAS-STING signalling and an interferon-induced SASP response (Simon et al., 2019, De Cecco et al., 2019). Similarly, loss of sirtuin activity in DNA repair-deficient models (e.g. XPA – (Fang et al., 2014)) demonstrates that DNA damage can induce mitochondrial dysfunction and additional ROS production. ROS damage both mitochondrial and nuclear genomes and deepen senescence through positive feedback mediated via p21 and persistent DDR signalling (Passos et al., 2010). Furthermore, ROS-mediated DNA damage (mainly at the telomeres, which are particularly sensitive to oxidative damage) leads to activation of p53, which limits transcription of PGC-1 α and PGC-1 β and mitochondrial biogenesis, driving further mitochondrial dysfunction (Hewitt et al., 2012). DNA damage can thus drive a positive feedback mechanism to propagate further dysfunction and senescence. Thus, supporting and enhancing cells' ability to repair DNA lesions and limit senescence (genoprotection) may be a valuable strategy for promoting healthy ageing.

1.3 Immunosenescence

Immunosenescence describes organism-level age-related dysfunction of both innate and adaptive immunity, which leads to increased susceptibility to pathogens (most recently exemplified by the COVID-19 pandemic (Cox et al., 2020)), poor vaccine responses (Lord,

2013), elevated cancer risk, and increased autoimmunity (Goronzy and Weyand, 2012). Additionally, immunosenescence is associated with a low-grade inflammatory environment that is generated, at least in part, by the SASP of senescent cells of both immune and non-immune origin (Teissier et al., 2022). Immunosenescence is partially underpinned by age-related changes in immune cells, leading to a variety of dysfunctional phenotypes, several of which are characteristic of senescence (Zhou et al., 2021). However, not all age-related changes to immune cells are indicative of cellular senescence – for example, T cells with an exhausted phenotype accumulate with age and/or chronic antigenic stimulation, such as cytomegalovirus (CMV) exposure (Fletcher et al., 2005). Furthermore, the age-related shifts in other immune cell subsets, particularly with a bias from anti-inflammatory to pro-inflammatory cells, may contribute to immune dysfunction consistent with immunosenescence (Marquez et al., 2020).

This section discusses the unique function of physiological DNA damage in lymphocytes. I then discuss how persistent immune DNA damage is associated with ageing and immunosenescence, with a particular focus on T cell senescence. I further describe the known sources of T cell DNA damage, and how genotoxic stress may cause the maladaptive phenotypes of senescent T cells that promote immunosenescence. Finally, I discuss how the senescence of non-immune cells can also suppress adequate antigen-specific responses and thus reduce older people's ability to respond effectively to novel antigens, leading to risk of severe illness or death from infectious diseases, (e.g. influenza (Reichert et al., 2012)).

1.3.1 DNA damage in the immune system

1.3.1.1 *'Intentional' DNA lesions generate receptor diversity*

DNA damage within the immune system is not always deleterious. In fact, the introduction of DNA lesions plays a vital role in generating receptor diversity in the B and T cells of the adaptive immune system; such physiological genome alterations do not trigger the irreversible cell cycle arrest characteristic of cell senescence (Gullickson et al., 2022). V(D)J recombination, employed by both cell types, relies on the induction of DSBs that allow the rearrangement of individual Variable, Diversity, and Joining segments to produce a unique repertoire of B and T cell receptors (BCRs and TCRs). BCRs undergo the critical processes of somatic hypermutation and class-switch recombination, both of which require the introduction of DNA lesions by activation-induced cytidine deaminase, and modification by canonical DNA repair pathways and factors – including DNA-PK, Ku, Artemis, DNA ligase IV, and ERCC4, as well as recombination-inducing RAG proteins (Gellert, 2002). Crucially, loss of either DSB-inducers, such as RAG1, or key DNA repair proteins, particularly those involved in DSB repair, leads to severe combined immunodeficiency characterised by a reduction of T and B cell populations and poor responses to immune challenge (Gullickson et al., 2022). Maintaining physiological DNA damage and repair processes is therefore central to normal immune function.

1.3.1.2 *Immune DNA damage mimics immunosenescence and accelerates ageing*

While appropriate DNA damage and repair is critical for normal immune function, a growing body of literature supports the hypothesis that increased DNA damage in the immune system drives accelerated ageing and immunosenescence. A recent meta-analysis which evaluated DNA damage in the circulating immune cells of 2,403 individuals, as measured by the comet assay, reported that higher levels of damage were associated with increased mortality (Bonassi et al., 2021). Further to this correlative association, immune DNA

damage has been linked causally to increased mortality and ageing. For instance, immune-specific genetic knockout of *Ercc1*, an endonuclease involved in multiple DNA repair pathways, is sufficient to phenocopy many aspects of immunosenescence and drive age-related changes in mice, including accelerated ageing of solid organs of non-immune lineage (Yousefzadeh et al., 2021b). *Ercc1*-deficient immune cells showed increased levels of the DNA damage marker γ H2AX and DNA oxidation product 8-oxo-dG, and their high expression of the cyclin kinase inhibitor, *Cdkn2a* (p16), indicated permanent exit from the cell cycle and senescence (Yousefzadeh et al., 2021b). Although it is not clear which haematopoietic cells drove this phenotype, the abundance of T cells in the immune system could contribute to a large extent, especially given that DNA damage accumulates in these cells and is associated with normal human ageing (discussed in section 1.3.1.3). Supporting the view that T cells may be the major cell type responsible is a recent study using *Tfam^{fl/fl} Cd4^{Cre}* mice, in which the nuclear-encoded mitochondrial transcription factor A (TFAM) is knocked out in T cells only (*TFAM* encodes a protein involved in mitochondrial DNA stability, replication, and transcription) (Desdin-Mico et al., 2020). These mice had significantly shorter lifespans compared to those of controls with functional TFAM, and exhibited premature onset of age-related muscular, cardiovascular, and cognitive dysfunction, as well as age-related susceptibility to an acute viral infection, indicating premature immunosenescence (Desdin-Mico et al., 2020).

Though these studies utilise mice which harbour non-physiological mutations driving a severe pro-ageing phenotype, they suggest that DNA damage and senescence in immune cells alone can be sufficient to drive age-related dysfunction. This does not exclude the possibility that DNA repair deficiency in other individual non-immune cell types may have progeroid effects. However, given that increased DNA damage in the immune system is observed during physiological ageing in T cells, and T cells are both numerous and readily

accessible from the blood of older adults, targeting DNA repair capabilities in these cells may serve as a valuable axis in designing therapeutics to promote healthy longevity.

1.3.1.3 Senescent T cells with DNA damage accumulate during ageing

Increased DNA damage during immunosenescence has been best characterised in the senescence of T cells during ageing. Naïve and memory T cells are long-lived cells of the adaptive immune system that become activated via their T cell receptor (TCR) to induce clonal proliferation and cytokine secretion. The specific functions of T cells are determined by their CD4⁺ or CD8⁺ subtype. Human senescent T cells are terminally-differentiated, antigen-experienced cells that are defined by their successive loss of expression of the co-stimulatory molecules, CD27 and CD28, together with high expression of CD57 and KLRG1 (Henson et al., 2014). T cell differentiation subsets can be determined by their relative expression of CD27 and CD45RA, thus defining four subsets: naïve (TN, CD27⁺CD45RA⁺), central memory (TCM, CD27⁺CD45RA⁻), effector memory (TEM, CD27⁻CD45RA⁻), and effector memory re-expressing CD45RA, known as TEMRA (CD27⁻CD45RA⁺). TEMRA cells are considered a senescent memory subset of T cells (Henson et al., 2014). Senescent T cells can also be identified by their high activity of senescence-associated β -galactosidase (SA β G), a classical senescence biomarker (section 1.2.4.3) (Martinez-Zamudio et al., 2021). Thus, senescent T cells have been defined in the literature as being CD27⁻CD28⁻, CD27⁻CD45RA⁺ (TEMRA), or more recently as SA β G^{high} cells, within the CD4⁺ or CD8⁺ subsets.

Though defined by differing markers, CD27⁻CD28⁻, TEMRA, and SA β G^{high} cells crucially share the characteristics both of being greatly expanded during chronological ageing and of displaying many features of senescence. The characteristics of these senescent T cell subsets are summarised in Table 1.1. SA β G^{high} senescent T cells accumulate in both CD4⁺

and CD8⁺ T cell compartments during ageing and are particularly enriched in the TEMRA terminally-differentiated subpopulation (Martinez-Zamudio et al., 2021). Compared to non-senescent SAβG^{low} cells, senescent SAβG^{high} CD8⁺ T cells have impaired proliferative ability and high expression of the cyclin kinase inhibitor, p16^{Ink4a}. Furthermore, increased T cell expression of p16^{Ink4a} is an accurate predictor of chronological age in humans (Liu et al., 2009). Compared with non-senescent cells, the functions of senescent TEMRA cells are severely impaired and include the inability both to stimulate appropriate downstream TCR signalling and to proliferate after activation – though, unlike senescent fibroblasts, their proliferative ability is not fully lost (Akbar et al., 2016, Henson et al., 2014). Senescent CD8⁺ TEMRA cells are highly cytotoxic and, much like senescent fibroblasts, produce a SASP with immune-active components (namely, TNF-α and IFN-γ) after short-term activation, that may contribute to the low-grade inflammatory environment in older people (Akbar et al., 2016, Henson et al., 2014, Henson et al., 2015). T cells from older adults have lower autophagic flux (Phadwal et al., 2012), contain dysfunctional mitochondria, and have higher levels of ROS (Sanderson and Simon, 2017, Henson et al., 2014) than cells from younger adults, all of which are hallmarks of ageing (Lopez-Otin et al., 2023). It is not clear if the accumulation of TEMRA (or senescent T cells) with age may be adaptive or maladaptive – for example, their hyper-cytotoxic and hyper-secretory phenotypes may beneficially compensate for the increased burden of malignant neoplastic cells during ageing, but bear the disadvantages of increased destruction of healthy self-tissues (Pereira and Akbar, 2016). Nevertheless, many of these age-related dysfunctional characteristics have been linked to the impaired function of senescent T cells, and reversing these characteristics could potentially promote immune resilience in old people.

Senescent T cells display markers of persistent DNA damage (Table 1.1). They have high expression of γH2AX, low telomerase expression, and critically short telomeres (Di Mitri et al., 2011, Henson et al., 2014, Sanderson and Simon, 2017). Even though T cells retain

telomerase expression for far longer than other somatic cells, telomere erosion is observed in T cells (and in peripheral blood mononuclear cells, PBMCs, more generally) during ageing (Sanderson and Simon, 2017, Lin et al., 2015). Similarly, compared to non-senescent SA β G^{low} cells, SA β G^{high} senescent T cells are enriched for the DNA damage marker, 53BP1, especially at telomeres (Martinez-Zamudio et al., 2021). Further evidence for the relationship between DNA damage and ageing T cells comes from DNA repair-deficient mice (*Ercc1*^{-Δ}), which prematurely experience an accumulation of highly-differentiated memory T cells akin to naturally aged wildtype mice (Pieren et al., 2021). Accumulation of senescent T cells with persistent DNA damage and a dysfunctional cytotoxic nature may therefore contribute to both immunopathology and immunosenescence during ageing (Akbar et al., 2016, Covre et al., 2020).

Table 1.1 Characteristics of senescent T cells

Phenotype	Description	Evidence in human ageing (defined T _{SEN} subset investigated and reference)	
		CD4 ⁺ T cells	CD8 ⁺ T cells
CD27-CD28 ⁻	Stimulatory co-receptors CD27 and CD28 are progressively lost during differentiation from naïve to memory T cell subsets	↑ %CD27-CD28 ⁻ (Moro-Garcia et al., 2013)	↑ %CD27-CD28 ⁻ (Plunkett et al., 2007)
CD27-CD45RA ⁺ (TEMRA)	CD27 and CD45RA define naïve, TN (27 ⁺ RA ⁺), central memory, TCM (27 ⁺ RA ⁻), effector memory, TEM (27 ⁻ RA ⁻), and senescent, TEMRA (27 ⁻ RA ⁺)	↑ %TEMRA (Libri et al., 2011) ↑ %TEMRA (Callender et al., 2020)	↑ %CD27-CD28 ⁻ (Henson et al., 2009) ↑ %TEMRA (Callender et al., 2020) ↑ %TEMRA (Riddell et al., 2015)
SAβG ^{high}	High senescence-associated β-galactosidase activity	↑ %SAβG ^{high} (Martinez-Zamudio et al., 2021)	↑ %SAβG ^{high} (Martinez-Zamudio et al., 2021)
KLRG1 ⁺	Killer Cell Lectin-like Receptor G1, co-inhibitory receptor	TEMRA (Di Mitri et al., 2011)	TEMRA (Henson et al., 2014, Henson et al., 2015, Pereira et al., 2020) CD27-CD28 ⁻ (Henson et al., 2009)
CD57 ⁺	Terminal differentiation marker	TEMRA (Libri et al., 2011)	TEMRA (Henson et al., 2014, Henson et al., 2015, Pereira et al., 2020) Old donor pan-CD8 ⁺ (Phadwal et al., 2012)
NKR ⁺	Natural Killer Receptors	Old donor pan-CD4 ⁺ (Alonso-Arias et al., 2011) TEMRA (Libri et al., 2011)	TEMRA (Pereira et al., 2020)
↑ Cytokine and cytotoxic granule production	Increased cytotoxicity and inflammatory cytokine production (e.g. IFNγ, TNFα, perforin and granzyme B)	TEMRA (Libri et al., 2011)	TEMRA (Henson et al., 2014, Henson et al., 2015, Callender et al., 2018)
↑ Sestrins	Sestrin-MAPK activation complex (sMAC) drives maladaptive T cell functions and NKR-mediated cytotoxicity	CD27-CD28 ⁻ (Lanna et al., 2017)	CD27-CD28 ⁻ (Pereira et al., 2020)
↓ autophagy	Decreased autophagic flux	Old donor pan-CD4 ⁺ (Bharath et al., 2020)	CD57 ⁺ (Phadwal et al., 2012) TEMRA (Henson et al., 2014) Old donor vaccine-specific cells (Alsaleh et al., 2020)
↓ proliferative ability	Decrease in number of cell divisions achieved after immune stimulation	TEMRA (Libri et al., 2011)	TEMRA (Henson et al., 2014, Henson et al., 2015) CD27-CD28 ⁻ (Plunkett et al., 2007)
↓ telomerase activity	Decrease in activity of telomerase (telomere-extending enzyme)	TEMRA (Di Mitri et al., 2011)	TEMRA (Henson et al., 2014, Henson et al., 2015) CD27-CD28 ⁻ (Plunkett et al., 2007)

↓ telomere length	Shorter telomeres destabilise the shelterin complex and trigger a persistent DDR	TEMRA (Di Mitri et al., 2011) (TEMRA had shorter telomeres than TN)	CD27-CD28 ⁻ (Plunkett et al., 2007) Old donor pan-CD8 ⁺ and TEMRA (Sanderson and Simon, 2017) TEMRA shorter than TN (Riddell et al., 2015)
↑ γH2AX	Phosphorylation of histone variant H2AX at Ser ¹³⁹ (γH2AX) accumulates at sites of DNA lesions	TEMRA (Di Mitri et al., 2011) CD27-CD28 ⁻ (Lanna et al., 2014) TEMRA (Callender et al., 2020) CD27-CD28 ⁻ (Lanna et al., 2014)	TEMRA (Henson et al., 2014, Henson et al., 2015) TEMRA (Callender et al., 2020)
↑ p-ATM (Ser ¹⁹⁸¹)	Phosphorylation/activation of DDR transducer		SAβG ^{high} (Martinez-Zamudio et al., 2021)
↑ 53BP1 ⁺	p53-binding protein 1, DNA damage sensor		TEMRA (Callender et al., 2018)
↑ p-p53 (Ser ¹⁵)	Phosphorylation/activation during DDR		TEMRA (Henson et al., 2014)
↑ ROS	Reactive Oxygen Species	CD27-CD28 ⁻ (Lanna et al., 2014)	Old donor pan-CD8 ⁺ (Sanderson and Simon, 2017)
↑ Dysfunctional mitochondria	Accumulation of mitochondria with a low membrane potential leads to inefficient oxidative phosphorylation and increase in ROS	Old donor pan-CD4 ⁺ (Bharath et al., 2020)	TEMRA (Henson et al., 2014) TEMRA (Callender et al., 2020)

T_{SEN} = senescent T cell

1.3.2 Causes of age-related accumulation of immune cells bearing DNA damage

The causes of DNA damage in aged T cells may be attributed both to endogenous and exogenous sources of genotoxins, and to the endogenous decline of DNA repair factor expression, leading to the loss of efficient DNA repair. This section discusses the sources of DNA damage which T cells experience across the lifespan, in addition to telomere attrition (discussed in section 1.2.5). These are summarised in Figure 1.4.

1.3.2.1 Impaired autophagy and removal of dysfunctional mitochondria

As discussed in section 1.2.4.5, autophagy is a process by which cells degrade excess or damaged macromolecules and larger cargo such as organelles; altered autophagy is recognised as a hallmark of ageing (Schmauck-Medina et al., 2022, Lopez-Otin et al., 2023). Autophagic flux decreases in both B and T cells during ageing (Zhang et al., 2019, Alsaleh et al., 2020), which correlates with increased DNA damage, as measured by γ H2AX (Phadwal et al., 2012). Autophagy is required for an adequate memory response to antigen challenge (Puleston et al., 2014). Reduced autophagy partly underlies the defective T cell responses in old people: much like senescent T cells, *Atg7*-deficient T cells, which are autophagy-deficient, are impaired in their proliferative capacity after antigen exposure (Zhou et al., 2022). Ineffective autophagy in T cells from old donors also underlies other senescent T cell phenotypes that are capable of driving T cell DNA damage, such as the accumulation of dysfunctional mitochondria.

Like senescent fibroblasts, senescent T cells from older people harbour enlarged mitochondria with disrupted bioenergetics that release excessive reactive oxygen species (ROS), which contribute to the positive feedback loops that deepen senescence (sections 1.2.4.6 and 1.2.5.1) (Sanderson and Simon, 2017, Henson et al., 2014, Bharath et al., 2020, Miwa et al., 2022). ROS also contribute to the telomere damage and attrition observed in T

cells cultured long term *in vitro*, which can be rescued by addition of a ROS scavenger, *N*-acetyl-L-cysteine, much like the autophagy-deficient senescent fibroblasts discussed in section 1.2.4.5 (Sanderson and Simon, 2017, Kang et al., 2011). Since autophagy is important in removing damaged mitochondria through mitophagy, the decline in basal autophagy observed in old lymphocytes is thought to contribute to accumulation of dysfunctional mitochondria that produce genotoxic ROS (Bektas et al., 2019). Accordingly, stimulating autophagy in T cells using either spermidine, metformin, or BIRB 796, a p38 MAPK inhibitor, reduced the presence of enlarged dysfunctional mitochondria and restored mitochondrial bioenergetics, indicating an overall improvement in mitochondrial quality (Alsaleh et al., 2020, Bharath et al., 2020, Henson et al., 2014). It is unclear, however, whether these effects are due solely to the specific induction of mitophagy or other autophagy-induced processes. This could be explored further – for example with pH-dependent fluorescent mitochondrial dyes; these dyes mark mitochondria but change their fluorescence profiles upon autophagosome-lysosome fusion, thus allowing for measurement of mitophagy (Bektas et al., 2019).

The ability of T cells to promote mitochondrial biogenesis after DNA damage may determine their propensity for seno-conversion. After DNA damage, AMPK activation causes CD4⁺ T cells to increase their expression of the transcription factor, GATA3 (Callender et al., 2021). By complexing with ATR, PGC1 α , and NRF2, GATA3 promotes the transcription of genes involved in mitochondrial biogenesis, thereby limiting ROS-mediated oxidative stress and preventing senescence (Callender et al., 2021). However, CD8⁺ T cells, unlike CD4⁺ T cells, do not increase GATA3 expression and mitochondrial biogenesis after DNA damage, which may partly explain their increased susceptibility to senescence and the higher proportion of senescent CD8⁺ than CD4⁺ T cells in peripheral blood (Callender et al., 2020, Czesnikiewicz-Guzik et al., 2008). This difference between CD8⁺ T cells and CD4⁺ T cells may be related to the fact that CD4⁺ T cells can differentiate into a variety of subsets

(e.g. T_h1 , T_h2 , T_h9 , T_h17 , T_{regs}) and mitochondrial respiration may play a role in this lineage flexibility, whereas $CD8^+$ T cells are mostly of the same subtype (cytotoxic) and therefore also more limited to one type of energy production. Overall, autophagy may limit genotoxic ROS production by removing dysfunctional mitochondria and ensuring mitochondrial quality is maintained. Hence age-related decreases in autophagy may contribute to both telomere attrition and oxidative DNA damage during T cell ageing and senescence.

1.3.2.2 Nutrient competition by regulatory T cells

Effector immune cell responses are fuelled by high levels of nutrients; cells which outcompete the effector cells for these nutrients can therefore restrict and regulate immune responses. While failure of mitochondrial biogenesis may be key to $CD8^+$ T cell senescence, nutrient competition has been suggested to induce DNA damage and senescence in $CD4^+$ T cells. Regulatory T cells (T_{regs}) play a role in restricting immune activation by inhibiting immune cell proliferation to prevent pathological hyperinflammatory responses. Naïve $CD4^+$ T cells, when co-cultured *in vitro* with $CD25^{high}FoxP3^+ CD4^+ T_{regs}$, displayed increased DNA damage markers ($\gamma H2AX$ and 53BP1) and active DDR signalling (p-ATM and p-Chk2) as well as other senescence phenotypes, including cell cycle arrest and high senescence-associated β -galactosidase activity (Liu et al., 2018, Ye et al., 2012). Since co-culturing in high glucose (25–35 mM) prevented these DNA damage phenotypes, this indicates that insufficient glucose, potentially due to the high consumption by T_{regs} , may be involved. One explanation for this could be that, in naïve T cells co-cultured with T_{regs} , the resulting low glucose levels and subsequent p-AMPK activation may stimulate mitochondrial biogenesis and a shift from glycolysis to oxidative phosphorylation, driving ROS production and DNA damage (section 1.2.5.1). T_{reg} -mediated immune regulation in this way could plausibly be a short-term advantageous mechanism, as induction of senescence in the effector T cells would limit their functions and therefore suppress an

overexuberant immune response. However, as senescent T cells display an inflammatory SASP and poor proliferative ability, this regulation may have longer-term drawbacks by impairing immune function. Furthermore, this process may be exacerbated in an ageing context where tissues are enriched with T_{regs} (Agius et al., 2009), which could plausibly lead to excessive induction of senescence of effector T cells.

1.3.2.3 *Exposure to infectious agents*

Infections can induce immune cell DNA damage and cellular senescence, directly and indirectly. Since immune cells accumulate at sites of infection, these cell types are ‘first responders’ which are repeatedly exposed to pathogen-derived genotoxic insults. Bacterial genotoxins, such as the typhoid toxin from *Salmonella* Typhi, induce a γ H2AX response induced by a genotoxin, or RING, in human fibrosarcoma cells, which is dependent on ATR-mediated hyperphosphorylation of RPA (Ibler et al., 2019). These RINGs comprise a non-canonical and persistent DDR that induces cellular senescence. Toxin-induced senescent cells have a unique SASP (txSASP) that further drives paracrine senescence and promotes *Salmonella* infection (Ibler et al., 2019). Furthermore, exposure of activated T cells to cytolethal distending toxin, a genotoxin produced by many pathogenic Gram-negative bacteria, induces DNA damage, senescence, and an ATM-induced SASP (Mathiasen et al., 2021). Overall, bacterial genotoxins may have evolved to enhance bacterial infection and transmissibility due to their pro-senescence effects.

Like some bacteria, virus infection is also an inducer of DNA damage and senescence (Lee et al., 2021, Gioia et al., 2023, Chuprin et al., 2013). Virus-induced senescence (VIS) may have evolved as an innate mechanism to inhibit viral exploitation of host replication machinery. However, some viruses evolved to exploit their pro-senescence effects to increase their infection ability. For example, coronavirus (e.g. SARS-CoV-2, which causes

COVID-19) induces cellular senescence and the SASP; this virus-induced SASP promotes upregulation of viral entry proteins, ACE2 and TMPRSS2, and downregulation of antiviral factors in adjacent cells, thus enhancing further infection susceptibility (Lee et al., 2021, Camell et al., 2021). SARS-CoV-2 infection causes DNA damage *in vitro* and *in vivo* in COVID-19 patients: distinct SARS-CoV-2 viral proteins both promote degradation of the DDR enzyme, Chk1, and outcompete 53BP1 for binding to DNA damage-induced long non-coding RNAs, thus preventing repair of DSBs (Gioia et al., 2023). Importantly, removing senescent cells improved the survival of mice after beta-coronavirus infection, suggesting a core role of VIS in the pathology of SARS-CoV-2 viral infection (Camell et al., 2021, Cox and Lord, 2021). Other pathogens, such as parasites belonging to the *Leishmania* genus, have exploited the physiological immunosuppressive impacts of T cell senescence in order to hijack T cell hyper-cytotoxic and hyper-secretory functions and further drive immunopathology (Covre et al., 2018, Covre et al., 2020). Furthermore, IFN- α , as part of the antiviral type I interferon response, may induce senescent phenotypes in T cells by inhibiting telomerase and driving telomere erosion (Lanna et al., 2013, Reed et al., 2004). Therefore, infections may drive immune DNA damage both by direct effects of the infectious agent, and by secondary effects of cytokine release on immune cell biology.

1.3.2.4 Chronic activation and DNA repair factor downregulation

Heightened inflammation with increasing age, termed 'inflammageing', is now recognised as a major hallmark of ageing (Schmauck-Medina et al., 2022, Lopez-Otin et al., 2023), and has been heavily implicated in driving immunosenescent phenotypes. Chronic activation of the immune system, such as in chronic viral infections and autoimmune diseases (e.g. rheumatoid arthritis), fosters a highly inflammatory environment. These disease conditions feature many of the ageing hallmarks, and have therefore been proposed to be conditions of accelerated ageing (Alsaleh et al., 2022). They also mirror the accumulation of senescent

CD28⁻ T cells observed during normal ageing, and so may provide valuable contexts to understand age-related immunosenescence (Alsaleh et al., 2022).

T cells from donors with a chronic virus infection (e.g. HBV, HCV or HIV) have short telomeres and display high levels of DNA damage (Ji et al., 2019); consistent with this, the severity of liver fibrosis negatively correlates with T cell telomere length in chronic HCV infection (Hoare et al., 2010, Hohensinner et al., 2011). T cells from chronically virus-infected donors are hypersensitive to DNA-damaging agents such as camptothecin, a topoisomerase I (TOP1) inhibitor (Ji et al., 2019). The decrease in T cell genome stability in these patients can be attributed to i) chronic activation-induced proliferation leading to telomere erosion and ii) decreased expression and activity of DNA repair factors, such as TOP1, ATM, and the telomere shelterin complex component, TRF2 (Zhao et al., 2018, Ji et al., 2019, Nguyen et al., 2018). Importantly, restoring expression of the DDR regulator, ATM, reduced IL-2 production by the T cells of patients with chronic HCV infection (Zhao et al., 2018).

Patients with autoimmune diseases, such as rheumatoid arthritis (RA), display an expansion of senescent T cells with a high burden of DNA damage (Hohensinner et al., 2011, Shao et al., 2009). These cells display critically short telomeres, which may be related to impaired upregulation of telomerase upon activation in naïve CD4⁺ T cells (Fujii et al., 2009). Furthermore, CD4⁺ T cells from RA patients display an increased burden of DNA damage, both at baseline and in response to ionising radiation (Shao et al., 2009). As with chronic HCV infection, the genome instability in T cells from RA patients is partly attributed to insufficiency of the DDR enzyme, ATM, and can be rescued by ectopic ATM overexpression (Shao et al., 2009). Furthermore, senescent CD4⁺ T cells from RA patients are deficient in their expression of the DNA repair nuclease, MRE11A (which forms part of the MRN complex in the early DDR, Figure 1.1), leading to their increased telomeric damage and cytotoxic phenotypes (Li et al., 2016).

Thus, in the contexts of chronic immune activation where accelerated immune ageing is observed, genome instability arises because of insufficiency in several DNA repair pathway enzymes. It is unclear why DNA repair factor expression and/or activities decrease in these conditions, and whether boosting DNA repair processes may be therapeutically advantageous. It remains to be assessed whether the phenotype of reduced DNA repair factor expression is present in normal cellular ageing. This is one of the focuses of my thesis.

1.3.3 DNA damage drives maladaptive senescent T cell phenotypes

T cells experience DNA damage across the human lifespan. Chronic DNA damage leads to persistent DDR signalling, but without completion of DNA repair this sustained DDR experienced by senescent T cells contributes to their maladaptive phenotypes. Activation of ATM during the DDR drives activation of the metabolic master regulator AMPK, which complexes with sestrin 2, a stress-response protein, to activate several downstream mitogen-activated protein kinases (MAPKs) – namely, ERK, JNK, and p38 (Lanna et al., 2017). Activation of these individual MAPKs induces distinct senescence phenotypes such as impaired proliferation, telomerase insufficiency, hypersecretion (i.e., SASP), low autophagic flux, and high ROS (Lanna et al., 2017). Knockdown of sestrin 2 or pharmacological inhibition of MAPKs rejuvenated senescent T cell phenotypes and, interestingly, reduced γ H2AX levels (Lanna et al., 2017, Henson et al., 2014). This indicates a two-way relationship between DNA damage-ATM activation, and AMPK-sestrin 2-MAPK activation, potentially mediated by the effects of reducing ROS levels through MAPK inhibition (Lanna et al., 2017).

DNA damage and ATM activation in senescent T cells also elicits gain-of-function characteristics, via sestrin 2, that may drive immunopathology in inflammatory contexts

such as ageing and autoimmune diseases. Senescent CD8⁺ T cells acquire an NK cell-like phenotype (Strauss-Albee et al., 2014), upregulating a range of NK receptors (NKR) such as the activating receptor NKG2D, which is dependent on the expression of sestrin 2 (Pereira et al., 2020). Senescent NKG2D⁺ T cells displaying defective TCR-dependent signalling are capable of an alternative NKG2D-mediated activation to elicit their cytotoxic functions (Pereira et al., 2020). This process could feasibly have evolved to compensate for the declining canonical T cell activation response in ageing. However, in the context of autoimmune disease, it has been suggested that NKR-activated T cells may drive the destruction of self-tissues, which upregulate NK ligands due to the high-stress inflammatory environment (Covre et al., 2020, Pereira et al., 2020). This has been hypothesised to drive immune-mediated tissue damage, implicating a causal role of senescent T cells in driving immunopathology. Hence DNA damage and ATM activation in senescent T cells can drive both loss-of-function (e.g. poor proliferation) and gain-of-function (e.g. NKR upregulation) phenotypes, both of which contribute to poor antigen-specific immunity and immunopathology during ageing.

1.3.4 Non-immune cell senescence promotes immunosenescence

Immunosenescence is also driven by the increasingly inflammatory niche of older tissues, due to the increased burden of senescent non-immune 'stromal' cells (Chambers et al., 2021a). Since persistent DNA damage is a major driver of senescence (section 1.2.5), efforts to promote DNA repair may attenuate senescent cell burden and the inflammatory tissue environment, with the potential to rejuvenate immune responses during ageing.

1.3.4.1 Mechanisms of senescent cell accumulation during ageing

Immunosenescence both drives, and is driven by, stromal cell senescence of non-immune cells. In healthy young individuals, turnover of senescent cells is homeostatically regulated: senescent cells are generated (for example, acutely at sites of wounds (Demaria et al., 2014)) and then killed by immune cells such as CD8⁺ T cells and Natural Killer (NK) cells, and cleared by macrophages through phagocytosis (Pereira et al., 2019, Oishi and Manabe, 2016). However, senescent cells accumulate during ageing (Tuttle et al., 2020), both due to an increase in stressors that generate senescent cells, and through failure of senescent cell clearance due to immunosenescence (Pereira et al., 2019, Ovadya et al., 2018). Impaired immunosurveillance with ageing is linked to intrinsic defects in immune system function; for example, heterochronic bone marrow transplantation (BMT) from old to young mice led to a marked increase in lung adenocarcinoma growth, compared to young-to-young or young-to-old BMT, suggesting intrinsic age-related defects in immunosurveillance of cancer neoantigens (Park et al., 2024). Furthermore, senescent cells escape immunosurveillance by increasing their expression of immune evasion molecules such as HLA-E, thus inhibiting their clearance by engaging the immunosuppressive T cell NKG2A receptor, an NKR which is upregulated upon T cell senescence (Pereira et al., 2019, Pereira et al., 2020). Similarly, senescent cell PDL-1 expression prevents clearance by engaging the immune checkpoint inhibitor, PD-1, on CD8⁺ T cells (Wang et al., 2022b). Furthermore, NK cells from older people are defective in their ability to deliver cytotoxic granules to the immune synapse (Hazeldine et al., 2012), which may impair their ability to kill senescent cells. The ability of macrophages to perform phagocytosis and efferocytosis also becomes defective during ageing (Solana et al., 2012, Stranks et al., 2015, De Maeyer et al., 2020). Notably, strategies that remobilise the immune system to target senescent cell (or 'seno'-) antigens using chimeric antigen receptor (CAR) T cells (Amor et al., 2020), seno-directed vaccines (Suda et al., 2021), and immune

checkpoint blockade (Wang et al., 2022b), have shown success in attenuating senescent cell burden and promoting health in ageing mice.

The aged immune system is also capable of inducing senescence of non-immune cells. In the *Vav-iCre^{+/+};Ercc1^{-fl}* immunosenescence model, DNA damage in haematopoietic cells alone led to accelerated ageing and increased senescent cell burden in non-immune tissues, indicating a potential role of bystander/paracrine senescence between the different cell types (Yousefzadeh et al., 2021b). Immune cells also perpetuate tissue senescence by inducing DNA damage. Senescent liver cells secrete IL-8/CXCL8 within their SASP, which recruits neutrophils that release ROS that in turn damage the telomeres of bystander hepatocytes in a process termed TelOxidation (Lagnado et al., 2021). Moreover, TelOxidation may be exacerbated during ageing through non-directional tissue infiltration by neutrophils with impaired chemotaxis, which may cause widespread collateral oxidative damage, through release of ROS and tissue-remodelling enzymes (e.g. elastase), leading to bystander senescence (Sapey et al., 2014). Furthermore, neutrophil adherence to senescent endothelial cells *in vivo* promotes microvascular permeability, leading to aberrant tissue inflammation and fibrosis (Rolas et al., 2024). Therefore, DNA damage of cells both within and outside of the immune system contributes to the increased senescent cell burden and subsequent inflammatory niche, which is capable of impairing immunity during ageing.

1.3.4.2 The SASP suppresses antigen-specific immunity

Senescent cells compromise adaptive immunity against pathogens in an aged context. The primary mechanism by which this occurs is through the secretion of components of the SASP. For example, the cytokine CCL2/MCP-1, produced by senescent fibroblasts in culture and the skin of old people, is an inflammatory monocyte chemoattractant (Pereira et al., 2019, Chambers et al., 2021a). Monocytes are recruited to the skin at sites of

cutaneous immune challenge in old people, and through their release of PGE₂, suppress the adaptive proliferative responses of local resident T cells (Chambers et al., 2021a). SASP components also impact the differentiation of effector T cells recruited during an immune response and promote an excessively suppressive environment. This may account for the observation that old mice and people exhibit poor clearance of influenza infection compared to that of younger individuals (Lorenzo et al., 2022, Thompson et al., 2004). TGF- β , a component of the SASP, can promote the differentiation of FoxP3⁺ regulatory T cells (T_{regs}). There are more T_{regs} in the lungs of old mice than young mice after influenza infection, and this can be attenuated by mAb-mediated blockade of TGF- β , supporting the importance of TGF- β in immune suppression (Lorenzo et al., 2022). To determine the role of senescent cells in dampening this influenza-specific immunity, old mice were treated prior to influenza infection with dasatinib and quercetin (D+Q), a senolytic combination that inhibits the pro-survival pathways that are hyperactivated in senescent cells. Clearance of senescent cells in this way reduced both TGF- β levels and FoxP3⁺ T_{reg} accumulation, and improved the response to influenza infection, suggesting that senescent cells were responsible for suppressing adequate immune responses during lung infection in old mice (Lorenzo et al., 2022). In younger life, this pathway may be advantageous in restricting an overexuberant response in the lung. However, an over-suppressive immune environment generated by accumulation of T_{regs} may contribute to the impaired clearance of influenza infection in an aged context. Interestingly, a further study reported that D+Q treatment did not improve the overall secondary memory response to influenza infection, suggesting there may be both positive and negative effects of senolytic treatment in this context (Torrance et al., 2023). Similarly to in influenza infection, senescent cells and their SASP drive mortality during coronavirus infection. As discussed earlier (section 1.3.2.3), *in vivo* senolysis of coronavirus-induced senescent cells by the senolytics D+Q or navitoclax reduced peripheral SASP levels, COVID-19-like immunopathology, and mortality in animal

models of the infection (Camell et al., 2021, Lee et al., 2021, Pastor-Fernandez et al., 2023).

It is not clear whether the senolytic removal of certain cell types, such as those either of immune or non-immune origin, would be sufficient to limit the SASP and senescence-related pathology in these diseases.

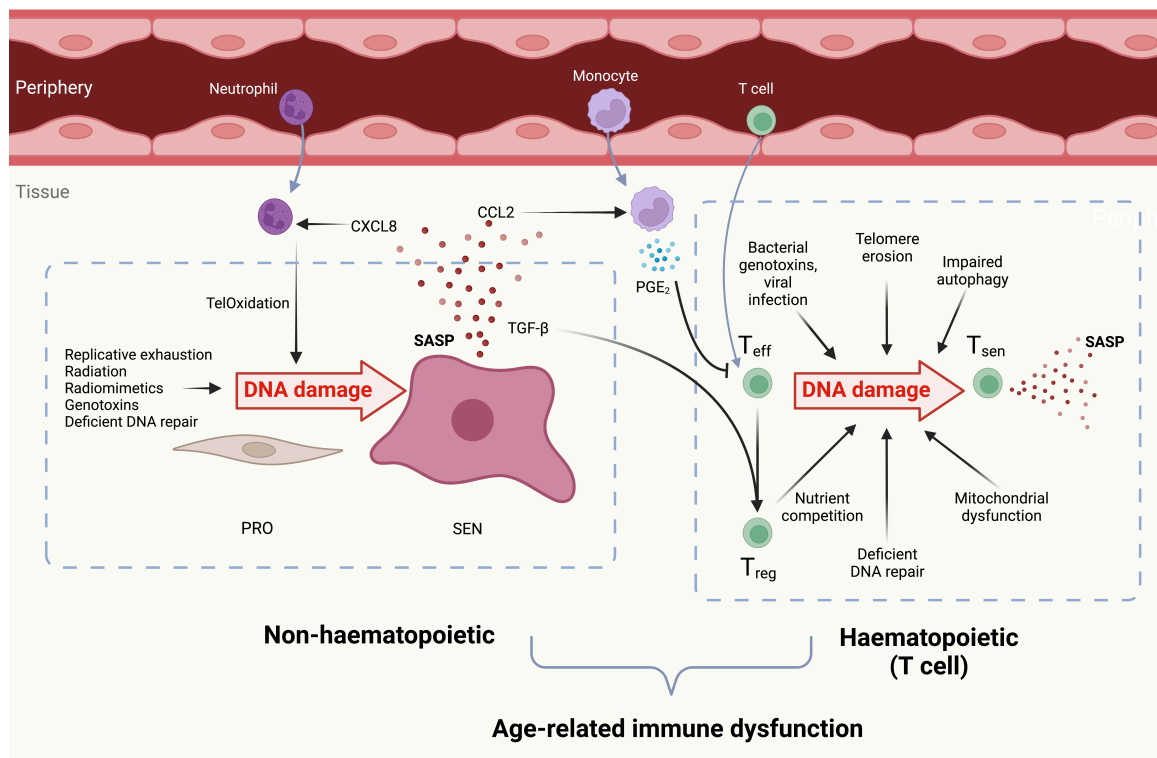


Figure 1.4 Overview of the causes and consequences of DNA damage in both cells of haematopoietic (T cell) and non-haematopoietic origins, which contribute to immunosenescence

DNA-damaged immune and non-haematopoietic cell senescence are linked. During ageing, senescent cells increase in both compartments due to reduced senescent cell clearance, deficient DNA repair processes, and chronic genotoxin abundance.

Non-haematopoietic origin: Proliferating cells (PRO) experience genotoxicity from radiation, replicative exhaustion, environmental pollutants and toxins, and radiomimetics to drive persistent DNA damage, proliferation arrest, and cellular senescence (SEN). The senescence-associated secretory phenotype (SASP) contains chemoattractants such as CXCL8 that draw in peripheral neutrophils, which induce oxidative damage in non-haematopoietic cell telomeric DNA, called TelOxidation, propagating further paracrine senescence. The SASP component CCL2 attracts monocytes which secrete PGE₂ and suppress T cell functions. TGF-β in the SASP favours T_{reg} generation and a pro-suppressive, inadequate immune response during ageing.

Haematopoietic origin: Effector T cells (T_{eff}) experience genotoxic injury from bacterial genotoxins, viral infections, short telomeres, reactive oxygen species (ROS) from dysfunctional mitochondria, and nutrient competition with regulatory T cells (T_{regs}). DNA-damaged T cells manifest senescence phenotypes in a sestrin- and ATM-dependent manner. Senescent T cells (T_{sen}) are dysfunctional and contribute to inflammageing with their inflammatory SASP, tissue cytotoxicity, and immune dysfunction during ageing.

1.4 mTOR inhibition as a geroprotective strategy

1.4.1 mTOR regulation

In addition to senolytic agents, senomodifiers (i.e. drugs which modulate senescent cell behaviour) have shown great promise in extending lifespan and healthspan (Harrison et al., 2009). One of the most well-known senomodifying and geroprotective interventions is inhibition of the mechanistic target of rapamycin (mTOR). mTOR is an atypical PI3K-like kinase which integrates multiple signalling pathways to drive anabolic processes such as protein synthesis, cell proliferation, and cell survival, in the form of two distinct complexes, mTORC1 and mTORC2. The mTOR complexes and their signalling pathways are summarised in Figure 1.5. mTOR signalling can be activated by the presence of the amino acids arginine and leucine, which relieve the inhibition of GATOR1, allowing it to be recruited to the lysosome by the KICSTOR complex, where it activates the RAG complex and RHEB-mediated activation of mTORC1 (Wolfson et al., 2017, Sahu and Ben-Sahra, 2023). RHEB, a GTPase, binds distally from mTOR's kinase active site, and allosterically re-aligns mTOR's active site residues to facilitate mTOR catalytic activity (Yang et al., 2017). Other upstream signals, such as growth factor signalling through Ras, converge upon the phosphorylation and inhibition of the tuberous sclerosis complex, TSC1/2, a negative regulator of mTOR. mTORC1 activity is prevented in low-energy, ATP-scarce contexts, where AMPK activation promotes TSC1/2 complex activity.

mTORC1 activity predominantly drives processes relating to biosynthesis pathways, through phosphorylation events of downstream targets p70S6K and 4EBP1 (Figure 1.5); these, as well as the phosphorylation of ribosomal protein S6, are commonly used readouts for mTORC1 activity (Wolfson et al., 2017). In addition, mTORC1 activation also drives pyrimidine and lipid biosynthesis pathways through the phosphorylation of CAD and lipin 1, respectively (Nakashima et al., 2013, Peterson et al., 2011). mTORC1 activation also

downregulates catabolic processes such as autophagy, through the inhibitory phosphorylations of TFEB and ULK1 (Mannick and Lamming, 2023). mTORC2 activity is often detected by phosphorylation of its target Akt at Ser⁴⁷³; though mTORC2 activity is less well understood, effects on ion transport, cytoskeletal organization, and cell survival are reported to be impacted (Figure 1.5) (Mannick and Lamming, 2023).

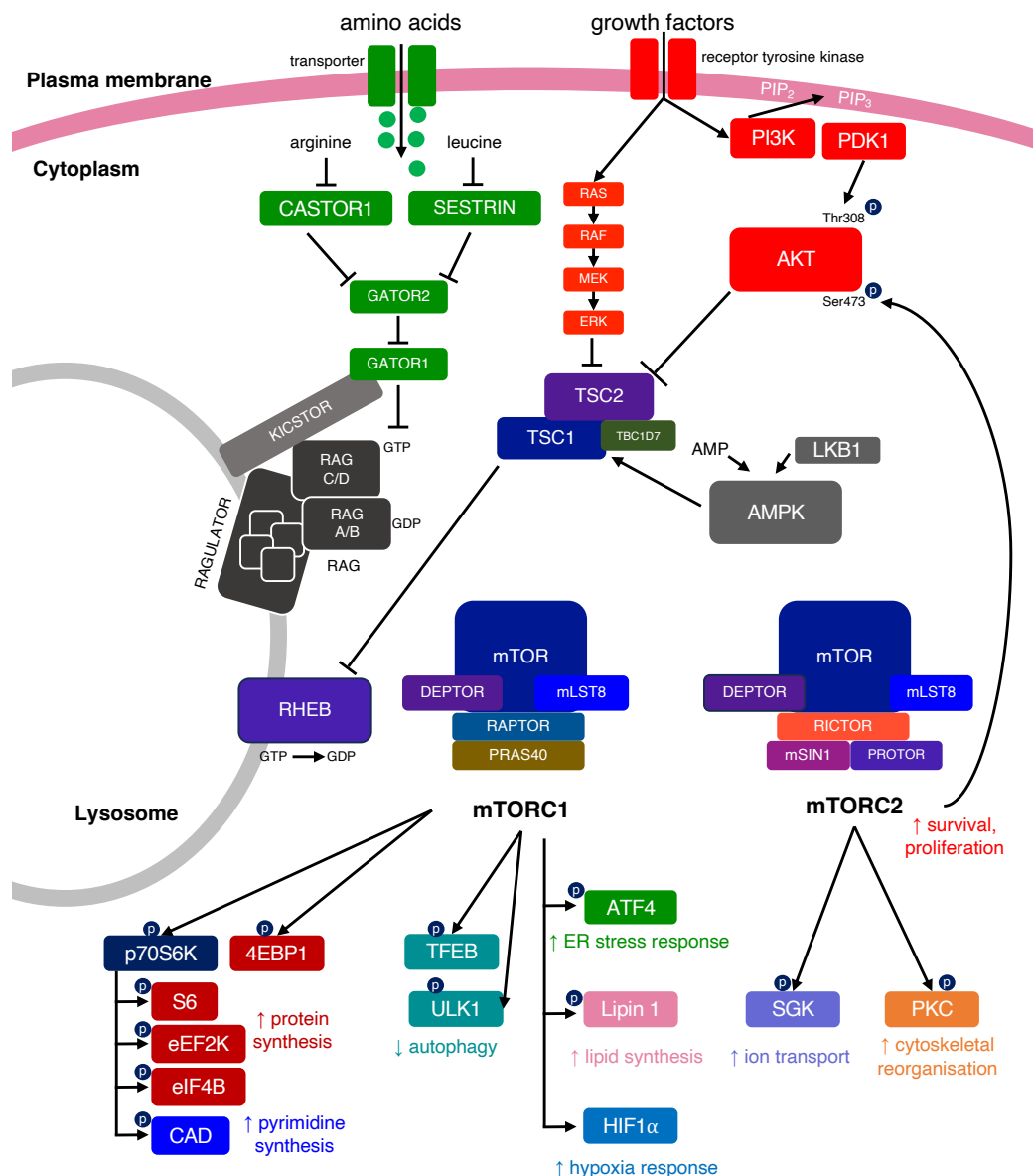


Figure 1.5 Summary of mTOR signalling and regulation

Mechanistic target of rapamycin (mTOR) is a master kinase regulator of nutrient signalling, driving anabolic processes including proliferation, macromolecule synthesis, growth, and survival.

1.4.2 mTOR inhibition attenuates ageing and senescence

mTOR inhibition is one of the most potent life- and healthspan extension interventions studied so far, even when performed late in life (Harrison et al., 2009). mTOR inhibition improves survival in a wide range of mouse disease models, (reviewed recently (Mannick and Lamming, 2023)). Though 6 trials using mTOR inhibitors in humans for age-related conditions have been completed, many age-related diseases which would be amenable to mTOR inhibition have yet to be tested (Walters and Cox, 2018, Mannick and Lamming, 2023).

mTOR inhibition is also one of the few interventions which has been shown to improve immunity in older people – i.e., to attenuate immunosenescence (discussed in greater detail in section 5.1.3). The mechanism behind the immunoprotection afforded by mTOR inhibition is not fully understood, though it has been found to increase peripheral antiviral gene expression (Mannick et al., 2021). Another mechanism may be its impact on cellular senescence. mTOR activity increases during the *in vitro* senescence of primary human fibroblasts (Walters et al., 2016, Carroll et al., 2017). Additionally, *Tsc2*^{-/-} mouse embryonic fibroblasts, which display constitutive mTORC1 activation (as measured by p-S6K), enter premature replicative cell senescence *in vitro* (Zhang et al., 2003). *In vivo*, mTOR activity increases in normal human muscle ageing (Markofski et al., 2015). Furthermore, long-lived mouse strains (e.g. Snell dwarf and GHR^{-/-}) display lower mTOR activity across multiple tissues compared to wildtype strains (Dominick et al., 2015). Consistent with a role of mTOR in ageing and senescence, mTOR inhibitors attenuate a variety of senescence phenotypes and extend replicative lifespan *in vitro* (Walters et al., 2016, Park et al., 2020a, Rolt et al., 2019). In humans, rapamycin (an mTORC1 inhibitor) reduced the presence of p16⁺ cells in the dermis when administered as a topical skin cream (Chung et al., 2019). Thus, mTOR inhibitors are a promising avenue for attenuating cellular senescence and immune resilience during ageing, though its mechanisms of action are not fully understood.

As discussed earlier, DNA damage is both a major driver and feature of cellular senescence and may be a significant causal factor in immunosenescence (Kell et al., 2023). However, how mTOR inhibition impacts DNA damage and repair mechanisms in the context of cellular senescence and immunosenescence has not been fully determined. This is the focus of my thesis.

1.5 Thesis aims

Hypothesis: *immunosenescence is underpinned, at least in part, by an increased burden of senescent cells of immune and non-immune lineage; treatments that limit senescence by targeting central ageing pathways (such as mTOR hyperactivation and chronic DNA damage signalling) may therefore boost immunity in older people.*

In this thesis, I aim to evaluate the effects of geroprotectors on DNA damage and cellular senescence in both non-immune cells (fibroblasts) and immune cells (primarily T cells), as both immune and non-immune compartments contribute to immunosenescence (Figure 1.4). I aim to investigate the roles of mTOR hyperactivity and expression of the DNA repair factor, *WRN*, in DNA damage-induced senescence in fibroblasts. Similarly, I aim to understand how features of cellular senescence, mTOR activity, and DNA damage, change in age-associated peripheral immune subsets. Accordingly, I aim to explore how mTOR inhibition affects senescence and survival after DNA damage using *in vitro* assays with both fibroblasts and human T cells. Finally, I aim to assess how *in vivo* treatment of older individuals with an mTOR inhibitor, rapamycin, affects broad features of immunosenescence. Overall, my goal is to understand how mTOR inhibition intersects with known nodes of immunosenescence, i.e. DNA damage and cellular senescence, and therefore to elucidate the mechanisms by which mTOR inhibitors promote immunoprotection in older people.

2

Materials and methods

2.1 Human research

2.1.1 Ethics

Healthy control blood was taken with fully informed consent under ethical approval from the Local Research Ethics Committee (REC) in Oxford, reference 11/H0711/7, to cover the use of human blood products purchased from National Health Services Blood and Transplant service (NHS England). Peripheral blood mononuclear cells (PBMCs) from donors with osteoarthritis were kindly provided by Professor Janet Lord under ethical approval by the University of Birmingham REC, reference ERN_12-1184R2. PBMCs from participants of the Rapamune Trial were obtained under ethical approval by the University of Nottingham FMHS REC, reference FMHS 90-0820, provided by Professor Philip Atherton.

2.1.2 *In vivo* rapamycin treatment

In the ongoing single-blind, placebo-controlled Rapamune trial (ClinicalTrials.gov identifier NCT05414292), older male participants (between 50-90 years old) are randomised into two groups and receive either 1 mg/day rapamycin (Pfizer, Belgium) or a placebo sucrose/lactose tablet (Hsconline). Only male participants were included initially in this pilot study to limit inter-participant variability; as the trial is ongoing, female participants will be included in the future. The primary outcome is change in muscle mass in response to unilateral leg extension resistance exercise training (RET) 3 times per week for 14 weeks at 75% of the participant's 1 repetition maximum. Secondary outcomes include changes in muscle strength, power, function, neuromuscular function, and assessment of muscle biopsies on protein synthesis and degradation. In the study reported in this thesis (section 5.2.3), PBMCs were isolated at 5 time points over 4 months of intervention (rapamycin and placebo). After 8 weeks of intervention, the blood concentration of rapamycin and white blood cell counts were measured by Prof. Dan Wilkinson at the University of Nottingham.

Inclusion criteria for the trial were that the participant could give informed consent to participate in the study, and that they were able to complete the RET. Exclusion criteria included having a BMI <18 or >35 kg/m², active cardiovascular, cerebrovascular, respiratory, or metabolic disease, clotting dysfunction, having a history of neurological or musculoskeletal conditions, having taken part in a recent study in the last 3 months, or contraindications either to MRI scanning or rapamycin. The study did not explicitly exclude participants if they were taking immunomodulatory drugs, since the addition of taking blood for immune analysis was an amendment to the approved trial design. However, given the strict disease exclusion criteria outlined above, participants were generally healthy and active.

2.2 Cell culture

2.2.1 Fibroblast subculture

Primary human neonatal foreskin fibroblasts, HF043 (Dundee Cell Products) were cultured in DMEM (Gibco) supplemented with 10% heat-inactivated FBS and 1x Glutamax (Gibco) without antibiotics at 37°C in a humidified incubator with 5% CO₂. Cells were observed using an EVOS Core microscope (Life Technologies) and harvested using TrypLE Express Enzyme (Invitrogen). Cell numbers were counted using a Cellometer T4 (Nexcelcom). Fibroblast population doublings were determined using the formula below. Cumulative population doublings (CPD) were determined by summing PD values over time.

$$\text{Population doubling (PD)} = \frac{\log_{10} \left(\frac{\text{total cells harvested}}{\text{total cells seeded}} \right)}{\log_{10} 2}$$

Details of all drugs used in this thesis are provided in Table 2.1.

2.2.2 PBMC isolation and culture

Fresh blood was either collected in EDTA tubes (9 ml) or in blood cones (10 ml) as concentrated by-products of the apheresis process, supplied by the National Health Service Blood and Transplant service (NHS England). NHS-BT blood cones were used as healthy controls (considered to be from donors <55 years old) throughout the *in vitro* experiments in Chapter 4. Blood cones were used as they provide a large number of cells which could be used across multiple different experiments to understand cell-intrinsic mechanisms while limiting effects due to donor variability; in this way, results from different techniques could be more easily cross-compared and interpreted as effects from the cell treatments rather than donor effects.

For PBMC isolation, PBMCs were isolated using standard Ficoll density gradient centrifugation. Briefly, blood was diluted 1:1 in sterile Dulbecco's PBS (DPBS, 1:5 for blood cones) (Sigma-Aldrich) and 15 ml gently pipetted over 20 ml Histopaque-1077 (Sigma) before centrifugation at 500g for 30 mins at room temperature with minimum deceleration. The PBMC layer was collected by aspiration and washed twice in DPBS. PBMC number was determined by mixing 1:1 (v/v) with Trypan Blue (Sigma) and counting using a haemocytometer. For cryopreservation, PBMCs were resuspended at 5×10^6 cells/ml in freezing medium (50% FBS, 40% RPMI 1640, 10% DMSO (all Sigma)) and placed at -80°C before transferral to a liquid nitrogen facility (-196°C) for long-term storage.

For *in vitro* experiments, PBMCs were cultured in 'R10' (RPMI 1640 (Gibco) containing penicillin (100 U/ml) and streptomycin (100 $\mu\text{g/ml}$) (both Sigma) and 10% FBS), in a humidified incubator with 5% CO_2 at 37°C .

Table 2.1 Details of drugs

Name	Target	Final concentration	Solvent	Manufacturer	Cat. code
Mitomycin C	Interstrand crosslinker	100 nM	DMSO	Abcam	ab120797-2mg
Zeocin	DSB inducer	200 µg/ml	H ₂ O	Invitrogen	R25001
Rapamycin	mTORC1 inhibitor	10 nM	DMSO	Alfa Aesar	J67452
AZD8055	Pan-mTOR inhibitor	70, 100 nM	DMSO	Strattech Scientific	A8214-APE-10mM
Bafilomycin A ₁	Autophagy inhibitor	10 nM	DMSO	Sigma	B1793-10UG
Hydrogen peroxide	Oxidative stress	25 µM	PBS	Sigma	H1009-100ML
NSC 19630	WRN helicase inhibitor	0.01 – 100 µM	DMSO	Merck Millipore	681647-5MG
Cycloheximide	Translation inhibitor	50 µg/ml	DMSO	Sigma	239763-M

N.B. DMSO vehicle controls always contained between 0.1-0.3% DMSO (v/v) in the cell culture media.

2.2.3 Fibroblast viability assay

Fibroblast viability (Figure 3.4E, Figure 3.7D) was assessed by replacing cell culture media with media containing 1X alamarBlue™ Cell Viability Reagent (Invitrogen). After incubating at 37°C for 3-5 hours, the cell culture media fluorescence at Ex/Em 540/590 nm was measured by a BMG PheraStar FS plate reader blanked against media and alamarBlue without cells. Drug cytotoxicity was calculated using the fluorescence values of treated cells normalised to negative untreated (taken as 0% cytotoxicity) and positive H₂O₂-treated (100% cytotoxicity) controls using the following formula:

$$\text{Normalised cytotoxicity} = 1 - \left(\frac{\text{Treated} - \text{H}_2\text{O}_2}{\text{Untreated control}} \right) * 100$$

2.2.4 Absorbance of phenol red in cell culture supernatants

To assess pH of cell cultures, phenol red was used as an indicator. After 3 days of cell culture as in Figure 4.3, 150 μ l of PBMC cell culture supernatant was transferred to wells of a 96-well plate and the ratio of absorbance at 450/560 nm was measured using a CLARIOstar microplate reader (BMG Labtech).

2.2.5 DNA damage assay in PBMCs

Cryopreserved PBMCs were thawed in R10 (RPMI 1640 (Gibco) containing penicillin (100 U/ml) and streptomycin (100 μ g/ml) (both Sigma) and 10% FBS, see section 2.2.2) (10 ml per 1 cryovial of cells) and centrifuged at 500g for 5 mins. The supernatant was removed, and cells resuspended in R10 at a concentration of 1×10^6 cells/ml and allowed to recover overnight in R10. The next day, PBMCs were subjected to T cell-specific activation using antibodies targeting CD3 (clone OKT3) and CD28 (clone CD28.2) at 1 μ g/ml final concentration (Table 2.4), in the presence of drug treatment or vehicle (Table 2.1), where appropriate, for 3 days. Where specified for individual experiments, CD4⁺ T cells were isolated by negative selection on a magnetic column using a kit (Miltenyi) and subsequently activated in wells of a 24-well plate (pre-coated with 1 μ g/ml anti-CD3 in PBS for 2 hours at 37°C) with 1 μ g/ml anti-CD28 in the culture media, for 3 days (0.5 - 1×10^6 cells/ml). After 3 days, cells were harvested by trituration, centrifuged at 500g for 5 mins, and resuspended in R10 with 200 μ g/ml zeocin, or untreated, and incubated for 2 hours in a 24-well plate. Cells were then washed and allowed to recover for a defined period (see individual figures) before being collected for further downstream analysis. For conventional flow cytometry 100-200,000 events were acquired on a BD LSRFortessa, and live CD4⁺ and CD8⁺ T cells were identified in FlowJo version 10.9.0 using the gating strategy outlined in Figure 2.1.

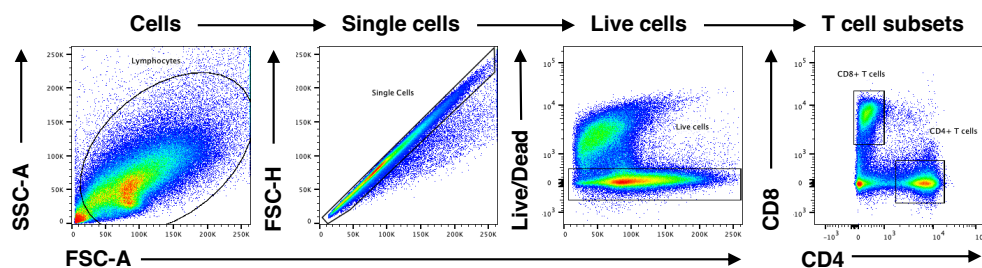


Figure 2.1 Gating strategy for CD4⁺ and CD8⁺ T cells using conventional flow cytometry after *in vitro* T-cell-specific activation of PBMCs

Total cells are gated based on size (forward scatter, FSC-A) and granularity (side scatter, SSC-A) to remove debris. Single cells are determined by their direct proportionality between FSC-area (FSC-A) and FSC-height (FSC-H). Live cells are identified by their absence of fluorescence of a membrane-permeable dye. T cell subsets are then identified by their high expression of either CD4 or CD8.

2.3 RNA and DNA work

2.3.1 RNA and DNA isolation

For RNA isolation of fibroblasts, 2-10x10⁵ cells were pelleted by centrifugation at 1200 rpm for 3 mins, and RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. DNA was isolated using a Quick-DNA Miniprep Plus Kit (Zymo Research). RNA and DNA yield and purity were determined by absorbance ratios at 260/280 and 260/230 nm respectively, blanked against elution buffer, using a DeNovix spectrophotometer. Values between 1.8 – 2.2 were deemed acceptable for further analysis.

2.3.2 qRT-PCR

For quantitative reverse transcription PCR (qRT-PCR) of RNA from fibroblasts, 20 ng of RNA obtained as above was used to evaluate the expression of target genes using TaqMan Real-Time PCR Assays (Thermo Fisher Scientific) in a one-step reaction using a QuantiTect Multiplex PCR Kit (Qiagen) in a StepOnePlus RT-PCR machine (Table 2.2) (Applied Biosystems). The expression of target genes was measured relative to the

expression of an internal housekeeping gene (*GUSB*), duplexed in the same well, using the $\Delta\Delta C_t$ method. *GUSB* was selected as a housekeeping gene as its expression has been shown previously to remain broadly constant during *in vitro* senescence (Gonzalez-Bermudez et al., 2019).

Table 2.2 (A) TaqMan assays and (B) cycling conditions for qRT-PCR

(A) PRIMERS				
	Target	Assay ID	Dye	Company
	<i>WRN</i>	Hs00172155_m1	FAM	Thermo Fisher Scientific
	<i>GUSB</i>	Hs00939627_m1	VIC	Thermo Fisher Scientific
	<i>CDKN1A</i>	Hs00355782_m1	FAM	Thermo Fisher Scientific
	<i>CDKN2A</i>	Hs00923894_m1	FAM	Thermo Fisher Scientific

(B) CYCLING CONDITIONS				
		Step	Time	Temperature
		Activation	15 mins	95°C
		Denaturation	60 s	94°C
	40 x 2-step cycling	Annealing/extension	60 s	60°C

2.3.3 Methylation-specific PCR

All methylation-specific PCR experiments were performed with Dr Ivan Boubriak. Bisulfite conversion of fibroblasts at high- or low- CPD, and genomic DNA standards (Zymo Research), was performed either directly from 2-10x10⁵ cells (using an EZ Methylation-Direct Kit) or from isolated DNA (EZ DNA Methylation-Lightning Kit) all according to manufacturer's instructions (both Zymo Research). Methylation-specific PCR was performed in a final volume of 50 μ l with JumpStart REDTaq ReadyMix Reaction Mix (Sigma) with 0.5 μ M forward and reverse primers using 50 ng of bisulfite-converted DNA (Table 2.3). Primers were complementary to sequences at -36 (sense) and +129 (antisense) from the transcription start site (Agrelo et al., 2006). DNA gel electrophoresis was performed in 2.2% agarose (Lonza) containing ethidium bromide (0.5 μ g/ml) in TBE buffer and bands imaged on an Alpha Innotech Gel Imaging System. Approximate sizes of the DNA products were determined using a Quick-Load® Purple Low Molecular Weight DNA Ladder (NEB).

Table 2.3 Details of methylation-specific PCR of the *WRN* promoter

(A)		PRIMERS	
	Target	Direction	Sequence (5'-3')
	Unmethylated	Forward	<i>gcgggtcccaaaagggtcagt</i> GTAGTTGGGTAGGGGTATTGTTTGT
	Unmethylated	Reverse	<i>gcgggtcccaaaagggtcagt</i> AAACAAAATCCACCACCCACCCC
	Methylated	Forward	<i>gcgggtcccaaaagggtcagt</i> CGGGTAGGGGTATCGTTCGC
	Methylated	Reverse	<i>gcgggtcccaaaagggtcagt</i> AACGAAATCCACCGCCCGCC
(B)		CYCLING CONDITIONS	
	Step	Time	Temperature
40 cycles	Activation	15 mins	94°C
	Denaturation	30 s	94°C
	Annealing	45 s	66°C
	Extension	30 s	72°C
	Final extension	10 mins	72°C

All primer sequences were designed and validated previously (Bosch et al., 2016). Lower case letters represent 5' GC-clamp sequences.

2.4 Phage display

2.4.1 Biopanning

Phage display was performed using a Ph.D.TM-12 Phage Display Peptide Library Kit (E8110S, NEB) to ascertain 12-mer peptides that bind to WRN helicase. Purified WRN helicase (residues 520-1242 with a C-terminal Avi-tag), was subsequently biotinylated and gifted by Opher Gileadi and Joseph Newman (Structural Genomics Consortium, University of Oxford). All phage display experiments were performed with Dr Ivan Boubriak.

Biopanning was performed in three successive rounds (I-III) with decreasing concentrations of biotinylated WRN (WRN-biotin). To control for phage bound to streptavidin or biotin and plastic, biopanning was also performed on streptavidin-coated plates ('Strep only') and streptavidin-coated plates that had been blocked with biotin ('Strep/biotin'). Any phage sequences obtained from these conditions were removed from the sequences obtained from WRN-biotin biopanning.

For biopanning I, 55 mm petri plates were first coated overnight in a humidified container at 4°C with 1.5 ml 100 µg/ml streptavidin in 0.1 M NaHCO₃, pH 8.6. The next day, the coating solution was removed and plates were blocked with 4 ml 0.1 M NaHCO₃, pH 8.6,

containing 5 mg/ml BSA, 0.1 μ g/ml streptavidin, and 0.02% NaN₃ (all Sigma), in a humidified container at 4°C for 1 hour. The blocking solution was discarded and plates were washed 6 times with TBS containing 0.1% (v/v) Tween-20 (0.1% TBS-T).

For biopanning I of WRN-biotin, 36 μ g of biotinylated WRN was combined with 5 μ l phage library at a final volume of 500 μ l in 0.1% TBS-T in a 1.5 ml low-bind microcentrifuge tube (Eppendorf) for 50 mins. Solution was then transferred to a blocked streptavidin-coated dish and incubated for 10 mins at room temperature on a shaking platform (80 rpm). 5 μ l of 10 mM biotin (final concentration 100 μ M) was subsequently added for a further 5 mins, to compete away any streptavidin-bound phage. For biopanning of control conditions, 5 μ l of the original Ph.D.-12 Library (NEB) in 500 μ l TBS-T was incubated on blocked plates for 1 hour at room temperature on a shaking platform (80 rpm).

Following incubation on plates, supernatant containing unbound phage was discarded, and plates were washed 10 times in 0.1% TBS-T. Bound phage were eluted in 1 ml 0.2 M Glycine-HCl, pH 2.5, containing 1 mg/ml BSA, for 15 mins at room temperature on a shaking platform (80 rpm). Phage eluates were then transferred to 1.5 ml microcentrifuge tubes and pH neutralised by addition of 200 μ l 1 M Tris-HCl, pH 8.8. Eluted phage numbers were assessed by 10-fold serial dilution titration (Figure 2.2) and amplified according to manufacturer's instructions. Briefly, overnight cultures of ER2738 *E. coli* were infected with 1 μ l phage eluates and left to shake at 37°C, 250 rpm for 4 -5 hours. Cells were removed by centrifugation at 4500g for 10 mins, the supernatant transferred to fresh tubes, and centrifugation repeated. 4 ml of 2.5 M NaCl/20 % PEG-8000 (w/v) was added to 16 ml of the phage-containing supernatant and left to precipitate overnight at 4°C. Amplified phage was then centrifuged at 12000g for 15 mins, and the pellet resuspended in 1 ml TBS and transferred to a 1.5 ml tube. After another brief centrifugation to remove debris, supernatant was transferred to another 1.5 ml tube and 200 μ l 2.5 M NaCl/20% PEG-8000 (w/v) was added and phage incubated on ice for 60 mins. Phage were then centrifuged at 12000-

14000 rpm in a benchtop centrifuge for 10 min, supernatant discarded, and phage pellet finally resuspended in 200 μ l TBS, in advance of the next round of biopanning.

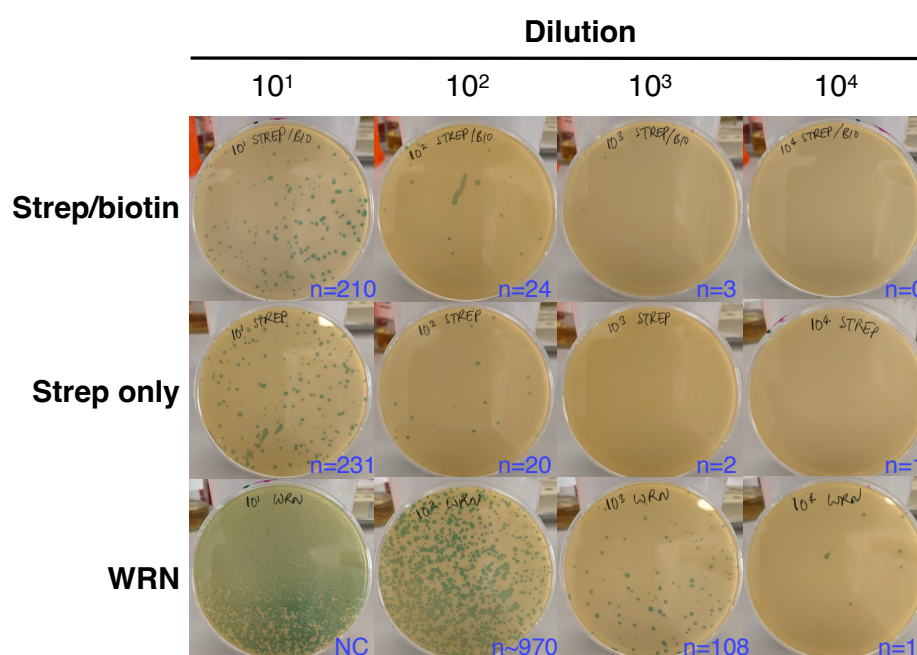


Figure 2.2 Example of phage eluate titering

Images of *E. coli* K12 ER2738 infected with increasing dilutions of phage display eluates from biopanning I. The number (n) of plaques counted at each dilution from the three conditions (streptavidin/biotin and streptavidin only controls, and WRN-biotin test) is shown. NC = not countable.

The following two rounds of biopanning (II and III) were performed as in biopanning I, with the following modifications: to increase stringency of selection conditions, the streptavidin concentration for coating plates was reduced 10-fold for each subsequent biopanning: i.e. 10 μ g/ml for biopanning II and 1 μ g/ml for biopanning III. The quantity of WRN-biotin was decreased 10-fold for each following biopanning: i.e. 3.6 μ g for biopanning II and 0.36 μ g for biopanning III. Finally, plates were washed more stringently with 0.5% Tween in TBS (v/v) for both biopanning II and III.

2.4.2 Elution and sequencing

After biopanning III, eluted phage were amplified and titered in the presence of chromogenic substrate, X-gal, as per the manufacturer's protocol. For each condition, blue plaques were selected, phage amplified, and ssDNA was subsequently purified and stored in 0.1X TE buffer (Thermo Scientific). Phage DNA concentration and purity was assessed by spectrophotometry (section 2.3.1) and sequenced with a -96 gIII primer (5'-CCCTCATAGTTAGCGTAACG-3') by SourceBioscience. Sequences reflecting the random 36-mer nucleotide inserts in phage coat gene III, expressed as 12-mer peptides on the phage surface, were analysed and conceptually translated *in silico* using SnapGene version 5.3.2. 12-mer motifs were aligned with human proteins using BLASTp, adjusted for short sequence input (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>).

2.5 Senescence assays

2.5.1 Sulphorhodamine B staining

Sulforhodamine B (SRB) staining of free amines to assess cell morphology was performed as described previously, without dye solubilisation (Vichai and Kirtikara, 2006). Briefly, ice-cold 10% trichloroacetic acid (w/v) was added to cell cultures (final concentration 3.3%) and fixed for 1 hour at 4°C, then cells were washed 4 times with H₂O. After air-drying at room temperature, cells were stained with 100 µl 0.057% SRB solution (w/v) dissolved in 1% acetic acid (v/v) for 30 mins. Cells were then washed 4 times with 1% acetic acid (v/v), air-dried, and imaged with an EVOS XL Core digital microscope.

2.5.2 Senescence-associated β -galactosidase

Senescence-associated β -galactosidase activity (SA β G) at pH 6.0 was assessed using a kit (#9860) according to manufacturer's instructions (Cell Signalling Technology).

2.5.3 Fluorescence microscopy

For immunofluorescence staining of adherent fibroblasts, cells in wells of a 96-well optical-bottom plate (Thermo Scientific) were washed 1 x in PBS and fixed for 10 minutes at room temperature (RT) in 3.7% formaldehyde in PBS-T (0.1% Tween-20 in PBS v/v). Cells were washed a further 3 x in PBS and permeabilised for 15 minutes at RT in 0.1% Triton X-100 in PBS, then washed 3 x PBS and blocked for 30 mins in PBS-T containing 1% BSA (w/v) and 300 mM glycine. Cells were then incubated with primary antibodies (Table 2.4) diluted in antibody diluent (1% BSA w/v in PBS-T) for 1 hour at RT, washed 3 x PBS, and incubated for 1 hour RT in the dark with fluorophore-conjugated secondary antibodies (Table 2.4) in antibody diluent. After 2 x PBS washes, nuclei were stained using NucBlue Live ReadyProbe Reagent (Thermo Fisher) diluted in PBS according to manufacturer's instructions for 5 minutes at RT. Cells were then washed two further times in PBS prior to imaging with a ZOE fluorescent microscope (Bio-Rad). Nuclear area was assessed by nuclear segmentation based on NucBlue Live fluorescence, and area measured using an optimised macro within Fiji version 2.3.0. The number of γ H2AX⁺ and 53BP1⁺ foci per nucleus was determined manually by a single investigator following nuclear segmentation in Fiji.

Analysis of fibroblast proliferation was achieved by labelling with 10 μ M 5-ethynyl-2'-deoxyuridine (EdU) for 18 hours and assessed with fluorophore conjugation via the Click-iT EdU kit (Invitrogen) according to manufacturer's instructions. For rhodamine-phalloidin staining of actin, cells were fixed for 15 mins at RT in 3.7% formaldehyde and washed twice

in 200 μ l PBS before permeabilisation for 5 mins at RT in 100 μ l 0.1% Triton X-100 in PBS (v/v). Cells were then washed 3 times in 200 μ l PBS and incubated in the dark for 10 mins in 100 μ l staining solution containing rhodamine-phalloidin (Thermo Fisher) diluted 1/1000 in PBS, before further washing 3 times in 200 μ l PBS. For assessment of lysosomal load, live cells were incubated for 30 mins with 50 nM LysoTracker Red (Invitrogen) diluted in complete DMEM. DNA was counter-stained with NucBlue Live ReadyProbes Reagent (Thermo Fisher). Cells were imaged either with a ZOE fluorescent microscope (Bio-Rad). Image analysis was performed using Fiji version 2.3.0.

2.6 Flow cytometry analysis

2.6.1 Staining for conventional flow cytometry

Cells were pelleted at 500g for 5 mins and resuspended in 50 μ l of a master mix of cell-surface-staining antibodies (Table 2.4) diluted in FACS buffer (0.2% BSA (w/v), 2 mM EDTA in PBS) and Zombie NIR Live/Dead viability dye (1/1000 dilution, BioLegend) with incubation for 30 minutes at 4°C. Cells were washed in FACS buffer and fixed for 20 minutes at 4°C in BD Cytofix Fixation Buffer (BD Biosciences). Permeabilisation of cells was performed by washing cells in 1X BD Phosflow Perm/Wash Buffer I (BD Biosciences), followed by incubation in the permeabilisation buffer for 10 minutes at RT in the dark. Intracellular antibody staining (Table 2.4) was performed overnight at 4°C in the dark in BD Phosflow Perm/Wash Buffer I. When necessary, staining of unconjugated primary antibodies (Table 2.4) with a fluorophore-conjugated secondary antibody was performed in BD Phosflow Perm/Wash Buffer I for 1 hour at RT in the dark. Cells were washed once more and stored in FACS buffer prior to acquisition of 100-200,000 events on a flow cytometer (BD LSRFortessa). Compensation analysis was performed using single-stained

compensation beads (Thermo Fisher or BioLegend). Gating and analysis were performed using FlowJo version 10.9.0 based on fluorescence minus one (FMO) controls.

2.6.2 Staining for spectral flow cytometry

PBMCs and fibroblasts were stained for analysis on an Aurora spectral flow cytometer (Cytek Biosciences) as above, with some modifications. First, cells were harvested and pelleted (500g for 5 mins) and incubated in 50 μ l solution containing LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit (1/400 dilution, Invitrogen) and FcR blocking reagent (1/400 dilution, Miltenyi) in PBS for 15 minutes at 4°C. Surface and intracellular antigen staining were then performed as above (Table 2.4) (section 2.6.1). After staining, cells were filtered through a 70 μ m Flowmi cell strainer (Sigma) to remove aggregates, before analysis on a 5-laser (R/B/V/YG/UV) Aurora spectral flow cytometer (Cytek Biosciences). For each experiment, all samples were processed in one batch and single-colour cell- and bead-based controls were generated in parallel alongside the sample staining for spectral unmixing. For the 27-colour spectral flow cytometry panel used in Chapter 5, the similarity of the spectral signatures for each fluorophore is encapsulated by the Similarity Index in Figure 2.3.

Similarity Index

	LIVE DEAD Blue	BUV496	BUV563	BUV615	BUV661	BUV737	BV421	BV480	BV510	BV570	BV605	BV650	BV711	BV786	Alexa Fluor 488	Alexa Fluor 532	PerCP-Cy5.5	PE	PE-Dazzle 594	Alexa Fluor 594	PE-Cy7	PE-Fire 810	APC	Spark NIR 685	Alexa Fluor 700	APC-Cy7	APC-Fire 810
LIVE DEAD Blue	1	0.57	0.13	0.06	0.04	0.04	0.29	0.23	0.24	0.08	0.04	0.05	0.04	0.05	0.02	0.09	0.02	0.01	0.01	0.03	0	0.01	0.01	0.02	0.03	0.01	0.02
BUV496	0.57	1	0.36	0.1	0.03	0.03	0.1	0.44	0.57	0.15	0.09	0.05	0.03	0.02	0.09	0.16	0.02	0.04	0.02	0.03	0	0.01	0.01	0.02	0.02	0.01	0.02
BUV563	0.13	0.36	1	0.38	0.06	0.02	0.02	0.1	0.3	0.42	0.23	0.06	0.02	0.01	0.08	0.32	0.04	0.49	0.24	0.18	0.02	0.15	0.02	0.01	0.01	0.01	0.01
BUV615	0.06	0.1	0.38	1	0.34	0.11	0.01	0.05	0.16	0.34	0.58	0.29	0.09	0.04	0.01	0.1	0.21	0.27	0.63	0.73	0.05	0.1	0.17	0.08	0.05	0.03	0.03
BUV661	0.04	0.03	0.06	0.34	1	0.35	0.01	0.01	0.05	0.08	0.2	0.49	0.29	0.08	0	0.02	0.4	0.03	0.15	0.24	0.05	0.04	0.81	0.63	0.41	0.16	0.16
BUV737	0.04	0.03	0.02	0.11	0.35	1	0.01	0.01	0.03	0.02	0.08	0.16	0.42	0.27	0	0.02	0.33	0.01	0.03	0.04	0.15	0.09	0.19	0.27	0.47	0.31	0.2
BV421	0.29	0.1	0.02	0.01	0.01	0.01	1	0.29	0.17	0.17	0.08	0.1	0.09	0.2	0	0.02	0	0	0	0.01	0	0	0	0	0	0	0
BV480	0.23	0.44	0.1	0.05	0.01	0.01	0.29	1	0.84	0.27	0.16	0.08	0.04	0.08	0.08	0.09	0.01	0.05	0.03	0.04	0	0.01	0.01	0.01	0.01	0	0.01
BV510	0.24	0.57	0.3	0.16	0.05	0.03	0.17	0.84	1	0.53	0.39	0.18	0.08	0.08	0.03	0.11	0.05	0.1	0.08	0.11	0	0.02	0.02	0.01	0.02	0.01	0.02
BV570	0.08	0.15	0.42	0.34	0.08	0.02	0.17	0.27	0.53	1	0.69	0.25	0.09	0.08	0.03	0.17	0.09	0.57	0.34	0.36	0.02	0.16	0.05	0.02	0.02	0.01	0.01
BV605	0.04	0.09	0.23	0.58	0.2	0.08	0.08	0.16	0.39	0.69	1	0.54	0.19	0.11	0.01	0.1	0.22	0.27	0.49	0.64	0.04	0.09	0.15	0.05	0.05	0.02	0.03
BV650	0.05	0.05	0.06	0.29	0.49	0.16	0.1	0.08	0.18	0.25	0.54	1	0.47	0.21	0.01	0.04	0.47	0.05	0.19	0.35	0.05	0.04	0.42	0.25	0.2	0.08	0.08
BV711	0.04	0.03	0.02	0.09	0.29	0.42	0.09	0.04	0.08	0.09	0.19	0.47	1	0.54	0.01	0.03	0.58	0.01	0.05	0.1	0.11	0.08	0.22	0.31	0.49	0.22	0.16
BV786	0.05	0.02	0.01	0.04	0.08	0.27	0.2	0.08	0.08	0.08	0.11	0.21	0.54	1	0	0.02	0.25	0.01	0.02	0.04	0.15	0.1	0.04	0.05	0.11	0.21	0.17
Alexa Fluor 488	0.02	0.09	0.08	0.01	0	0	0	0.08	0.03	0.03	0.01	0.01	0.01	0	1	0.51	0.01	0.08	0.04	0.01	0	0.02	0	0	0	0	0
Alexa Fluor 532	0.09	0.16	0.32	0.1	0.02	0.02	0.02	0.09	0.11	0.17	0.1	0.04	0.03	0.02	0.51	1	0.06	0.4	0.27	0.08	0.02	0.12	0.01	0.01	0.01	0	0.01
PerCP-Cy5.5	0.02	0.02	0.04	0.21	0.4	0.33	0	0.01	0.05	0.09	0.22	0.47	0.58	0.25	0.01	0.06	1	0.05	0.29	0.29	0.24	0.18	0.34	0.38	0.38	0.16	0.13
PE	0.01	0.04	0.49	0.27	0.03	0.01	0	0.05	0.1	0.57	0.27	0.05	0.01	0.01	0.08	0.4	0.05	1	0.49	0.32	0.03	0.3	0.03	0.01	0.01	0	0.01
PE-Dazzle 594	0.01	0.02	0.24	0.63	0.15	0.03	0	0.03	0.08	0.34	0.49	0.19	0.05	0.02	0.04	0.27	0.29	0.49	1	0.85	0.06	0.18	0.15	0.05	0.04	0.02	0.02
Alexa Fluor 594	0.03	0.03	0.18	0.73	0.24	0.04	0.01	0.04	0.11	0.36	0.64	0.35	0.1	0.04	0.01	0.08	0.29	0.32	0.85	1	0.08	0.15	0.25	0.09	0.08	0.03	0.04
PE-Cy7	0	0	0.02	0.05	0.05	0.15	0	0	0	0.02	0.04	0.05	0.11	0.15	0	0.02	0.24	0.03	0.06	0.08	1	0.71	0.05	0.05	0.09	0.3	0.22
PE-Fire 810	0.01	0.01	0.15	0.1	0.04	0.09	0	0.01	0.02	0.16	0.09	0.04	0.08	0.1	0.02	0.12	0.18	0.3	0.18	0.15	0.71	1	0.04	0.03	0.05	0.17	0.26
APC	0.01	0.01	0.02	0.17	0.81	0.19	0	0.01	0.02	0.05	0.15	0.42	0.22	0.04	0	0.01	0.34	0.03	0.15	0.25	0.05	0.04	1	0.7	0.44	0.18	0.18
Spark NIR 685	0.02	0.02	0.01	0.08	0.63	0.27	0	0.01	0.01	0.02	0.05	0.25	0.31	0.05	0	0.01	0.38	0.01	0.05	0.09	0.05	0.03	0.7	1	0.68	0.29	0.22
Alexa Fluor 700	0.03	0.02	0.01	0.05	0.41	0.47	0	0.01	0.02	0.02	0.05	0.2	0.49	0.11	0	0.01	0.38	0.01	0.04	0.08	0.09	0.05	0.44	0.68	1	0.39	0.26
APC-Cy7	0.01	0.01	0.01	0.03	0.16	0.31	0	0	0.01	0.01	0.02	0.08	0.22	0.21	0	0	0.16	0	0.02	0.03	0.3	0.17	0.18	0.29	0.39	1	0.69
APC-Fire 810	0.02	0.02	0.01	0.03	0.16	0.2	0	0.01	0.02	0.01	0.03	0.08	0.16	0.17	0	0.01	0.13	0.01	0.02	0.04	0.22	0.26	0.18	0.22	0.26	0.69	1

Complexity™ Index: 9.24

Figure 2.3 Similarity index for 27-colour spectral flow cytometry panel

Theoretical similarity of each fluorophore's spectrum. Each pairwise comparison is given a value between 0-1 to denote similarity. An overall Complexity Index of 9.24 is indicated (source: cloud.cytexbio.com), which measures how distinguishable a collection of spectral signatures is from each other when unmixed. It calculates this by looking at the ratio of the Similarity Index of the worst overlapping combination of signatures to the best overlapping combination of signatures (source: Cytex Aurora User Guide, 2021, Cytex Biosciences, Inc).

2.6.3 Autophagic flux analysis

Measurement of autophagic flux in cells was performed using an antibody-based LC3 assay kit (Cytex Biosciences) with the following modifications. 2 hours prior to LC3 staining, each sample of cells was divided for treatment either with 10 nM bafilomycin A₁ (Table 2.1) or vehicle (0.1% DMSO final concentration) in R10. Cells were harvested and incubated in Live/Dead viability dye (1/1000 dilution, BioLegend) and FcR blocking reagent (1/400

dilution, Miltenyi) in PBS for 15 minutes at 4°C, then surface staining with antibodies (Table 2.4) was performed as described above, followed by washing in 1X assay buffer (Cytex Biosciences) diluted in dH₂O. Cells were subsequently permeabilised using 0.05% saponin (w/v) in PBS for 3 minutes at RT and incubated with an LC3-FITC conjugated antibody (Table 2.4) in 1X assay buffer for 1 hour at 4°C. Co-staining with anti-LC3 antibody and anti-γH2AX antibodies was optimised (section 4.2.15). After staining, cells were then washed in 1X assay buffer, and fixed in 2% PFA for 10 minutes at RT before being finally resuspended in FACS buffer and analysed on an LSRFortessa cytometer (BD). Autophagic flux was calculated from the mean fluorescence intensity (MFI) of LC3 in bafilomycin A₁- and vehicle-treated conditions ($LC3_{BafA}$ and $LC3_{Veh}$ respectively) using the formula:

$$Autophagic\ flux = \frac{LC3_{BafA} - LC3_{Veh}}{LC3_{Veh}}$$

2.6.4 Cell cycle phase analysis

To assess cell cycle phase distribution using flow cytometry, cells were incubated for 4 hour with 10 μM final concentration of EdU (Invitrogen). Cells were then harvested and incubated in Live/Dead viability dye (1/1000 dilution, BioLegend) and FcR blocking reagent (1/400 dilution, Miltenyi) in PBS for 15 minutes at 4°C. After this, surface antigen staining for flow cytometry was performed as described above (section 2.6.1). DNA-incorporated EdU was identified via a click chemistry reaction according to the manufacturer's instructions (Invitrogen), followed by overnight staining for intracellular γH2AX as described above (section 2.6.1). Cells were then washed and incubated with FxCycle™ Violet Stain for DNA (1/1000 dilution, Invitrogen) in permeabilisation buffer (from EdU kit, Invitrogen) and analysed on an LSR Fortessa X-20 cytometer (BD).

2.6.5 Protein synthesis assay

Nascent protein synthesis was measured using the Click-iT™ Plus OPP Alexa Fluor™ 594 Protein Synthesis Assay Kit (Invitrogen). Cells were incubated with 20 μ M O-propargyl-puromycin (OPP, from the kit) for 30 mins, and then collected and stained for surface antigens. Cells treated for 1 hour with 50 μ g/ml cycloheximide served as a positive control for protein synthesis inhibition. Cells were subsequently fixed, permeabilised, and polypeptide-incorporated OPP was detected, according to the manufacturer's instructions. Cells were analysed on a Fortessa X-20 flow cytometer (BD).

2.7 Measuring DNA lesions

2.7.1 Alkaline comet assay

SuperFrost Plus Adhesion slides (VWR) were first pre-coated with normal melting point agarose (NMPA) (1% (w/v) in dH₂O, Sigma) and allowed to dry overnight. Following treatments (section 4.2.13), cells were harvested by trituration and resuspended in PBS at a concentration of 2×10^5 cells/ml. 250 μ l of the cell suspension was mixed with 1 ml of low melting point agarose (1% w/v in PBS, Sigma) pre-warmed to 37°C, and 1 ml of the mixture was pipetted onto one NMPA-coated slide. Coverslips were then placed on the slides and left to gel on ice. For the lysis of cells, coverslips were removed, and slides were incubated overnight at 4°C in fresh, ice-cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base containing 1% DMSO (v/v) and 1% Triton X-100 (v/v), pH 10.5). The next day, slides were incubated for 30 mins in the dark with fresh alkaline electrophoresis buffer (300 mM NaOH, 1mM EDTA and 1% DMSO (v/v), pH > 13). Slides were then electrophoresed in the dark for 25 mins at 1 V/cm (distance between electrodes), at a constant current of 300 mA. Slides were neutralised during 3 x 5 mins incubations in neutralisation buffer (500 mM Tris-HCl,

pH 8.1) and subsequently left to dry overnight. The next day, slides were rehydrated for 30 mins in dH₂O, and DNA was stained for 30 mins with 1X SYBR™ Gold Nucleic Acid Gel Stain (Invitrogen) in dH₂O. Comets were visualised with a Zeiss Axio Imager and analysed using the OpenComet plugin in Fiji version 2.3.0.

2.8 Statistical tests and figures

All statistical and (log)normality testing of data was performed using GraphPad Prism version 10.0.0. *P*-values indicating statistical significance are represented as: ns (not significant) $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Unless otherwise stated, bar graph data are always represented as mean $\pm$ SEM. Box-and-whisker plots always show minimum to maximum values, with the median, 25th, and 75th percentiles indicated. Figures were made using either BioRender (biorender.com) or Microsoft PowerPoint version 16.88

Table 2.4 Details of antibodies

Use	Target	Fluorophore	Dilution	Supplier	Catalogue code	Clone
CC	CD3	n/a	1 µg/ml	BioLegend	317326	OKT3
CC	CD28	n/a	1 µg/ml	BioLegend	302934	CD28.2
FC	CD25	FITC	1/100	BioLegend	302604	BC96
FC	CD25	BV785	1/100	BioLegend	302638	BC96
FC	CD27	PE	1/100	BioLegend	302808	O323
FC	CD27	PE-Cy7	1/100	BioLegend	302838	O323
FC	CD28	BV510	1/100	BioLegend	302936	CD28.2
FC	CD4	APC	1/100	BioLegend	317416	OKT4
FC	CD4	FITC	1/100	BioLegend	317408	OKT4
FC	CD45RA	BV605	1/100	BioLegend	304134	HI100
FC	CD8	AF700	1/100	BioLegend	344724	SK1
FC	LC3	FITC	1/20	Cytex Biosciences	Part of kit FCCH100171	4E12
FC	p-Akt (Ser ⁴⁷³)	PE	1/50	Cell Signalling Technology	4060S	D9E
FC	p-Chk1 (Ser ³⁴⁵)	n/a	1/100	Cell Signalling Technology	2348	133D3
FC	p-Chk2 (Thr ⁶⁸)	n/a	1/100	Cell Signalling Technology	2197	C13C1
FC, SFC	CD57	BV421	1/100 (FC), 1/400 (SFC)	BD Biosciences	563896	NK-1
FC, SFC	KLRG1	BV711	1/100 (FC), 1/400 (SFC)	BioLegend	138427	2F1
FC, SFC	p-S6 (Ser ²³⁵ , Ser ²³⁶)	APC	1/100 (FC), 1/400 (SFC)	eBioscience	17-9007-42	cupk43k
FC, SFC	γH2AX (Ser ¹³⁹)	PerCP-Cy5.5	1/100 (FC), 1/400 (SFC)	BD Biosciences	564718	N1-431
IF	53BP1	n/a	1/200	Invitrogen	PA1-16566	pc
IF	Mouse IgG	AF488	1/200	Invitrogen	A21202	pc
IF	Rabbit IgG	AF488	1/200	Invitrogen	A21206	pc
IF	γH2AX (Ser ¹³⁹)	n/a	1/200	Cell Signalling Technology	9718T	4E2
IF, FC	Anti-rabbit IgG (H+L)	AF488	1/200 (IF), 1/500 (FC)	Invitrogen	A-11034	pc
SFC	CD11b	BV785	1/400	BioLegend	101243	M1/70
SFC	CD14	APC-Fire810	1/400	BioLegend	367155	63D3
SFC	CD16	PE-Dazzle594	1/400	BioLegend	302053	3G8
SFC	CD19	BUV615	1/400	BD Biosciences	612990	SJ25C1
SFC	CD27	BV650	1/400	BioLegend	302827	O323

SFC	CD28	BUV661	1/400	BD Biosciences	741635	CD28.2
SFC	CD3	AF700	1/400	BioLegend	300324	HIT3a
SFC	CD38	Spark NIR 685	1/400	BioLegend	303551	HIT2
SFC	CD4	PE-Fire810	1/400	BioLegend	344677	SK3
SFC	CD45RA	BUV563	1/400	BD Biosciences	612927	HI100
SFC	CD56	BV570	1/400	BioLegend	362540	5.1H11
SFC	CD8	APC-Cy7	1/400	BioLegend	300925	HIT8a
SFC	HLA-DR	BUV496	1/400	BD Biosciences	752493	L203.rMAb
SFC	IgD	BV480	1/400	BD Biosciences	566187	IA6-2
SFC	LAG3	BV605	1/400	BioLegend	369323	11C3C65
SFC	NKG2A	BUV737	1/400	BD Biosciences	749682	131411
SFC	NKG2D	BV510	1/400	BioLegend	563266	1D11
SFC	p-Akt (Ser ⁴⁷³)	AF532	1/100	Cell Signalling Technology	26580	D9E
SFC	p16 ^{Ink4a}	PE	1/400	Abcam	AB209579	EPR1473
SFC	p21 ^{Cif1/Waf1}	AF594	1/400	Cell Signalling Technology	11850S	12D1
SFC	p53	AF488	1/400	Abcam	AB224943	E26
SFC	PD-1	PE-Cy7	1/400	BioLegend	329917	EH12.2H7

CC = cell culture; FC = conventional flow cytometry; SFC = spectral flow cytometry; IF = immunofluorescence; pc = polyclonal.

3

Results:

Investigating pan-mTOR inhibition
and WRN protein for geroprotection
of primary human fibroblasts
undergoing DNA damage-induced
senescence

3.1 Introduction

Since it was first described by Hayflick and Moorhead over 60 years ago (Hayflick and Moorhead, 1961), cellular senescence has been found to play pleiotropic roles in development, disease, and ageing (van Deursen, 2014). Senescence describes a cell's proliferative arrest in response to persistent stress or unrepaired damage. As discussed earlier (section 1.2.4), cell senescence is associated with a wide range of biomarkers, though no single biomarker is unique to senescent cells. Of these, increased expression of cyclin kinase inhibitors, particularly p16^{Ink4a} and p21^{Cip1/Waf1}, is often used to define and manipulate senescent cells *in vivo*. High levels of genome instability and unrepaired DNA damage are also characteristic of senescence (Schumacher et al., 2021). By assessing biomarkers including these (section 1.2.4), senescent cells are observed to accumulate in tissues with ageing, both in animal models (Yousefzadeh et al., 2020) and in humans (Tuttle et al., 2020). Senescent non-immune cells, such as fibroblasts, actively suppress adaptive immune responses in an aged context, primarily through their immunomodulatory SASP (section 1.3.4.2). Therefore, identifying geroprotectors which attenuate non-immune cell senescence is a promising avenue of research for targeting immunosenescence. Furthermore, since DNA damage-induced senescence (DDIS) is particularly relevant to immunosenescence and ageing (sections 1.2.5 and 1.3.1.3), identifying geroprotectors using *in vitro* models of DDIS may be especially informative.

3.1.1 Strategies targeting senescent cells

3.1.1.1 First- and second-generation senolytic drugs

The reported roles of senescent cells in driving ageing pathologies have spurred drug discovery campaigns to identify compounds that modulate senescent cell biology. Generally, these drugs either selectively kill senescent cells (senolytics) or modulate an

aspect of their physiology without killing them (called senomodifiers, or senomorphics). The first senolytics were identified through hypothesis-driven drug discovery methods, which observed that senescent cells upregulate pro-survival pathways to resist apoptosis, called Senescent Cell Anti-Apoptotic Pathways (SCAPs) (Kirkland and Tchkonja, 2020). Drugs such as the tyrosine kinase inhibitor dasatinib (D) and the polyphenol quercetin (Q), or their combination, D+Q, preferentially kill senescent cells over proliferating or quiescent cells (Zhu et al., 2015). Later research identified that ABT263 (navitoclax), an inhibitor of the anti-apoptotic proteins BCL-2 and BCL-xL, selectively kills some senescent cell types and led to improved survival and healthy ageing of irradiated mice (Chang et al., 2016). Furthermore, inhibition of the FOXO4-p53 interaction with a small peptide induces senescent cell apoptosis and limits frailty in progeroid and naturally aged mice (Baar et al., 2017). Other compounds such as bisphosphonates (e.g. zoledronate) have shown senolytic activity and are particularly promising due to their good safety profile in older people as treatments for osteoporosis (Samakkaranthai et al., 2023).

While drugs that directly kill senescent cells are classed as first-generation senolytics, second-generation senolytic treatments involve modulation of the immune system, or more targeted delivery of drugs to senescent cells (e.g. using drug-loaded nanoparticles that are preferentially taken up by senescent cells (Munoz-Espin et al., 2018)). Senolytic treatments manipulating the immune system generally involve the identification of novel cell-surface, senescent cell-specific (seno-) antigens, such as the urokinase plasminogen activator receptor (uPAR); chimeric antigen receptor (CAR) T cells can then be engineered to recognise and remove these uPAR-expressing senescent cells *in vivo* (Amor et al., 2020). Additionally, senescent CD153⁺ T cells accumulate in the adipose tissue of obese individuals; treating high-fat diet-induced obese mice with a CD153-targeted vaccine, with a CpG adjuvant, significantly reduced the accumulation of senescent T cells in their adipose tissue and improved both insulin resistance and glucose tolerance (Yoshida et al., 2020).

Finally, glycoprotein nonmetastatic melanoma protein B (Gpnmb) is another candidate seno-antigen, against which vaccination robustly increased survival in progeroid *Zmpste24*^{-/-} mice (Suda et al., 2021).

Though senolytic drugs have shown major promise in mouse models of progeria, natural ageing, and specific age-related diseases, their effects on immunity and homeostasis across all tissues is less well understood. For example, despite the established role of senescent cells in suppressing acute influenza-specific pathological lung responses, senolytic D+Q treatment did not improve secondary influenza-specific memory responses in aged mice (Torrance et al., 2023, Lorenzo et al., 2022). Furthermore, when senescent cells are not replaced after they have been removed, such as is the case with senescent liver sinusoidal endothelial cells, senolysis leads to severe liver pathology and decreased mouse healthspan (Grosse et al., 2020). More generally, where some tissues, such as the pancreas, accumulate up to 35% p16⁺ cells in humans with ageing (Idda et al., 2020), senolysis may have severe deleterious effects. Hence, senolytics may not have universal benefits across all ageing tissues and organs.

3.1.1.2 Senomodifiers

Senomodifiers, which modulate senescent cell functions rather than remove the cells themselves, therefore provide an additional avenue for therapeutic targeting of senescent cells. Many senomodifiers primarily act to suppress the SASP, as this is the axis by which senescent cells mostly exert their detrimental effects. The activity of intracellular inflammatory signalling pathways, such as the IKK/NF- κ B pathway, increases in tissues with ageing; inhibiting NF- κ B with a small peptide, NBD, delays ageing in progeroid *Ercc1*^{-/-} mice (Tilstra et al., 2012). p38 MAPK activity is also upregulated in cell senescence; inhibiting p38 MAPK signalling with SB203580 or vitamin D₃ reduces the SASP (Sayegh et

al., 2024, Alimbetov et al., 2016). While senomodifiers modulate the phenotypes of cells that are already senescent, geroprotectors also protect cells from entering senescence in response to damage (termed seno-conversion). Identifying strategies that prevent deleterious seno-conversion are also valuable, not only as an anti-ageing strategy, but also in the context of the off-target, senescence-inducing effects of genotoxic cancer chemotherapy.

3.1.2 Werner syndrome protein as a potential geroprotector

One potential avenue for identifying geroprotectors is by studying genes essential for healthy ageing. Werner syndrome (WS) is a segmental progeroid disease where patients experience early onset of age-related phenotypes such as hair greying, inflammageing, atherosclerosis, cataracts, and high cancer incidence (Cox and Faragher, 2007). WS arises from loss-of-function mutations in *WRN*, a gene involved in DNA recombination, replication, and repair; loss of WRN protein leads to high levels of DNA damage and failure of lagging strand synthesis. Fibroblasts from WS patients undergo premature senescence in culture (Rodriguez-Lopez et al., 2002, Bagley et al., 2010), and young WS fibroblasts are transcriptionally highly similar to fibroblast cell lines derived from old people (Kyng et al., 2003). Thus, WRN's normal role is to prevent stresses that would otherwise drive senescence. Interestingly, the replicative lifespan of WS fibroblasts can be extended with known geroprotective treatments, such as p38 MAPK inhibitors (Davis et al., 2005) and mTOR inhibitors (Saha et al., 2014). Recently, our group found that overexpression of WRN extended both lifespan and healthspan in *C. elegans* (Lees et al., manuscript in preparation). This suggests that elevating WRN levels may be a geroprotective strategy. It is currently unclear how WRN levels change with senescence in normal cells, either during replicative senescence or DNA damage-induced senescence, though early studies report a reduction in *WRN* in PBMCs with age (Polosak et al., 2011). Furthermore, whilst WRN

interacts with many other proteins in the cell relevant to DNA metabolism (Cox and Faragher, 2007), it is less understood how WRN interacts with pathways that are relevant to senescence, such as mTOR hyperactivation. The observation that the proliferative capacity of WRN-deficient cells is extended by rapamycin (Saha et al., 2014), an mTORC1 inhibitor, suggests a functional relationship between WRN and mTOR.

3.1.3 mTOR inhibitors as modulators of ageing and senescence

mTOR inhibitors are senomodifiers, i.e. molecules that modulate senescent cell biology. mTOR is a kinase that forms two distinct complexes, mTORC1 and mTORC2, involved in anabolic signalling for cell growth and biosynthesis; the activity of either complex can be measured by assessing the levels of downstream phospho-proteins, such as p-S6 and p-4EBP1 (mTORC1) and p-Akt (mTORC2) (section 1.4.1) (Walters and Cox, 2018).

Much has been published on the geroprotective effects of the first mTOR inhibitors, such as rapamycin, a natural macrolide antibiotic lactone produced by *Streptomyces hygroscopicus*. Mechanistically, rapamycin allosterically inhibits mTORC1 by binding FKBP12, forming a complex that binds and partially occludes the kinase active site of the mTORC1 complex (Yang et al., 2013a). Second-generation mTOR inhibitors, such as AZD8055 and BEZ235 (also named RTB101), target mTOR by competing with ATP at the kinase active site (Chresta et al., 2010); in this way, these inhibitors target both mTORC1 and mTORC2 signalling complexes (Walters and Cox, 2018). The ATP-competitive mTOR inhibitor, RTB101, has shown particular efficacy for long-term protection against respiratory infections in older people, especially when combined with rapalogues (Mannick et al., 2018). AZD8055 has been shown previously in our group to modify a wide range of phenotypes in human dermal fibroblasts undergoing replicative senescence (Walters et al., 2016, Rolt et al., 2019). However, how this second-generation mTOR inhibitor affects DNA

damage-induced senescence, which is particularly relevant to ageing and immunosenescence (Schumacher et al., 2021, Yousefzadeh et al., 2021a, Kell et al., 2023), has not yet been determined.

3.1.4 Chapter aim and objectives

In this chapter, I aim to identify potential senomodifiers or geroprotectors against DNA damage-induced senescence.

Objectives:

1. To establish a model of DNA damage-induced senescence (DDIS) in human dermal fibroblasts, validated against known phenotypes of replicatively senescent (RS) fibroblasts.
2. To determine whether changes in a known ‘anti-ageing’ DNA repair factor, *WRN*, are involved in DDIS and RS.
3. To identify potential pathways for the role of WRN in DNA damage protection through isolation of novel WRN-interacting peptides by phage display.
4. To determine the potential of mTOR inhibition in genoprotection, by:
 - i. Initially establishing mTOR activity in DDIS.
 - ii. Analysing DNA damage and senescence phenotypes in DDIS upon treatment with an mTOR inhibitor.

3.2 Results

3.2.1 Validating a human dermal fibroblast model of replicative senescence

First, I aimed to characterise the dynamics and phenotypes of replicative senescence by longitudinally culturing HF043 human dermal fibroblasts. Over a period of 100 days, the cumulative population doublings (CPD) of the fibroblasts steadily increased from CPD 45 to CPD 80 (Figure 3.1A). Concomitantly with cell population age, the proliferative capacity, measured as population doublings achieved per day (PD/day), steadily decreased, reaching as low as 0.1 PD/day at CPD 80 (Figure 3.1B). At this high CPD, the cells lost their fibroblastic spindle morphology and adopted a classically senescent morphology, becoming rounder and greatly enlarged compared with low-CPD fibroblasts (Figure 3.1C). Consistent with their slow proliferation rate and senescent morphology, these high-CPD fibroblasts expressed significantly higher levels of the cyclin kinase inhibitor genes, *CDKN1A* and *CDKN2A*, than low-CPD fibroblasts, as assessed by qRT-PCR (Figure 3.1D). Taken together, these features are suggestive that the fibroblasts had entered a replicatively senescent (RS) state.

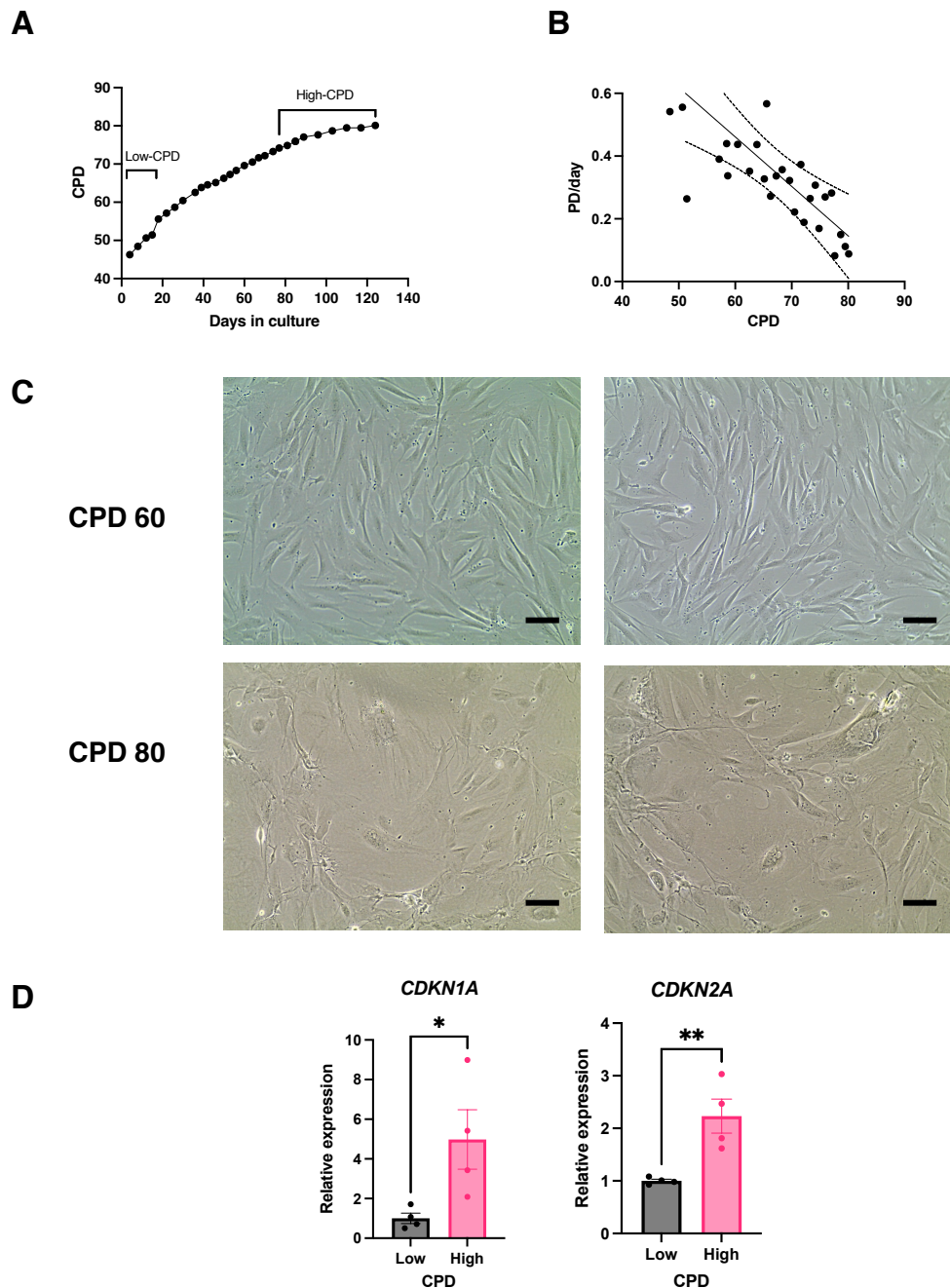


Figure 3.1 Replicative senescence in human dermal fibroblasts

(A) Cumulative population doubling (CPD) of HF043 human dermal fibroblasts throughout continuous cell culture. (B) Population doublings (PD) per day (PD/day) of HF043 human dermal fibroblasts at increasing CPDs. Simple linear regression analyses: solid line indicates line of best fit, with dotted lines representing 95% confidence intervals. (C) Representative brightfield images of HF043 human dermal fibroblasts at different CPDs. Scale bar represents 100 μm . (D) qRT-PCR gene expression of *CDKN1A* (left) and *CDKN2A* (right) in low-CPD (45-51) and high-CPD (74-81) HF043 human dermal fibroblasts, relative to internal housekeeping gene (*GUSB*). $N=4$ samples per condition, p -value from an unpaired student's t-test.

3.2.2 Optimising conditions for DNA damage-induced senescence in human dermal fibroblasts

Having established these phenotypes of RS in the HF043 fibroblasts, I set out to optimise a protocol for the induction of DNA damage-induced senescence (DDIS), since DNA damage is both a major trigger and feature of physiological senescence (section 1.2.5) (Schumacher et al., 2021). Mitomycin C (MMC) is a chemotherapeutic drug which binds to guanine nucleotides, leading to DNA bi-adducts and interstrand crosslinks (McKenna et al., 2012). Chronic exposure to non-lethal levels of MMC induces senescence in human lung adenocarcinoma A549 cells (McKenna et al., 2012). To identify an optimised concentration of MMC for induction of DDIS, I treated primary HF043 human dermal fibroblasts with MMC at 30, 60, or 150 nM for 7 days compared to vehicle-only control, and analysed cellular and nuclear morphology by fluorescence microscopy (Figure 3.2A). Staining for nuclear DNA together with filamentous (F-) actin using rhodamine-phalloidin revealed that all concentrations of MMC induced cellular enlargement and the presence of actin stress fibres, characteristic of senescence (Figure 3.2B), as well as an increase in nuclear area, another feature of fibroblast senescence (Figure 3.2C) (Duran et al., 2024). In summary all concentrations of MMC tested induced morphological features of senescence.

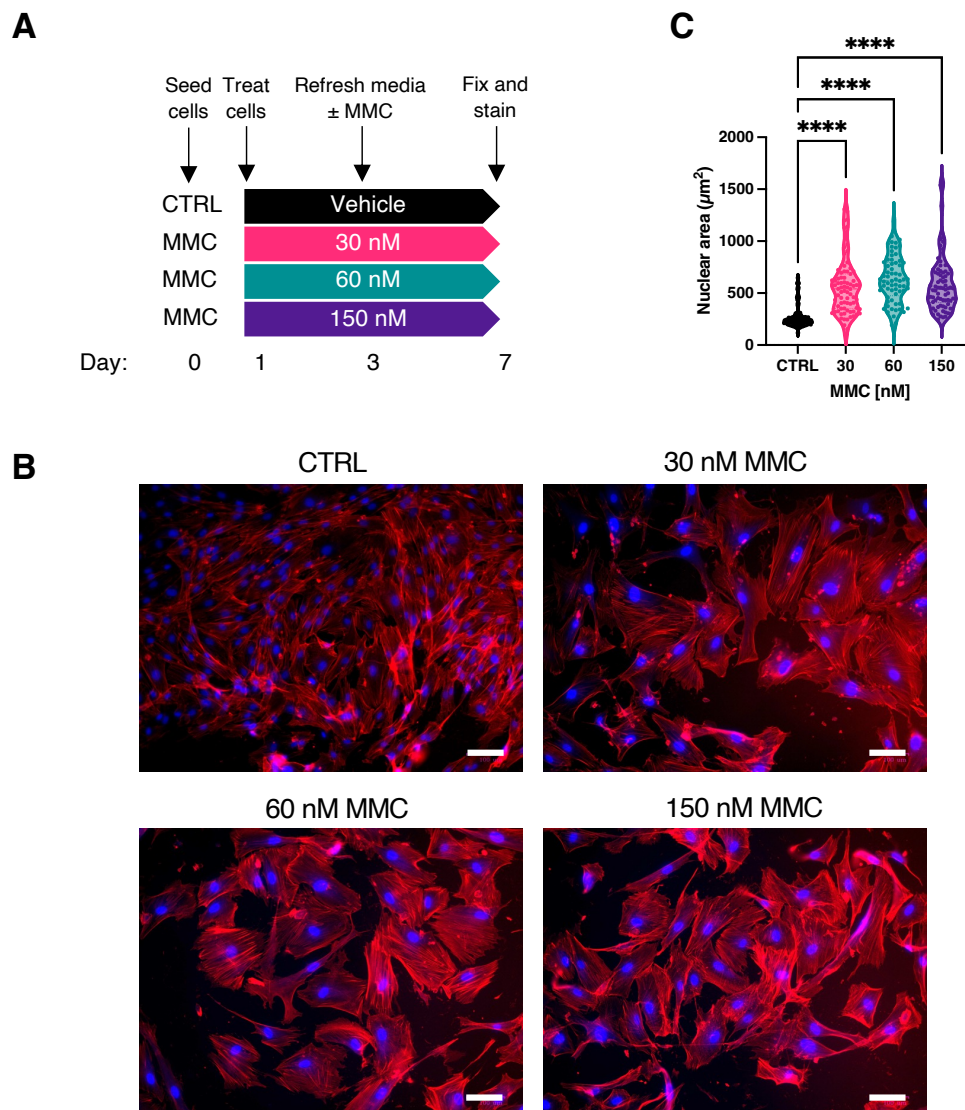


Figure 3.2 Optimising conditions for DNA damage-induced senescence in human dermal fibroblasts

(A) Experimental design for optimising the induction of DNA damage-induced senescence in human dermal fibroblasts (at CPD 66.6) with vehicle-only control (CTRL) or mitomycin C (MMC) at 30, 60, or 150 nM. (B) F-actin (rhodamine-phalloidin, red) and nuclear (NucBlue Live, blue) staining of treated fibroblasts, scale bar represents 100 μm . (C) Nuclear area of 50-100 nuclei analysed per condition. *P*-values are derived from a one-way ANOVA with Dunnett's multiple comparisons test.

3.2.3 Senescence biomarkers are strongly induced in MMC-induced DDIS

Following preliminary trials (Figure 3.2), subsequent studies used 100 nM treatment for 7 days, assessing a wider panel of senescence features. To ensure that phenotypes reflected senescence rather than transient responses to DNA damage, MMC was removed after 7

days and cells incubated without drug for a further 7–14-day recovery period (Figure 3.3A). Consistent with observations for RS in these fibroblasts, DDIS was associated with significantly higher gene expression of *CDKN1A* and *CDKN2A* (as assessed by qRT-PCR), in addition to their respective protein products, p21^{Cip1/Waf1} and p16^{Ink4a} (as assessed by flow cytometry), indicating cell cycle arrest (Figure 3.3B–C). The fold increase of *CDKN1A*/p21 expression was higher than *CDKN2A*/p16 expression in DDIS compared to control fibroblasts (30-fold vs 10-fold), consistent with previous reports that DDIS is achieved through hyperactivation of the p53-p21 pathway (Yosef et al., 2017). Sulphorhodamine B staining of free amines confirmed a marked cellular enlargement in DDIS fibroblasts (Figure 3.3D), which corresponded with the presence of actin stress fibres (Figure 3.3E), higher cell diameters (measured in suspension after trypsin harvesting) (Figure 3.3F) and nuclear areas (measured *in situ*) (Figure 3.3G).

High activity of lysosomal senescence-associated β -galactosidase (SA β G) activity at a stringent pH of 6.0 is a hallmark of senescence (Dimri et al., 1995). Accordingly, DDIS following MMC treatment elicited strong SA β G positivity, measured by the development of a dark blue perinuclear stain (Figure 3.3H). Staining for lysosomes with LysoTracker further confirmed lysosomal hypertrophy in MMC-induced DDIS fibroblasts (Figure 3.3I). Finally, I assessed DNA damage by the staining of γ H2AX⁺ and 53BP1⁺ nuclear foci, and found that these were both significantly enriched in DDIS fibroblasts compared with untreated controls (Figure 3.3J–K). In summary, DDIS of fibroblasts using MMC elicited a wide range of senescence biomarkers and may be used as a model for investigating geroprotection *in vitro*.

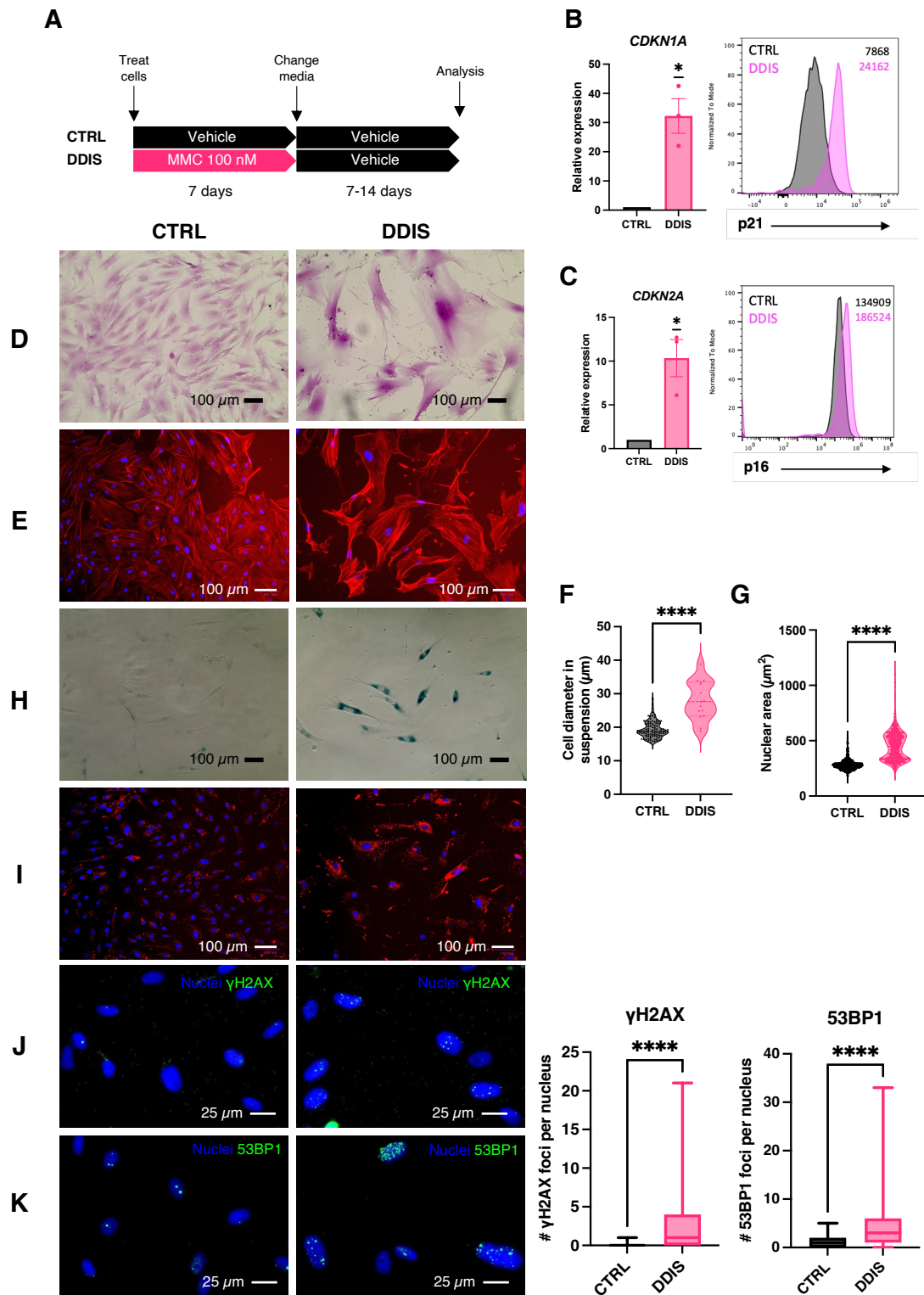


Figure 3.3 Measuring senescent cell biomarkers in DNA damage-induced fibroblast senescence

(A) Experimental design for induction of DNA damage-induced senescence (DDIS) by MMC treatment alongside negative controls (CTRL) in HF043 human dermal fibroblasts. (B–C) (Left) qRT-PCR for relative expression of cyclin kinase inhibitor genes (B) *CDKN1A* or (C) *CDKN2A* in control (CTRL) vs DDIS fibroblasts. Student's one-sample t-test, n=3 biological repeats. (Right) Fluorescence histograms of p21 (B) or p16 (C) with geometric mean

fluorescence intensities indicated. **(D—E, H—I)** Representative staining of **(D)** cell biomass (free amines) using dye sulphorhodamine B, **(E)** F-actin (red) and nuclei (blue), **(H)** senescence-associated β -galactosidase activity (blue), and **(I)** lysosomes (red) and nuclei (blue). **(J—K)** (Left) Nuclear (blue) and DNA damage foci (green) staining of **(J)** γ H2AX and **(K)** 53BP1. Number of foci per nucleus is quantified to the right. *P*-values are derived from a Mann-Whitney test. **(F)** Cell diameter in suspension. *P*-value derived from an unpaired t-test. **(G)** Nuclear areas of 150-200 nuclei analysed per conditions. *P*-value derived from a Mann-Whitney test. Data are representative of three independent experiments. All scale bars represent distances as indicated.

3.2.4 *WRN* gene expression is increased in both DDIS and RS

The *WRN* gene encodes Werner protein (WRN), a member of the RecQ DNA helicase family, with a unique N-terminal exonuclease domain. WRN plays a central role in maintaining genome stability in DNA replication, recombination and repair (Cox and Faragher, 2007). Recent work from the Cox and Woollard labs found that overexpression of WRN helicase in the nematode worm, *C. elegans*, extended lifespan and healthspan above that of wild-type animals, and could protect worms from UV-induced DNA damage (Lees *et al.*, manuscript in preparation), suggesting that WRN can be geroprotective, and its levels may be limiting during ageing.

To determine whether WRN levels may be limiting in human cell ageing, I used qRT-PCR to measure the expression of *WRN* in both the RS and DDIS of human dermal fibroblasts, relative to a housekeeping gene, *GUSB*, whose expression has been found previously to remain constant during *in vitro* senescence (Gonzalez-Bermudez *et al.*, 2019). *WRN* mRNA expression increased in both DDIS (>2-fold) and RS (>1.5-fold) compared with proliferating cells of the same lineage (Figure 3.4A—B). Furthermore, in linear regression analyses, the expression of *WRN* positively correlated with the expression of the cyclin kinase inhibitor genes, *CDKN1A* (Figure 3.4C) and *CDKN2A* (Figure 3.4D), indicating that *WRN* upregulation was related to senescence. This was contrary to my hypothesis that *WRN* expression decreases with cell ageing, but may reflect an upregulation of this DNA repair

factor in an attempt to respond to the high levels of DNA damage in RS and especially in DDIS (Figure 3.3J—K).

To investigate whether reduced WRN activity might contribute to senescence, I tested the impact of treating fibroblasts with the WRN helicase inhibitor, NSC 19630. Using a half-log dilution series, I identified a range of NSC 19630 concentrations (0.01—3 μ M) that did not decrease viability, as measured by the alamarBlue reduction assay (Figure 3.4E). Cell proliferation in the presence of 0.1 μ M NSC 19630 was determined by incorporation of the nucleoside analogue, 5-ethynyl-2'-deoxyuridine (EdU), into nascent DNA. WRN helicase inhibition decreased the proportion of EdU⁺ cells relative to untreated controls (Figure 3.4F), indicating slower proliferation, suggesting that WRN activity may be a limiting factor for cell proliferation. Potentially, therefore, WRN overexpression may be geroprotective against DDIS and RS in fibroblasts.

To understand further whether WRN overexpression might affect senescence, I attempted to overexpress the endogenous *WRN* gene in primary fibroblasts. I chose to use a CRISPR-ON system, involving the transfection of three separate plasmids to increase transcription from the endogenous *WRN* promoter. This system uses an enzyme-deactivated Cas9 (dCas9) as a scaffold for a *WRN*-specific gRNA which is co-transfected with transcriptional activating proteins that localise at the endogenous *WRN* promoter, driving gene transcription (Konermann et al., 2015). Unfortunately, the transfection of the three plasmids was completely cytotoxic to the primary human fibroblasts (data not shown). I attempted to minimise this cytotoxicity by titrating decreasing amounts of plasmid DNA for transfection: though this successfully prevented cell death, none of the cells survived the antibiotic selection subsequently, suggesting an absence of stable transfection (data not shown). This was therefore not a viable approach for overexpressing WRN in sensitive primary human fibroblasts and was not pursued further.

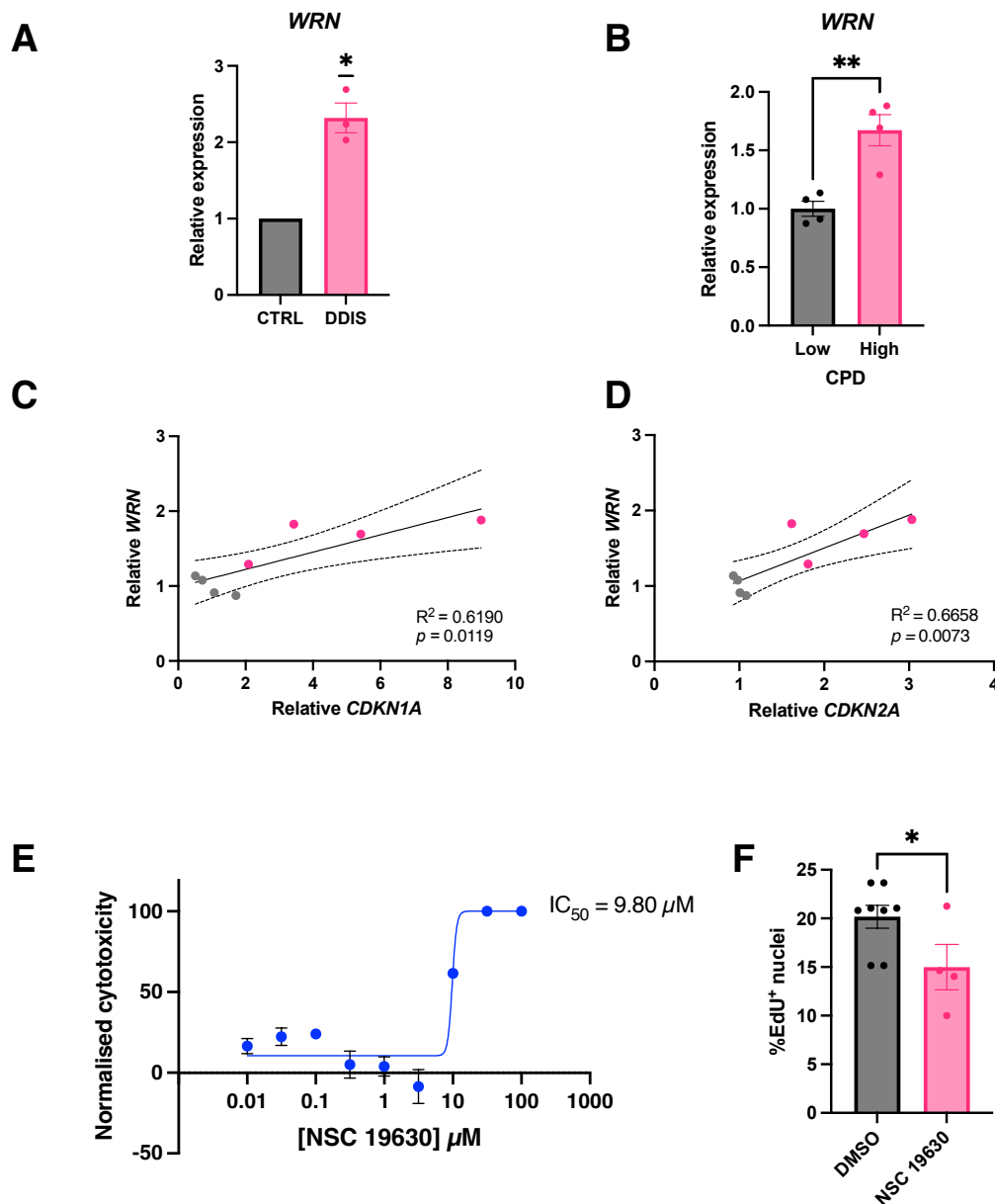


Figure 3.4 *WRN* mRNA levels are upregulated in DDIS and RS

(A–B) qRT-PCR for expression of the *WRN* gene relative to internal housekeeping gene, *GUSB*. (A) Control (CTRL) vs DNA damage-induced senescent (DDIS) HF043 dermal fibroblasts. *P*-value derived from a student's one-sample t-test (theoretical mean = 1), *n*=3 per condition. (B) Low-CPD (45–51) and high-CPD (74–81) HF043 human dermal fibroblasts, *n*=4 per condition, *p*-value from an unpaired student's t-test. (C–D) Simple linear regression analyses of *WRN* expression vs (C) *CDKN1A* or (D) *CDKN2A* in low-CPD (grey dots) and high-CPD (pink dots) fibroblasts, as in (B). Solid line indicates line of best fit, with dotted lines representing 95% confidence intervals. (E) Cytotoxicity curve of human dermal fibroblasts treated for 3 days with half-log dilutions of the *WRN* helicase inhibitor, NSC 19630, using the alamarBlue reduction assay, *n*=3 technical replicates per condition. (F) EdU incorporation of human dermal fibroblasts treated for 3 days with 0.1 μ M NSC 19630, a *WRN* helicase inhibitor. *P*-value derived from an unpaired t-test, *n*=5–8 technical replicates.

3.2.5 Methylation-specific PCR of the *WRN* promoter region

WRN expression is known to be regulated by methylation at its promoter, and hypermethylation is associated with certain cancers (Agrelo et al., 2006). To probe potential mechanisms underpinning the observed increase in *WRN* mRNA levels in RS, I used methylation-specific PCR to assess cytosine methylation within the *WRN* gene promoter. Here, DNA is subjected to bisulfite conversion, where unmethylated (but not methylated) cytosines are deaminated to generate uracils. This yields distinct DNA templates based on the original methylation status of cytosines. The bisulfite-converted DNA can be subsequently discriminated by primer sets specific to the *WRN* promoter, targeting a region -31 bp to +128 bp relative to the transcription start site (Agrelo et al., 2006, Bosch et al., 2016) (Figure 3.5B), specific to cytosine residues which were unmethylated and hence converted ('U primers') compared with those where cytosines were methylated and protected from bisulfite conversion ('M primers'). Presence of PCR products with 'U primers' indicates absence of methylation, whereas PCR products generated with 'M primers' indicate promoter methylation (Figure 3.5A).

Primer specificity was tested using unmethylated and methylated genomic DNA standards. After bisulfite conversion, U primers produced a product with only the unmethylated standard, whereas M primers only produced a product with the methylated standard, thereby demonstrating the methylation specificity of the primers (Figure 3.5C). Next, I tested whether *WRN* promoter methylation changed as cells tended towards replicative senescence by comparing HF043 fibroblasts at mid- and high-population doublings (CPD<50, proliferative; CPD>75, peri-senescent). Interestingly, I observed PCR products with both low- and high-CPD fibroblasts with both U and M primer sets (Figure 3.5D), suggesting a heterogeneous mix of both unmethylated and methylated *WRN* promoter regions in these cell populations. In the absence of demonstrable *WRN* promoter methylation changes with senescence, the increase in *WRN* transcript abundance

observed in RS and DDIS fibroblasts (Figure 3.4) is likely due to factors other than altered promoter methylation.

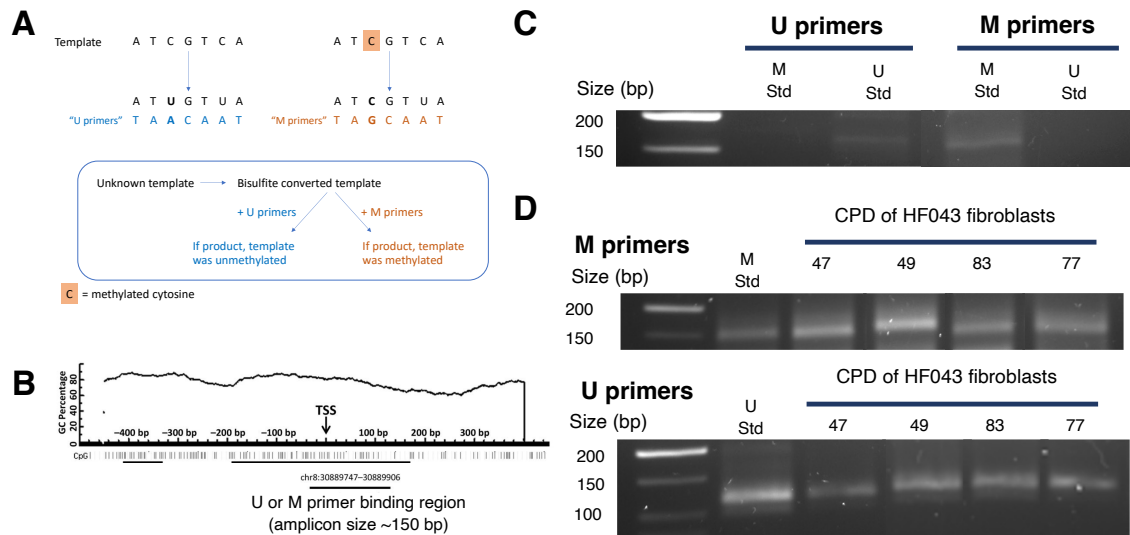


Figure 3.5 Methylation-specific PCR of the WRN promoter region in RS fibroblasts

(A) Schematic illustrating how methylation-specific PCR can be used to distinguish the methylation status of cytosines at a region of interest. (B) Graph adapted from Bosch *et al.* 2016, indicating the region of the *WRN* promoter at which the U or M primers are predicted to bind. TSS = transcription start site. (C) Agarose gel of genomic standards (methylated, M Std; Unmethylated, U Std) that have undergone bisulfite conversion and subsequent MSP with either U or M primers. (D) MSP of genomic DNA extracted from HF043 fibroblasts at different cumulative population doublings (CPDs). Bisulfite-converted samples underwent MSP either with primers specific to unmethylated DNA (U primers) or methylated DNA (M primers), with unmethylated and methylated genomic DNA standards used as the internal controls.

3.2.6 Phage display of WRN helicase to identify WRN-interacting peptides

The observation that *WRN* overexpression increases lifespan and healthspan in *C. elegans* (Lees *et al.*, manuscript in preparation), and that *WRN* mRNA levels are increased in both RS and DDIS (Figure 3.4A–B), raises the question of what WRN may be interacting with in order to exert its potentially geroprotective and DNA repair effects. To investigate this, I used phage display to identify short peptide motifs that bound to WRN residues 520–1242. This truncated protein contained the helicase, RQC, and HRDC domains of WRN, with a

C-terminal Avi-tag to allow for biotinylation and hence protein capture with streptavidin (Figure 3.6A), and was kindly provided by Dr Joseph Newman and Professor Opher Gileadi (Newman et al., 2021). A commercial M13 phage library displaying random 12-mer peptides fused to the N-terminus of the minor coat protein (pIII) was screened for peptide-ligand binding against i) biotinylated WRN (to identify WRN-interacting peptides), ii) biotin alone (to identify biotin- and plastic-specific phage binders ('Strep/biotin')), and iii) buffer alone (to identify streptavidin- or plastic-specific phage binders ('Strep only')). Any control peptide sequences identified in both the WRN test and control (Strep/biotin or Strep only) conditions were deemed as non-specific to WRN (Figure 3.6B). Three rounds of biopanning were performed, each followed by phage elution and amplification, with increasing stringency in each subsequent round of biopanning (I-III) by decreasing the amount of WRN 10-fold each time and increasing stringency of wash steps (see section 2.4). Figure 3.6C shows phage titres after each elution and amplification step.

Sequencing of a subset of the eluted and amplified phage for each condition after the third biopanning round revealed the presence of 'Strep/biotin'-interacting sequences in all conditions; 'Strep only' binding sequences were also selected in WRN target conditions. Overall, I obtained nine 12-mer sequences that were unique to the WRN test condition (Figure 3.6D).

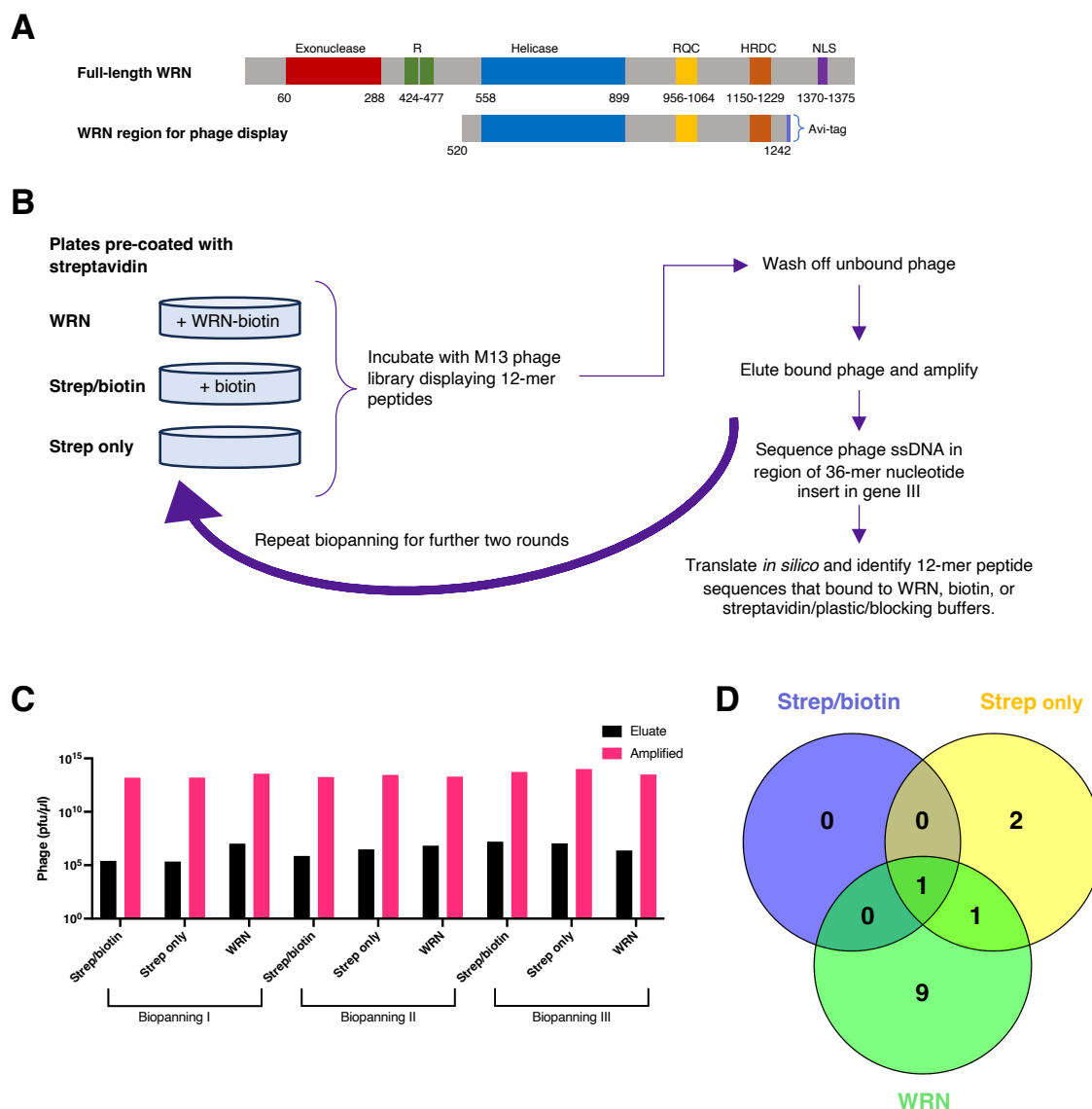


Figure 3.6 Phage display of WRN helicase

(A) WRN protein regions with amino acid positions indicated. The region of WRN used in the phage display experiments is indicated. (B) Experimental design for phage display of WRN protein. (C) Number of plaque-forming units per μl (pfu/ μl) obtained from each biopanning, either directly after elution or after amplification, across biopannings I-III. (D) Venn diagram showing the number of unique 12-mer peptide sequences obtained from the three different biopanning conditions

To identify homology to known human proteins, I aligned these 9 sequences against the human proteome using BLASTp, adjusted for short sequence input. These alignments, along with their top BLASTp hit, are shown in Table 3.1. Of note, each showed significant matches with a wide range of proteins. Strikingly, 4/9 peptide sequences (in bold) match those in human proteins involved in protein translation and anabolic processes. Of these,

Ribosomal Protein S6 Kinase C1, Ribosomal protein S6 kinase-like 1, and KICSTOR complex protein SZT2, all have roles in the mechanistic target of rapamycin (mTOR) pathways (Karlsson et al., 2015, He et al., 2019, Peng et al., 2017). This leads to the possibility that WRN physically interacts with components of the mTOR pathway and is especially interesting given the potentially opposing roles of WRN and mTOR in ageing: while WRN deficiency leads to progeria (Lautrup et al., 2019), increased mTOR activity is observed in aged tissues (Markofski et al., 2015). Since mTOR activity is strongly implicated in senescence and ageing phenotypes, and loss of WRN promotes premature aging, the potential that WRN protein may affect signalling through the mTOR pathway is provocative. It has recently been shown that overexpression of WRN reduces DNA damage in Hutchinson-Gilford progeria cells potentially through interacting with and inhibiting progerin (Kang et al., 2021), and that mTOR inhibition overcomes proliferative defects in WRN-deficient cells (Saha et al., 2014). Therefore, I explored the role of mTOR and mTOR inhibitors in DNA damage responses, particularly in DNA damaged-induced senescence in fibroblasts (below) and in immune cells (Chapters 4—5).

Table 3.1 WRN-binding 12-mer peptide sequences identified by phage display

12-mer peptide sequence	No. of plaques with sequence	Gene name	BLASTp hit	BLASTp alignment			
AWKHPAGVTTLN	1	RPS6KC1	Ribosomal Protein S6 Kinase C1	Query	4	HPAGV---TTLN	12
				Sbjct	407	HPAG+ TTLN	418
						HPAGINTHTTLN	
TSLETWILSGPS	1	RPS6KL1	Ribosomal protein S6 kinase-like 1	Query	1	TSLET-----WILSGP	11
				Sbjct	519	TSLET W LSGP	535
						TSLEHLRLTIHW-LSGP	
ATMRGDQSVRIF	1	SZT2	KICSTOR complex protein SZT2	Query	3	MRGDQSVRIF	12
				Sbjct	2568	M GD SVRIF	2577
						MAGDTSVRIF	
VSVPGIITGTLR	2	TUFM	Elongation Factor Tu	Query	2	SVPG---IITGTL	11
				Sbjct	267	SVPG ++TGTL	279
						SVPGRGTVVTGTL	
FSQSWKAPTWLN	1	REXO4	RNA exonuclease 4	Query	1	FSQSWKA---PTW	10
				Sbjct	66	FSQ WKA P W	79
						FSQNWKALQEVPRW	
GFHLSWKPYTQD	1	ADAMTS9	ADAM metalloproteinase with thrombospondin type 1 motif	Query	6	WKPYTQ	11
				Sbjct	530	WKPYTQ	535
						WKPYTQ	
GVLNSSPSTRFV	1	CRADD	CASP2 and RIPK1 domain containing adaptor with death domain	Query	2	VLNSSPSTR	10
				Sbjct	110	+LNSSPS R	118
						ILNSSPSDR	
CFAGTPSILMPA	1	MIPEP	Mitochondrial intermediate peptidase	Query	2	FAGTPSILM	10
				Sbjct	522	FA PSILM	530
						FAEVPISILM	
TEGRGLSMPVLW	1	DUOXA1	Dual oxidase maturation factor 1	Query	6	LSMPVL	11
				Sbjct	198	LSMPVL	203
						LSMPVL	

Hits in bold represent potential WRN-interacting proteins that are involved in mTOR signalling and/or protein synthesis. Query = phage peptide; Subject (subject) = human protein alignment.

3.2.7 DNA damage-induced senescent fibroblasts display high mTOR activity

To investigate whether mTOR activity changed during DDIS, I used MMC to induce DDIS in primary human fibroblasts (Figure 3.7A) and measured the levels of two major mTOR complex downstream targets, p-S6 (mTORC1) and p-Akt (mTORC2), by phosflow cytometry (Figure 3.7B—C). The fluorescence histograms for both p-S6 and p-Akt were shifted towards higher values in DDIS compared to control cells, strongly suggesting that both mTORC1 and mTORC2 activity was higher in DDIS (Figure 3.7B—C).

To assess whether this was a phenotype of DDIS, rather than an effect either specific to MMC or the type of DNA damage induced (interstrand crosslinks), I induced DDIS with zeocin, a double-strand break-inducer related to bleomycin (Hu et al., 2018). As the response of HF043 fibroblasts to zeocin is unknown, I first conducted a toxicity test on human dermal fibroblasts treated for 3 days with zeocin concentrations from 1 nM to 1 mM and measured cell viability (Figure 3.7D). To drive DDIS, cells were treated for a short period (24 hours) with 35 μ M zeocin, followed by a 7-day recovery period (Figure 3.7E). Zeocin treatment increased total p16 and γ H2AX levels, in addition to the percentage of cells positive for γ H2AX, p53, and p16, as measured by flow cytometry, indicating cell cycle arrest, persistent DNA damage, and DDIS (Figure 3.7F). Measuring phosphorylated S6 (p-S6) as a readout for mTORC1 activity, I found elevated p-S6 levels in zeocin-induced DDIS fibroblasts compared with control cells (Figure 3.7G). Since DDIS fibroblasts display high mTOR activity when induced to undergo senescence by either DSB formation (zeocin) or interstrand crosslinks (MMC), it is likely that elevated mTORC1/2 activity reflects DDIS rather than being specific to the type of inducer.

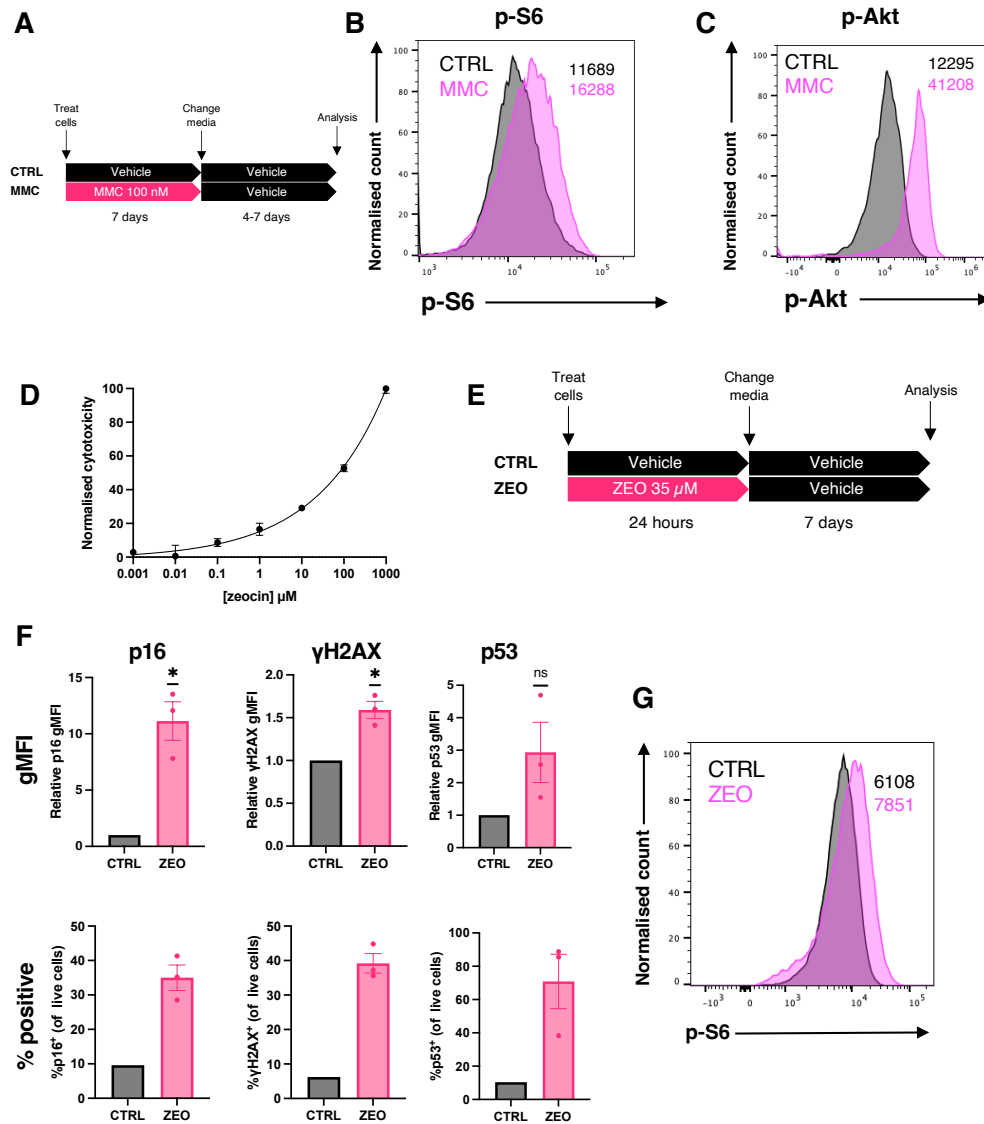


Figure 3.7 DNA damage-induced senescent fibroblasts display high mTOR activity

(A) Induction of MMC-induced DDIS. (B–C) Fluorescence histograms of (B) p-S6 or (C) p-Akt in CTRL (grey) and MMC-induced senescent fibroblasts (pink), measured by flow cytometry (D) Dose response curve of human dermal fibroblast viability after 3-day treatment with 10-fold dilutions of zeocin, measured using the alamarBlue viability assay, $n=3$ technical replicates per concentration. (E–G) (E) Experimental design for zeocin-induced DDIS (F) Geometric mean fluorescence intensity (gMFI) (upper) or percentage of cells positive (lower) for p16, γH2AX , and p53, measured by flow cytometry, in zeocin-induced senescent fibroblasts. gMFI data are normalised to CTRL fibroblasts. P -values are derived from a one-sample t-test, $n=3$ technical replicates. (G) Representative fluorescence histogram of p-S6 in CTRL (grey) and zeocin-induced senescent fibroblasts (pink). Geometric mean fluorescence intensity (gMFI) of p-S6 in each condition is indicated.

3.2.8 Pan-mTOR inhibition is geroprotective in DDIS

3.2.8.1 *Screening the effects of pan-mTOR inhibition before, during, or after induction of DDIS*

Since mTOR activity is elevated in DDIS, I tested whether pan-mTOR inhibition would be able to protect against senescence induction by DNA damage using the drug AZD8055, an ATP-competitive inhibitor of mTOR that blocks both mTORC1 and mTORC2 (Chresta et al., 2010). Fibroblasts were treated with 70 nM AZD8055 for 7 days either before, during, or after 7-day treatment with MMC to induce DDIS, for 14 days in total (Figure 3.8A). Nuclear area was assessed by staining for nuclei *in situ* with NucBlue Live, and cellular morphology was assessed by staining for actin with rhodamine-phalloidin. In the absence of mTOR inhibition (MMC→DMSO and DMSO→MMC conditions), MMC induced nuclear and overall cellular enlargement consistent with senescence (Figure 3.8B—C, Figure 3.3). Whereas 7-day AZD8055 pre-treatment limited the increase in cell size induced by MMC (AZD→MMC), it did not significantly limit a senescent-like increase in nuclear area (Figure 3.8C). Co-treatment with both MMC and AZD8055 between days 8-14 significantly prevented nuclear enlargement (DMSO→AZD/MMC), but not if drugs were administered in days 1-7 and then removed for a further 7 days (AZD/MMC→DMSO) (Figure 3.8C). Post-treatment of MMC-treated cells with AZD8055 (MMC→AZD) limited both cellular and nuclear enlargement by day 14 (Figure 3.8B—C). However, AZD8055 was most effective at limiting both senescence-associated morphological phenotypes when administered continuously throughout the 14 days of the experiment (AZD/MMC→AZD and AZD→AZD/MMC conditions, Figure 3.8B—C). Continuous treatment with AZD8055 was therefore performed in subsequent experiments to explore further the geroprotective effects of mTOR inhibition.

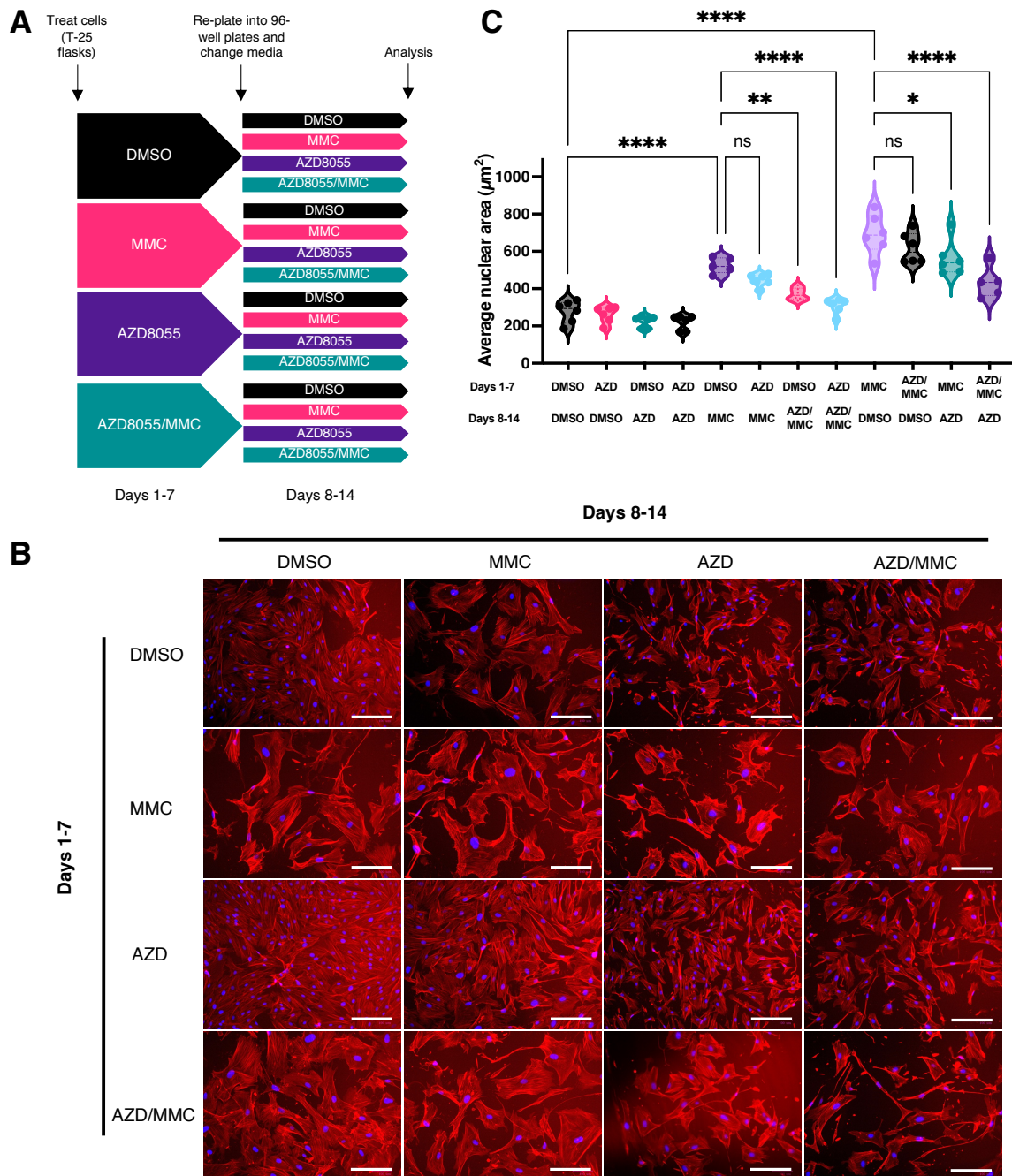


Figure 3.8 Screening for the effects of mTOR inhibition before, during, and after DDIS

(A) Experimental design treating cells with DMSO (vehicle), 100 nM mitomycin C (MMC), and/or the 70 nM of the pan-mTOR inhibitor, AZD8055 (AZD) at 70 nM, across consecutive 7+7 days (days 1-7, 8-14). (B) Rhodamine-phalloidin staining for F-Actin (red) and NucBlue Live staining for nuclei (blue). Scale bars represent 100 μm . (C) Average nuclear areas of across different conditions, $n=6$ technical replicates (average of 50-100 nuclei per replicate). P -values are derived from a one-way ANOVA with Tukey's multiple comparisons test.

3.2.8.2 Pan-mTOR inhibition protects against multiple phenotypes of senescence induced by DNA damage

To investigate the effects of pan-mTOR inhibition on additional phenotypes of DDIS, I treated fibroblasts for 7 days with MMC, followed by a 7-day recovery period without MMC, in the continuous presence or absence of 70 nM AZD8055, and measured multiple senescence features including cellular and nuclear morphology, SA β G activity, lysosomal load, and the presence of γ H2AX⁺ and 53BP1⁺ DNA damage foci (Figure 3.9A). Consistent with my previous observations (Figure 3.8), treatment with AZD8055 (AZD8055/MMC→AZD8055) profoundly limited the induction of enlarged, senescent morphologies (Figure 3.9B) and actin stress fibres visualised by rhodamine-phalloidin (Figure 3.9C). AZD8055-treated cells maintained a non-senescent diameter (measured in cell suspension, Figure 3.9D) and nuclear area in adherent cells (Figure 3.9E). AZD8055 treatment also significantly reduced dark blue SA β G⁺ staining compared to MMC alone (MMC→DMSO), indicating decreased lysosomal SA β G activity (Figure 3.10A). Consistent with this reduction in lysosomal SA β G activity, AZD8055 treatment also diminished lysosomal hypertrophy induced by MMC, as measured by LysoTracker Red fluorescence (Figure 3.10B).

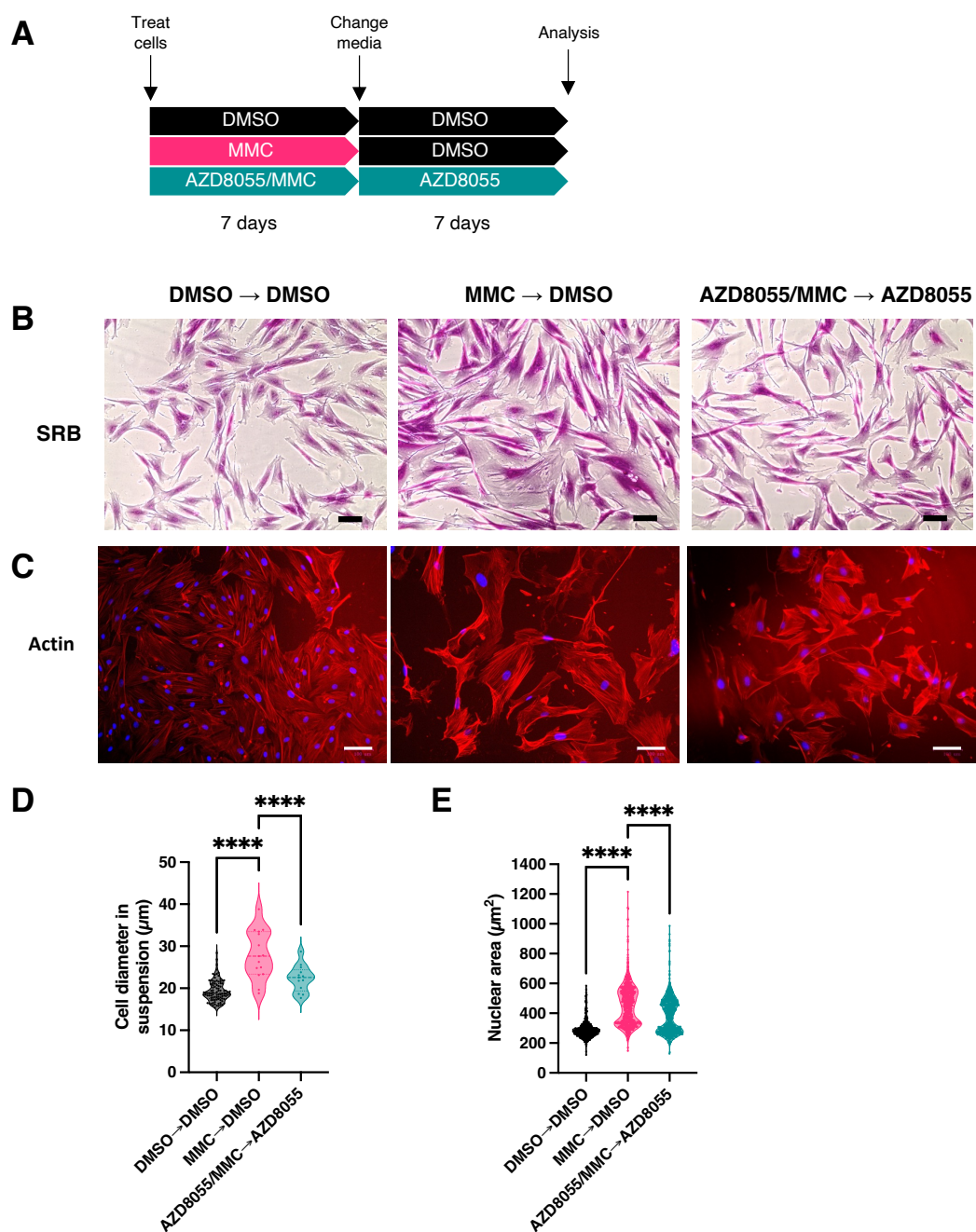


Figure 3.9 mTOR inhibition limits the ability of MMC treatment to induce senescence morphology in DDIS

(A) Experimental design for DNA damage-induced senescence with 100 nM mitomycin C (MMC) in the presence of the pan-mTOR inhibitor, AZD8055, at 70 nM. (B–C) Staining with (B) sulphorhodamine B for cellular biomass (free amines) and (C) rhodamine-phalloidin for F-actin (red) and NucBlue Live for nuclei (blue). Scale bars represent 100 μm . (D–E) (D) Cell diameter in suspension (30-100 cells analysed per condition) and (E) nuclear area (150-200 nuclei analysed per condition). *P*-values derived from a one-way ANOVA with Dunnett's multiple comparisons test. Data are representative of three independent experiments.

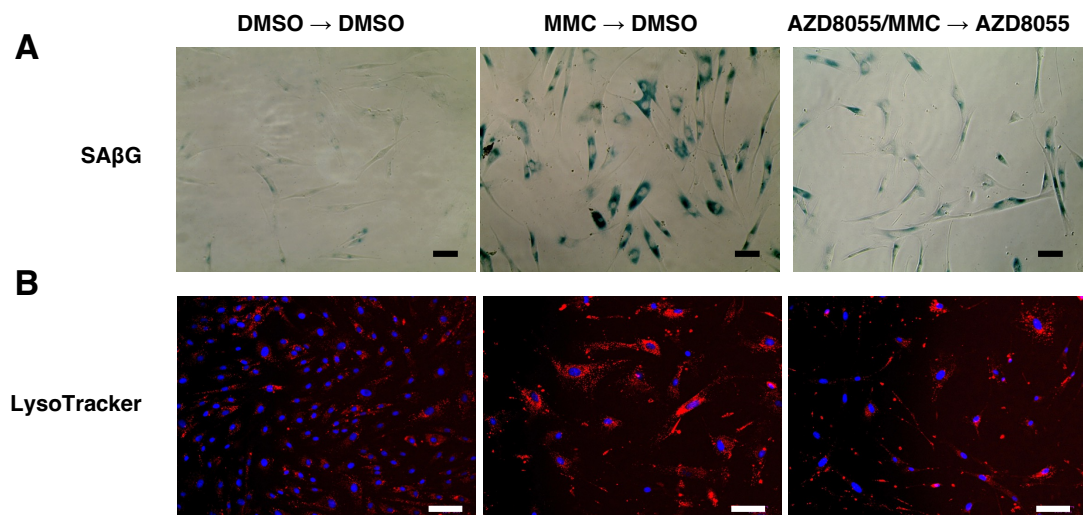


Figure 3.10 Pan-mTOR inhibition limits SAβG activity and lysosomal hypertrophy in DDIS

Experimental design as in Figure 3.9 with staining at day 14 for (A) senescence-associated β -galactosidase activity (blue) and (B) lysosomes with LysoTracker Red and NucBlue Live for nuclei (blue). Scale bars represent 100 μ m. Data are representative of three independent experiments.

The reduction in SAβG activity and lysosomal stress with AZD8055 treatment suggests that mTOR inhibition may reduce DDIS phenotypes. I therefore tested whether AZD8055 treatment could affect the presence of DNA damage markers, γ H2AX and 53BP1, by immunofluorescence microscopy (Figure 3.11A–B) after 14 days of treatment in total, as in Figure 3.9A. Intriguingly, AZD8055 significantly reduced the number of both γ H2AX⁺ and 53BP1⁺ foci per nucleus (Figure 3.11A–D), suggesting that pan-mTOR inhibition may limit either DNA damage signalling or the accumulation of DNA lesions during DDIS. In summary, pan-mTOR inhibition by AZD8055 greatly attenuated features of DDIS, including DNA damage, suggesting it may be a geroprotective treatment.

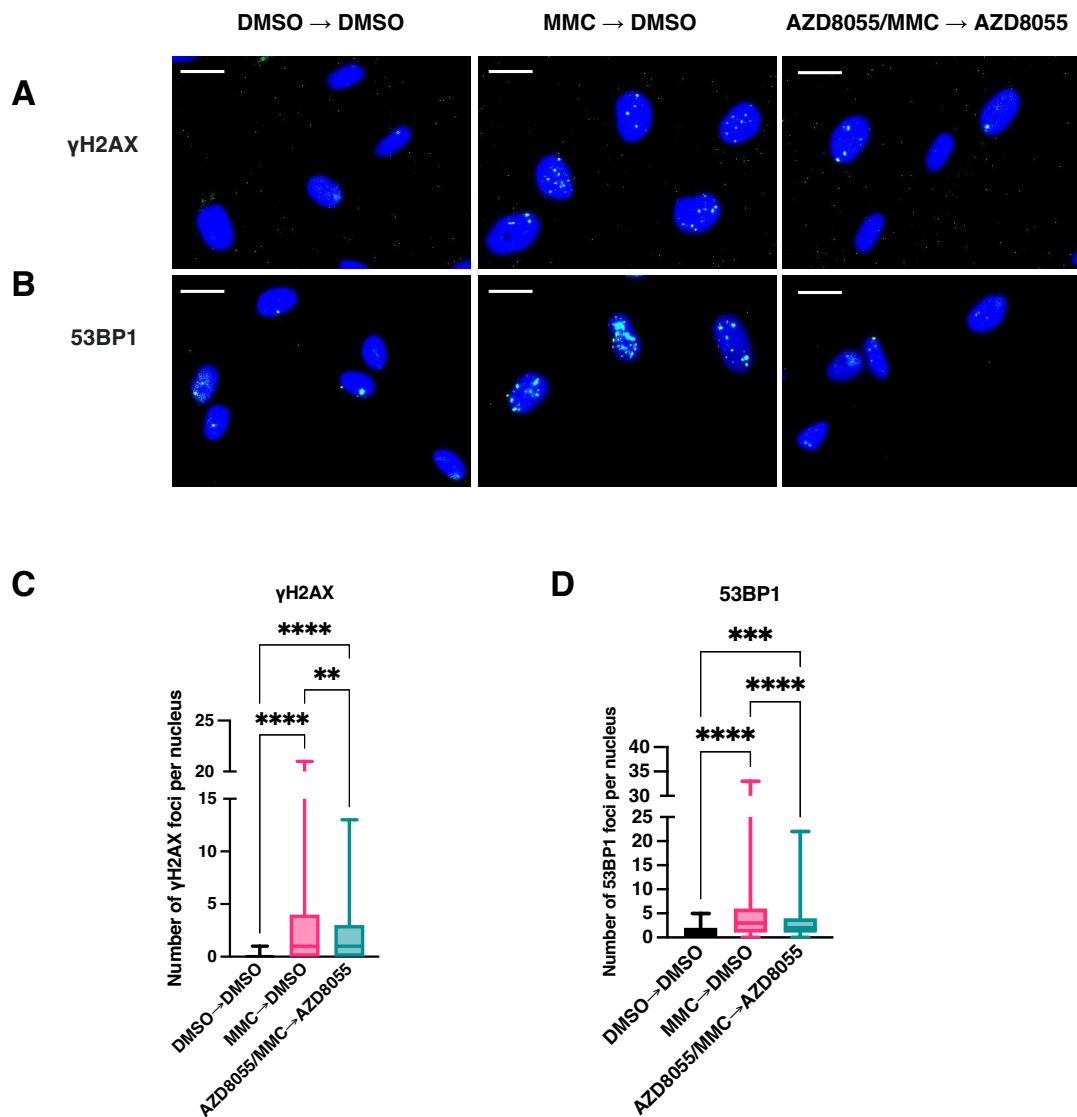


Figure 3.11 Pan-mTOR inhibition decreases presence of DNA damage foci in DDIS

Cells were exposed to 100 nM mitomycin C (MMC) $\pm$ 70 nM AZD8055 as in Figure 3.9, with immunofluorescence microscopy at day 14, staining with (A–B) NucBlue Live for nuclei (blue) and DNA damage foci (AF488, green) for (A) γ H2AX and (B) 53BP1. Scale bars represent 25 μ m. (C–D) Quantification of the number of (C) γ H2AX or (D) 53BP1 foci per nucleus in fibroblasts. *P*-values are derived from a one-way ANOVA with Tukey's multiple comparisons test, 150-200 nuclei analysed per condition. Data are representative of three independent experiments.

3.3 Discussion

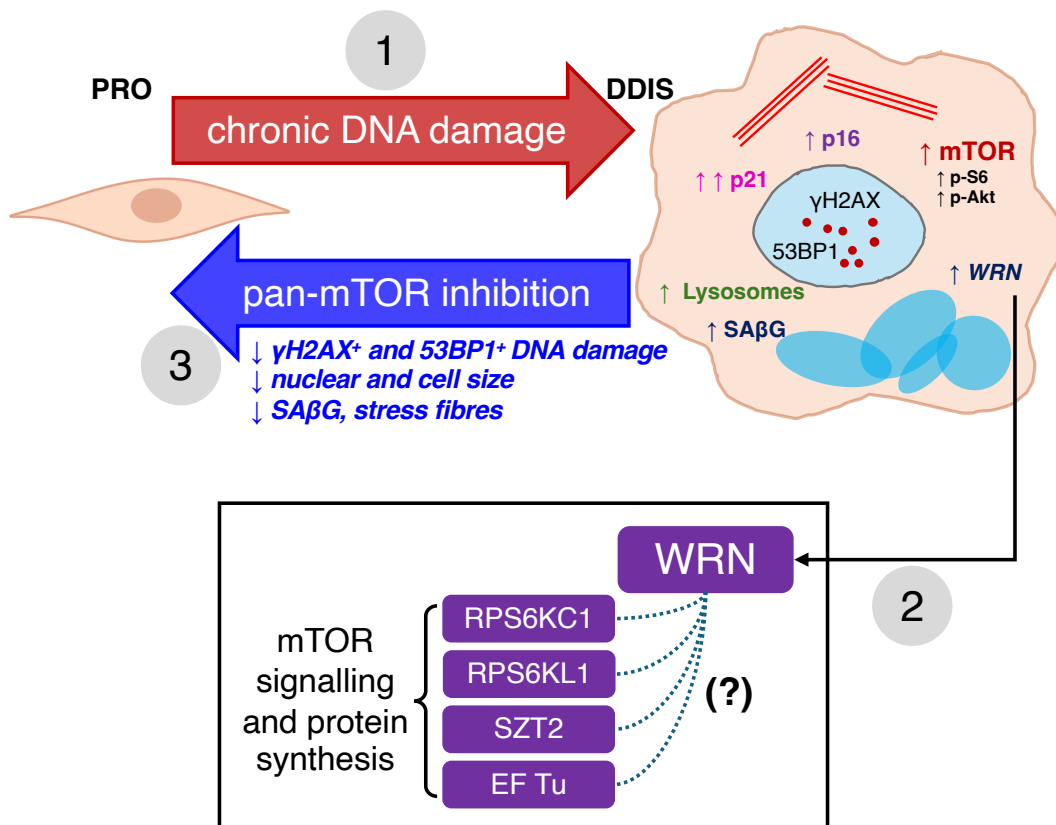


Figure 3.12 Summary of major findings from Chapter 3

1) Chronic mitomycin C treatment of proliferating (PRO) human dermal fibroblasts induced DNA damage-induced senescence (DDIS). DDIS fibroblasts displayed many senescence biomarkers, including high levels of cyclin kinase inhibitor expression, lysosomal hypertrophy, SAβG activity, cellular and nuclear enlargement, and persistently high levels of 53BP1+ and γH2AX+ DNA damage foci. **2)** DDIS fibroblasts expressed high levels of *WRN* mRNA. Phage display of purified WRN helicase suggests potential interactions with human proteins involved in mTOR signalling and protein synthesis pathways. **3)** Continuous pan-mTOR inhibition with AZD8055 restored youthful phenotypes in DDIS fibroblasts, including decreased DNA damage foci.

The major findings from this chapter are summarised in Figure 3.12. As HF043 human foreskin dermal fibroblasts were cultured until they reached replicative senescence (RS), they acquired senescent morphological features and high cyclin kinase inhibitor levels. These dermal fibroblasts displayed a long replicative lifespan, reaching CPD>80. Dermal fibroblasts appear to have a longer proliferative lifespan compared with fibroblasts from other tissue origins, such as WI-38 lung fibroblasts, which reach replicative senescence at

CPD 50 (Hayflick and Moorhead, 1961). This is likely due to the different roles of these cell types in the body: dermal fibroblasts must replenish the skin barrier, which constantly receives abrasive impacts that are not experienced by lung fibroblasts (Gauthier et al., 2023). Nevertheless, this indicates that these different fibroblast types display differing proclivities towards replicative exhaustion, which may be driven by underlying anti-senescence pathways. Of note, all experiments in this chapter were performed with human male foreskin fibroblasts, and therefore may not capture any sexually dimorphic phenotypes of cell senescence. Therefore, future work would benefit from increasing the variety of cell types utilised, and the sex of their donor origins.

While the impact of mTOR inhibition on RS has been described (Walters et al., 2016), in this chapter I characterised DNA damage-induced senescence (DDIS) in human dermal fibroblasts, recapitulating a number of the senescence-associated phenotypes observed in RS. I observed high levels of *CDKN1A* (p21^{Cip1/Waf1}) and *CDKN2A* (p16^{Ink4a}) mRNA, with a higher fold increase of *CDKN1A* than *CDKN2A* in both RS and DDIS fibroblasts compared to non-senescent cells. This suggests that these skin fibroblasts may have preferentially undergone p21-dependent senescence: while this is expected in the context of DDIS, where sustained DDR activation of p53 leads to upregulation of p21, this suggests that RS may also be established predominantly by DNA damage. Notably, p21- and p16-dependent senescence play different roles in age-related disease pathology. A comparison of *p16-INK-ATTAC* and *p21-ATTAC* mouse models, which allows for targeted ablation of p16- and p21-expressing senescent cells respectively, found that only removal of p21⁺ cells was able to alleviate radiotherapy-induced osteoporosis, whilst removal of p16⁺ cells had no effect, though both p21⁺ and p16⁺ senescent cells were induced by the radiotherapy treatment (Chandra et al., 2022). Both p21 and p16 positivity are often used somewhat synonymously in the context biomarkers of senescence – however, due to their divergent roles in

senescence induction and disease pathology, understanding the differences between these two major pathways for senescence induction in a range of cell types is important.

Previous data from our group identified that overexpression of WRN, a dual helicase and exonuclease with many roles in maintaining genome stability, strongly increased lifespan and healthspan in *C. elegans* (Lees *et al.*, manuscript in preparation). I aimed to assess the hypothesis that WRN levels were limiting during human cell ageing. I found by qRT-PCR that *WRN* expression increased by ~1.5-2-fold in RS and DDIS (Figure 3.4A—B) compared to non-senescent control fibroblasts, though attempts to assess WRN on a protein level were unsuccessful as commercially available antibodies failed to give a signal at WRN levels found in primary cells. While my data suggest that *WRN* mRNA levels increase with senescence *in vitro*, this is in contrast to an earlier study which found that *WRN* transcript levels decrease with human ageing in peripheral blood mononuclear cells (PBMCs) (Polosak *et al.*, 2011). These differences may reflect cell type-specific *WRN* expression in fibroblasts vs PBMCs and/or the difference between *in vitro* senescence and chronological human ageing (Polosak *et al.*, 2011). This increase in *WRN* mRNA levels in fibroblasts undergoing RS could not be attributed to changes in the methylation status of the *WRN* promoter, according to my methylation-specific PCR data (Figure 3.5). Possibly, *WRN* mRNA abundance may be higher either due to decreased mRNA degradation, or due to other mechanisms such as increased transcription factor engagement at the promoter. To determine whether increased *WRN* mRNA levels could be a compensatory mechanism in response to the levels of DNA damage and decrease in proliferative potential in senescence, I attempted to overexpress WRN using a CRISPR-ON system (Konermann *et al.*, 2015), to test the hypothesis that higher WRN levels could protect cells from DDIS. However, due to the sensitivity of these primary cells to transfection with high quantities of exogenous DNA, this was not a viable approach and was not pursued further. Future approaches could utilise simpler constitutive overexpression systems, perhaps using a

CMV-based promoter. I explored the consequences of loss of WRN function by inhibiting WRN helicase activity using NSC 19630 and measuring the proliferative capacity of fibroblasts (Figure 3.4E—F). As expected, NSC 19630 decreased fibroblast proliferation, consistent with the use of this drug as a chemotherapeutic agent for hyperproliferative cancer cells (Moles et al., 2016).

WRN protein interacts with a wide range of proteins, predominantly those with roles in DNA metabolism and repair (Lachapelle et al., 2011). Since I had observed that *WRN* mRNA levels increased with senescence and is reported to be geroprotective in *C. elegans* (Lees et al., manuscript in preparation), I used phage display to identify WRN-interacting peptide motifs (Figure 3.6). Intriguingly, several of the phage peptide sequences that uniquely bound WRN bore similarity to proteins involved in the mTOR signalling and protein synthesis pathways – namely, Ribosomal Protein S6 Kinase C1, Ribosomal protein S6 kinase-like 1, KICSTOR complex protein SZT2, and Elongation Factor Tu (Table 3.1). This suggests potential novel WRN interactions with both the mTOR and protein synthesis pathways. Previous mass spectrometry identification of proteins interacting with YFP-tagged WRN identified ribosomal proteins, such as RPS3, RPS9, RPS3A, RPL5 and RPL11, as known WRN-binding proteins (Lachapelle et al., 2011). Subsequent pathway analysis found that these and other WRN interactors identified were significantly enriched in protein biosynthesis and processing pathways (Lachapelle et al., 2011). It is unclear if, in this context, WRN is exerting its canonical role in DNA repair – since some ribosomal proteins, such as RPS3, have established roles in binding oxidative DNA damage lesions (Yadavilli et al., 2007). Alternatively, WRN may be exhibiting non-canonical functions in mTOR signalling and protein synthesis. In support of this non-canonical role of WRN, is that WRN-binding ribosomal proteins were detected despite nuclease treatment (i.e., binding was not dependent on the presence of DNA) (Lachapelle et al., 2011). Furthermore, WRN protein associates with a complex of cytoplasmic ribosomal proteins (Pedraz et al., 2016),

and WRN depletion significantly decreased *de novo* protein synthesis, suggesting WRN positively regulates protein production (Iglesias-Pedraz et al., 2020). Intriguingly, lentiviral depletion of WRN also caused an increase in p-p70S6 levels, indicating high mTORC1 activity, which may possibly arise as a compensatory mechanism for loss of WRN-mediated aid of protein synthesis (Iglesias-Pedraz et al., 2020). Previous studies demonstrate that the premature senescence of WRN-deficient fibroblasts can be alleviated by long-term treatment with the mTORC1 inhibitor, rapamycin (Saha et al., 2014). Interestingly, DDIS fibroblasts also displayed high levels of mTOR activity (Figure 3.7), despite high *WRN* expression; future experiments could elucidate further the interactions between WRN helicase and mTOR signalling by chemical knockdown and overexpression techniques.

This phage display study is limited due to the low throughput of sequencing of phage plaques. Only 9 unique WRN-interacting peptide sequences were identified, and most of these were selected from one plaque each. Future work would benefit from screening more plaques from the WRN test condition, ideally in a high throughput manner, such as bulk DNA sequencing. Furthermore, future work would need to confirm an *in vivo* interaction of WRN with the targets identified from the phage display, as the alignments of phage peptide sequences and human proteins provides evidence only for a putative interaction.

DDIS fibroblasts exhibited high levels of mTOR activity (Figure 3.7). The pan-mTOR inhibitor, AZD8055 strongly attenuated phenotypes of DDIS (Figure 3.9), extending previous work from our group that reported on the geroprotective effects of AZD8055 in the replicative senescence of skin fibroblasts (Walters et al., 2016, Rolt et al., 2019). One particularly interesting observation was that continuous AZD8055 treatment reduced the number of γ H2AX⁺ and 53BP1⁺ DNA damage foci in cells undergoing DDIS (Figure 3.11), suggesting that AZD8055 either decreases DDR signalling or the number of lesions induced by the DNA-damaging agent. Since mTOR is an atypical PI3K-like kinase, it is possible that AZD8055 may inhibit signalling by PI3K-like kinases involved in the DDR pathway, such as

DNA-PK, ATM, and ATR (Huang et al., 2020b) – as evidence against this, however, AZD8055 shows 1000-fold selectivity for mTOR over all other PI3K and PI3K-like isoforms (Chresta et al., 2010). An alternative explanation is that mTOR inhibitors may directly decrease DNA damage, perhaps by promoting DNA repair. This explanation is supported by evidence that mTOR inhibition by rapamycin increases protein expression of the DNA repair factors, MGMT and NDRG1, via a post-transcriptional mechanism, both *in vitro* and *in vivo* (Dominick et al., 2017). Furthermore, continuous rapamycin treatment reduced the number of 53BP1⁺ DNA damage foci in WRN-deficient fibroblasts, again suggesting that mTOR inhibition increases genome stability in conditions of DNA repair deficiency (Saha et al., 2014). Future experiments to understand whether AZD8055 treatment in fibroblast DDIS reduces genuine DNA lesion burden, such as by using the alkaline comet assay, could be informative. Since DNA damage is a major trigger and feature of senescence and ageing more generally (Schumacher et al., 2021, Yousefzadeh et al., 2021a), the possibility that mTOR inhibition may be both geroprotective and genoprotective is provocative (Kell et al., 2023).

4

Results

mTOR inhibitors limit the induction of DNA
damage in primary human T cells

4.1 Introduction

4.1.1 Senescent T cells as a target for immunosenescence interventions

Circulating leukocytes with high levels of damage, either in the DNA or the mitochondria, leads to accelerated immunosenescence and organismal ageing in mice (Yousefzadeh et al., 2021b, Desdin-Mico et al., 2020). T cells are an abundant immune cell type that accumulate DNA damage with chronological ageing, particularly in the CD27⁻CD45RA⁺ TEMRA subset, and contribute to tissue deterioration (Callender et al., 2020, Soto-Herederó et al., 2023). Like senescent fibroblasts (Chapter 3), senescent T cells are proliferatively impaired, secrete pro-inflammatory cytokines, and possess high levels of DNA damage (Table 1.1) (Akbar et al., 2016). Furthermore, the master regulator of the DNA damage response (DDR), ATM, drives p38 MAPK activation and the acquisition of dysfunctional features of T cell senescence (section 1.3.3). T cells are therefore an attractive cellular target for both gero- and genoprotective strategies to avoid or overcome immunosenescence (Kell et al., 2023).

4.1.2 The role of mTOR in T cells

mTOR plays important roles in T cell biology. T cells activated for 24 hours increase their expression of over 50% of their proteome to increase their rate of mitosis, protein content, and cytokine production (Howden et al., 2019). Accordingly, activated T cells dramatically increase their protein synthesis rate to ~800,000 proteins/min, a rate 13 times higher than that of naïve T cells (Wolf et al., 2020). mTOR activity contributes, at least in part, to aspects of proteome remodelling after T cell activation, as mTORC1 inhibition by rapamycin limited protein expression in activated T cells by 20%, including the expression of cytokines (IFN γ , granzyme B, IL-2, and perforin), and proteins involved in mitochondria, ribosomes, and

glycolysis (Howden et al., 2019). However, such activation may accelerate immunosenescence, as CD8⁺ T cells from T-*Tsc2*^{-/-} mice, which experience T-cell-specific mTOR hyperactivity, display high positivity for the T cell senescence marker, KLRG1, are very cytotoxic, and exhibit proliferative dysfunction after antigenic challenge, which could be reversed with rapamycin treatment (Pollizzi et al., 2015). These data suggest that, though mTOR activity is required for T cell activation, mTOR hyperactivity drives a terminally differentiated T cell phenotype (Pollizzi et al., 2015). One intervention for improving immune system function in older people is low-dose mTOR inhibition with a rapalogue (RAD001 (Mannick et al., 2014)) or second-generation mTOR inhibitor (RTB101 (Mannick et al., 2018)), either as a dual or monotherapy. It is unclear how mTOR inhibition improves immunity, though effects on decreasing %PD-1⁺ T cells and increasing peripheral antiviral gene expression have been observed (Mannick et al., 2014, Mannick et al., 2018, Mannick et al., 2021). Overall, mTOR inhibition in older people may have wide-ranging effects on T cell biology to elicit immunoprotection.

In Chapter 3, I found that DDIS fibroblasts displayed high levels of mTORC1 and mTORC2 activity, and that treatment with a second-generation pan-mTOR inhibitor, AZD8055, attenuated many senescence phenotypes. Intriguingly, AZD8055 treatment also reduced γ H2AX⁺ and 53BP1⁺ DNA damage, though it is unclear whether this was due to decreased DDR signalling or a lower DNA lesion burden. Based on these results, the question arises as to whether low-dose mTOR inhibition may boost immunity in older people by modifying senescent immune cell phenotypes, such as persistent DNA damage. The effect of mTOR inhibition on T cell DNA damage, senescence, and survival, has not been fully explored.

4.1.3 Chapter aim and objectives

In this chapter, I therefore aim to explore the relationship between DNA damage and mTOR activity both in T cell senescence and in response to genotoxic challenge.

Objectives:

1. To assess whether DNA damage burden and mTOR activity differ in different T cell memory subsets.
2. To evaluate whether mTOR inhibition affects T cell activation.
3. To investigate whether mTOR activity changes in response to acute DNA damage in human CD4⁺ and CD8⁺ T cells.
4. To analyse if mTOR inhibition affects DNA damage responses and lesion burden in CD4⁺ and CD8⁺ T cells.

4.2 Results

4.2.1 Senescent CD4⁺ T cells display high DNA damage levels and mTORC1 activity

To investigate human T cell senescence, I used flow cytometry to identify T cell differentiation subsets in blood from healthy donors from the NHS blood transfusion (NHS-BT) service, based on their cell surface marker expression. Naïve (TN, CD27⁺CD45RA⁺), central memory (TCM, CD27⁺CD45RA⁻), effector memory (TEM, CD27⁻CD45RA⁻), and effector memory re-expressing CD45RA (TEMRA, CD27⁻CD45RA⁺) T cells were identified (Figure 4.1A). The proportions of naïve (TN) and effector memory (TEM) cells were the same in CD4⁺ and CD8⁺ T cells (Figure 4.1B); however, CD8⁺ T cells comprised a lower percentage of central memory (TCM) cells, and a greater percentage of TEMRA cells than did CD4⁺ T cells (Figure 4.1B). TEMRA cells are established both to accumulate with ageing faster in CD8⁺ than in CD4⁺ T cells, and to display characteristics of senescence (Callender et al., 2020). TEMRA populations were enriched for cells expressing the terminal differentiation markers, CD57 (Figure 4.1C) and KLRG1 (Figure 4.1D), in both CD4⁺ and CD8⁺ T cells. Next, I assessed γ H2AX levels (indicative of unrepaired DNA damage) in the different T cell memory subsets, finding that CD4⁺ TEMRA cells displayed significantly higher levels of γ H2AX than their naïve counterparts (Figure 4.1E). However, γ H2AX levels were the same in all CD8⁺ T cell subsets (Figure 4.1E).

To assess mTOR activity, phospho-specific antibodies were used to detect p-S6 (mTORC1 target) and p-Akt (mTORC2 target) levels in the T cell memory subsets. CD4⁺ TEMRA cells had significantly higher levels of p-S6, but not p-Akt, than naïve cells, indicating high mTORC1 activity (Figure 4.1F—G). As with γ H2AX levels, CD8⁺ TEMRA cells did not show any increase. These data indicate that both DNA damage (γ H2AX) and mTORC1 (p-S6) activity is higher in senescent than early-differentiated, naïve CD4⁺ T cells, similar to my findings in Chapter 3 in senescent dermal fibroblasts.

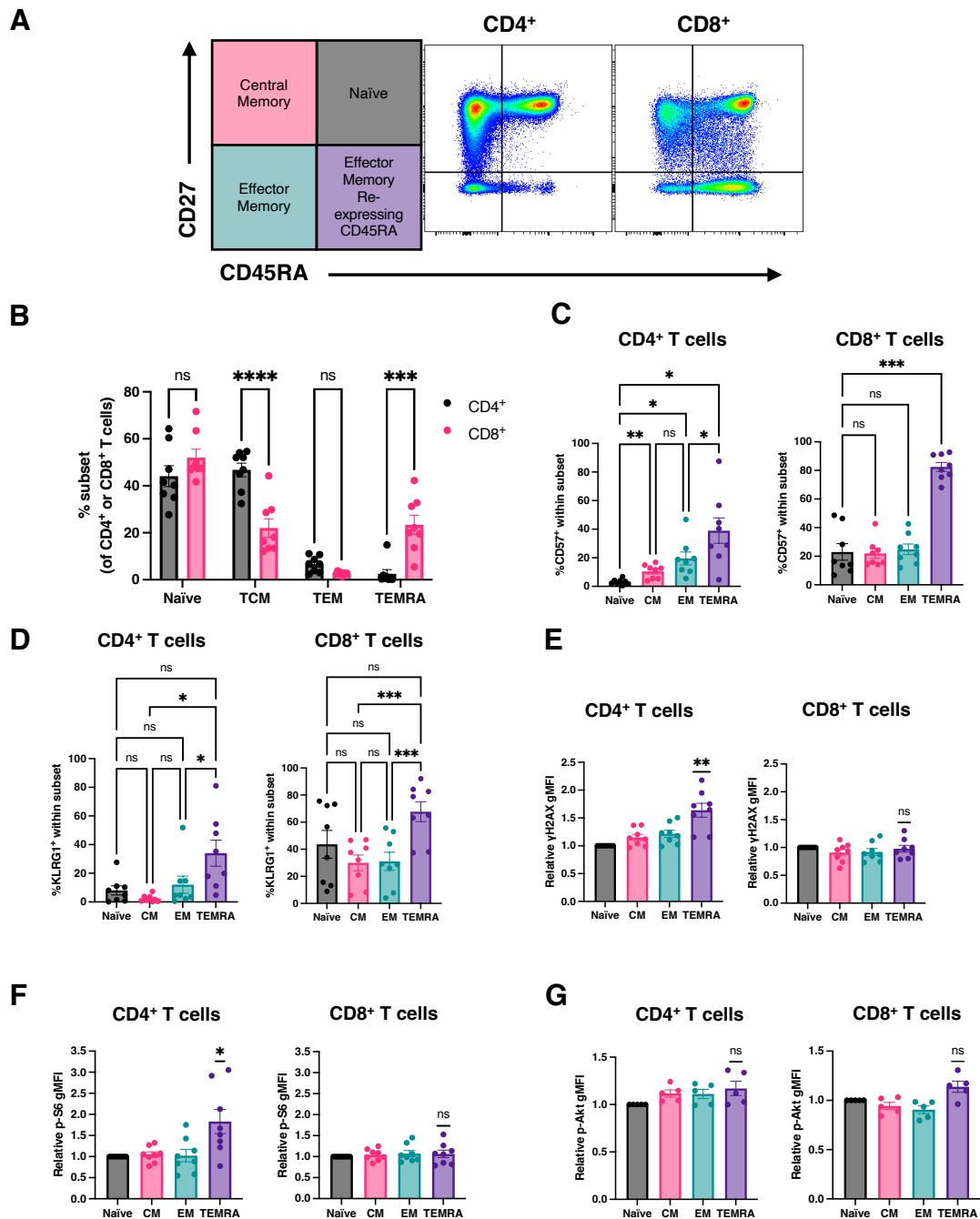


Figure 4.1 Identifying senescent T cells from healthy human blood by flow cytometry

(A) (Left) Gating of CD27/CD45RA-defined T cell memory subsets using flow cytometry. Representative flow cytograms of CD4+ and CD8+ T cells from one healthy donor *ex vivo*, gated on: Cells/Single cells/Live cells/CD4+ or CD8+ T cells (see Figure 2.1). (B) Proportion of memory subsets in CD4+ and CD8+ T cells in $n=8$ healthy donors, two-way ANOVA with Šídák's multiple comparisons test. (C–D) Proportion of positive (C) CD57 and (D) KLRG1 cells within differentiation subsets. P -values derived from a one-way ANOVA with Tukey's multiple comparisons test, $n=8$ healthy donors. (E–G) Geometric mean fluorescence intensity (gMFI) of (E) γ H2AX, (F) p-S6, and (G) p-Akt, in differentiation subsets, relative to naïve cells for each donor. P -values derived from a Wilcoxon Signed Rank Test (theoretical median = 1), $n=8$ healthy donors.

4.2.2 Dynamics of mTOR activation across 3-day T cell activation

Low-dose mTOR inhibitors improve vaccine responses in humans (Mannick et al., 2018, Mannick et al., 2021, Mannick et al., 2014). To test whether this could be recapitulated *in vitro*, PBMCs from healthy donors were subjected to 3-day T-cell-specific activation with anti-CD3 and anti-CD28 antibodies to mimic antigen-specific engagement of the T cell receptor (TCR) and co-stimulation, in the presence 10 nM rapamycin, 100 nM AZD8055, or DMSO control. Cells were harvested at various points throughout the 3-day activation (Figure 4.2A) to determine the effects of the mTOR inhibitors on T cell phenotypes by flow cytometry. These mTOR inhibitor concentrations were chosen as previous studies show the geroprotective properties of rapamycin at either 10 or 100 nM (Houssaini et al., 2018, Park et al., 2020a). Since low-dose (1 mg/day) oral rapamycin in older adults leads to mean blood rapamycin concentration of 7.88 nM (range 5.14 – 12.91) (Kraig et al., 2018), 10 nM rapamycin was therefore selected to mimic this *in vivo* dose *in vitro*. As preliminary investigations in activated human T cells showed that 100 nM AZD8055 limited p-S6 to levels equivalent to 10 nM rapamycin (data not shown), AZD8055 at 100 nM was selected for further experiments.

Gating on T cell subsets revealed that CD4⁺ T cells displayed bimodal activation of mTORC1 activity across the 3 days, as measured by p-S6 levels, peaking at 6 and 48 hours (Figure 4.2B). By contrast, p-S6 in CD8⁺ T cells peaked strongly at 24 hours of activation and declined thereafter (Figure 4.2B). The peak of p-S6 in CD8⁺ T cells was much higher than in CD4⁺ T cells (Figure 4.2B). Treatment with either rapamycin or AZD8055 strongly inhibited levels of p-S6 at all time points (Figure 4.2B). Gating further on the T cell CD27/45RA subpopulations showed that in CD4⁺ T cells, naïve (TN) and central memory (TCM) subpopulations contributed to the early (6 hour) increase in p-S6 levels, while TEM and TEMRA cells contributed to the later peak in p-S6 at 48 hours of activation (Figure 4.2C). CD8⁺ T cells differed from CD4⁺ T cells, as p-S6 induction in TN, TCM, and TEM all

occurred maximally at 24 hours, in contrast to TEMRA cells, which showed a small p-S6 peak at 48 hours (Figure 4.2C). In both CD4⁺ and CD8⁺ T cells, both the speed and extent of p-S6 signal after TCR engagement decreased with increasing T cell differentiation status (i.e. TN>TCM>TEM>TEMRA). This may be due to the nature of TCR engagement, as TCR downstream signalling, which activates mTOR, is impaired in senescent T cells (Henson et al., 2009).

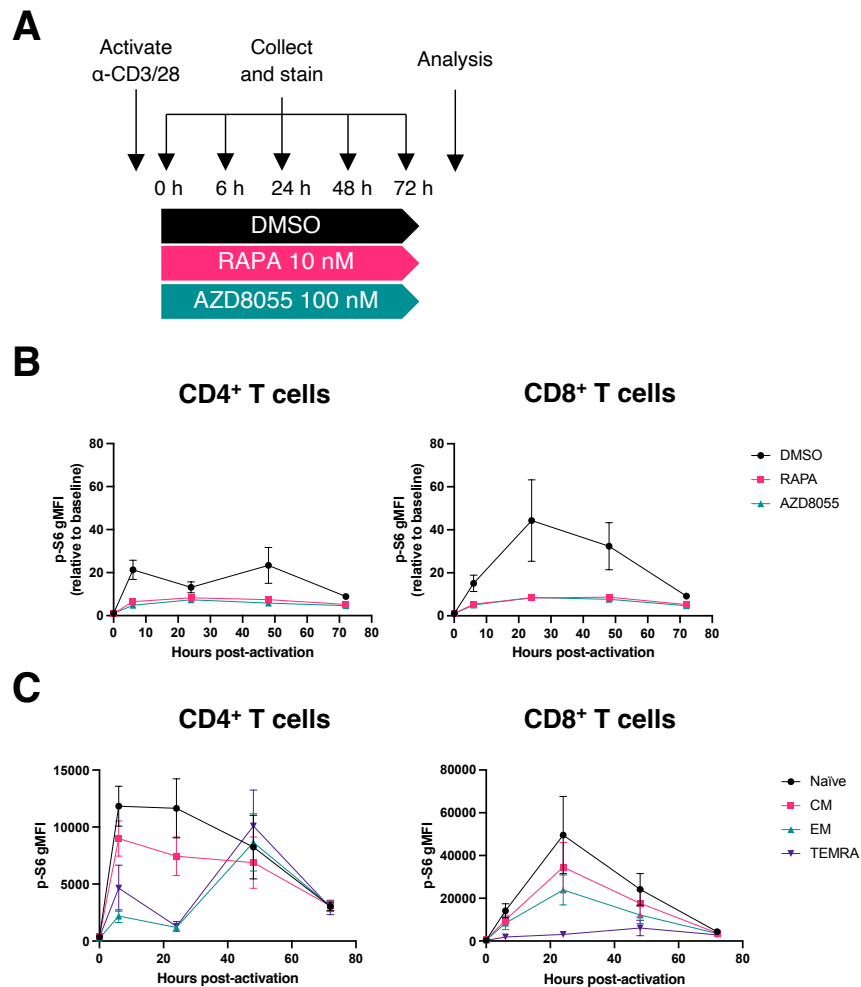


Figure 4.2 Effects of mTOR inhibition on p-S6 across 3-day T cell activation

(A) Experimental design for 3-day T-cell-specific activation of PBMCs from healthy donors and flow cytometric analysis. (B–C) p-S6 geometric mean fluorescence intensity (gMFI) relative to baseline, throughout 72 hours of activation in (B) total CD4⁺ or CD8⁺ T cells with 10 nM rapamycin (RAPA), 100 nM AZD8055, or DMSO control, or (C) in DMSO-treated T cell memory subsets (naïve, central memory (CM), effector memory (EM), or effector memory re-expressing CD45RA (TEMRA), in CD4⁺ (left) and CD8⁺ (right) T cells, n=4 healthy donors per time point.

These changes in phosphorylation of mTOR target proteins were reflected in pH changes in the cell culture supernatants, indicative of metabolic activity. At low pH, the phenol red indicator is converted to yellow, which can be measured by an increase in the 450/560 nm absorbance ratio of the culture supernatants (Figure 4.3A) (Wang et al., 2003). The culture supernatants of PBMCs undergoing 3-day T-cell-specific activation in the presence of vehicle control (DMSO) displayed a significantly higher 450/560 nm absorbance ratio than cells treated with rapamycin or AZD8055, whose culture supernatants appeared more like those of media only (no cells) (Figure 4.3B). This indicates that mTOR inhibition decreases T cell metabolism in these culture conditions.

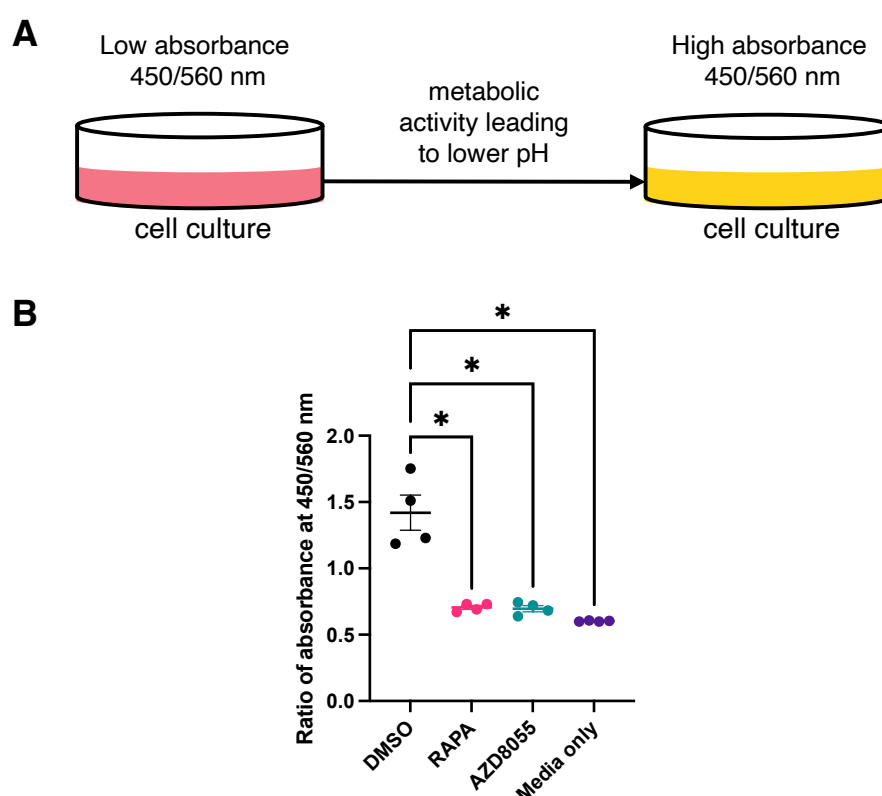


Figure 4.3 Effects of mTOR inhibition on culture supernatant pH (assessed by phenol red absorbance) across 3-day activation of healthy donor T cells (A) Schematic diagram of phenol red colour changes with decreasing pH. (B) Ratio of 450/560 nm absorbance of PBMC culture supernatants across conditions from the experiment in Figure 4.2A, measured after 72 hours of activation. *P*-values are derived from a one-way ANOVA with Dunnett's multiple comparisons test, *n*=4 healthy donors.

4.2.3 Cellular effects of mTOR inhibition across 3-day T cell activation

To further assess the impact of mTOR inhibition in stimulated T cells, cells were labelled with an antibody against IL-2R α (CD25, a marker upregulated upon early T cell activation), and T cell senescence markers, KLRG1 and CD57, were analysed by flow cytometry. In CD4⁺ and CD8⁺ T cells over 3 days of activation, the percentage of CD25⁺ cells reached an average ($\pm$ SD) of $5.25 \pm 1.75\%$ at 6 hours, $63.0 \pm 20.8\%$ at 24 hours, $80.5 \pm 7.99\%$ at 48 hours, and almost 100% at 72 hours of activation (Figure 4.4A1). Though the induction of CD25⁺ T cells occurred slightly earlier with rapamycin and AZD8055 treatment, the proportion of CD25⁺ T cells was the same as in DMSO-treated cells at 3 days (Figure 4.4A). The percentage of CD57⁺ of CD4⁺ T cells steadily increased throughout activation but was significantly lower in AZD8055-treated cells at 3 days (Figure 4.4B). In CD8⁺ T cells, however, CD57 expression was higher in mTOR inhibitor-treated cells at 24 and 48 hours of activation, but lower in AZD8055-treated cells at 3 days (Figure 4.4B). AZD8055 treatment significantly reduced the proportion of KLRG1⁺ CD4⁺ T cells at 3 days activation, but not CD8⁺ T cells (Figure 4.4C). To assess whether mTOR inhibition throughout activation affected the level of DNA damage, I measured the proportion of cells positive for γ H2AX by flow cytometry in both CD4⁺ and CD8⁺ T cells. γ H2AX levels increased throughout activation, peaking at 48 hours (Figure 4.4D). Interestingly, the mTOR inhibitors suppressed the γ H2AX signal in both T cell subtypes: in CD4⁺ T cells, both rapamycin and AZD8055 limited γ H2AX at 48 and 72 hours of activation; in CD8⁺ T cells, both rapamycin and AZD8055 significantly inhibited γ H2AX at its peak levels, at 48 hours of activation, and this was also observed at 72 hours with rapamycin only (Figure 4.4D). In summary, whereas an early marker of T cell activation (CD25) was unchanged with mTOR inhibition, rapamycin and AZD8055 modulated several features of T cell senescence across 3-day activation: positivity for CD57, KLRG1, and γ H2AX⁺ DNA damage.

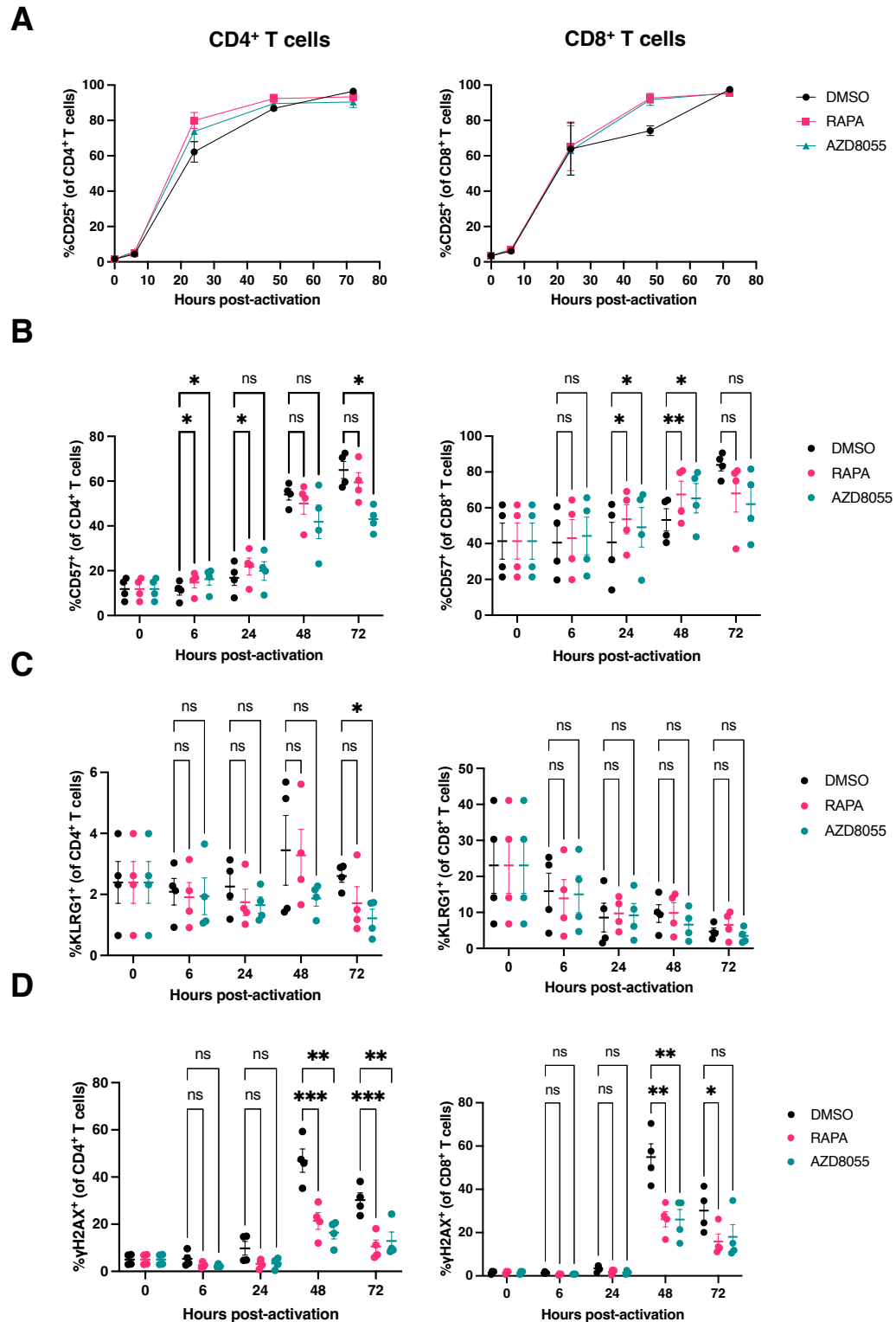


Figure 4.4 Cellular effects of mTOR inhibition across 3-day T cell activation in PBMCs from healthy NHS-BT donors

T cell activation was carried out as in Figure 4.2A. (**A–D**) Proportion of cells positive for (**A**) CD25, (**B**) CD57, (**C**) KLRG1, or (**D**) γH2AX in CD4⁺ (left) or CD8⁺ (right) T cells throughout 72-hour activation with 10 nM rapamycin, 100 nM AZD8055, or DMSO control. *P*-values derived from a two-way ANOVA with Dunnett's multiple comparisons test, *n*=4 healthy NHS-BT donors.

4.2.4 Developing a DNA damage assay in human T cells

To examine the effects of mTOR inhibition on acute DNA damage, I developed an assay for the *in vitro* induction of DNA damage with measurement by flow cytometry in PBMCs activated for 3 days with T-cell-specific anti-CD3/28, prior to induction of DNA damage. T cell activation was performed since others have shown that activation is required for T cells to recruit DNA damage sensors to sites of DNA lesions, in order to mount a DDR (Hu et al., 2018). After activation, cells were treated for 2 hours with 200 $\mu\text{g/ml}$ zeocin, a DNA double-strand break inducer (see Figure 3.7D—G), and then allowed to recover for 0, 2, 4, and 24 hours, before analysis of γH2AX in gated CD4^+ and CD8^+ T cells using flow cytometry (Figure 4.5A). Zeocin was used to induce specific DNA breaks, as previous work suggests that immune cells from older people accumulate SSBs and DSBs (Bonassi et al., 2021); zeocin may therefore induce DNA lesions more relevant to T cell ageing than other genotoxic agents. Acute treatment with 25 μM H_2O_2 was also used to induce non-specific DNA damage as a positive control. Like H_2O_2 treatment, zeocin induced a defined population of γH2AX^+ cells (Figure 4.5B). This γH2AX^+ population was higher in zeocin-treated (ZEO) compared to untreated (UT) cells across all recovery time points, but was significant and maximal in both CD4^+ and CD8^+ T cells after 4 hours recovery from zeocin (Figure 4.5C), consistent with a previous study (Hu et al., 2018). As these experiments were performed using cells from a single healthy donor, I assessed whether zeocin induced a γH2AX^+ signal at this 4-hour recovery time point using PBMCs from 6 NHS-BT donors, confirming that this population comprised between 18-50% of zeocin-treated T cells (Figure 4.5D). This *in vitro* DNA damage assay was then used to explore the role of mTOR in DNA damage induction.

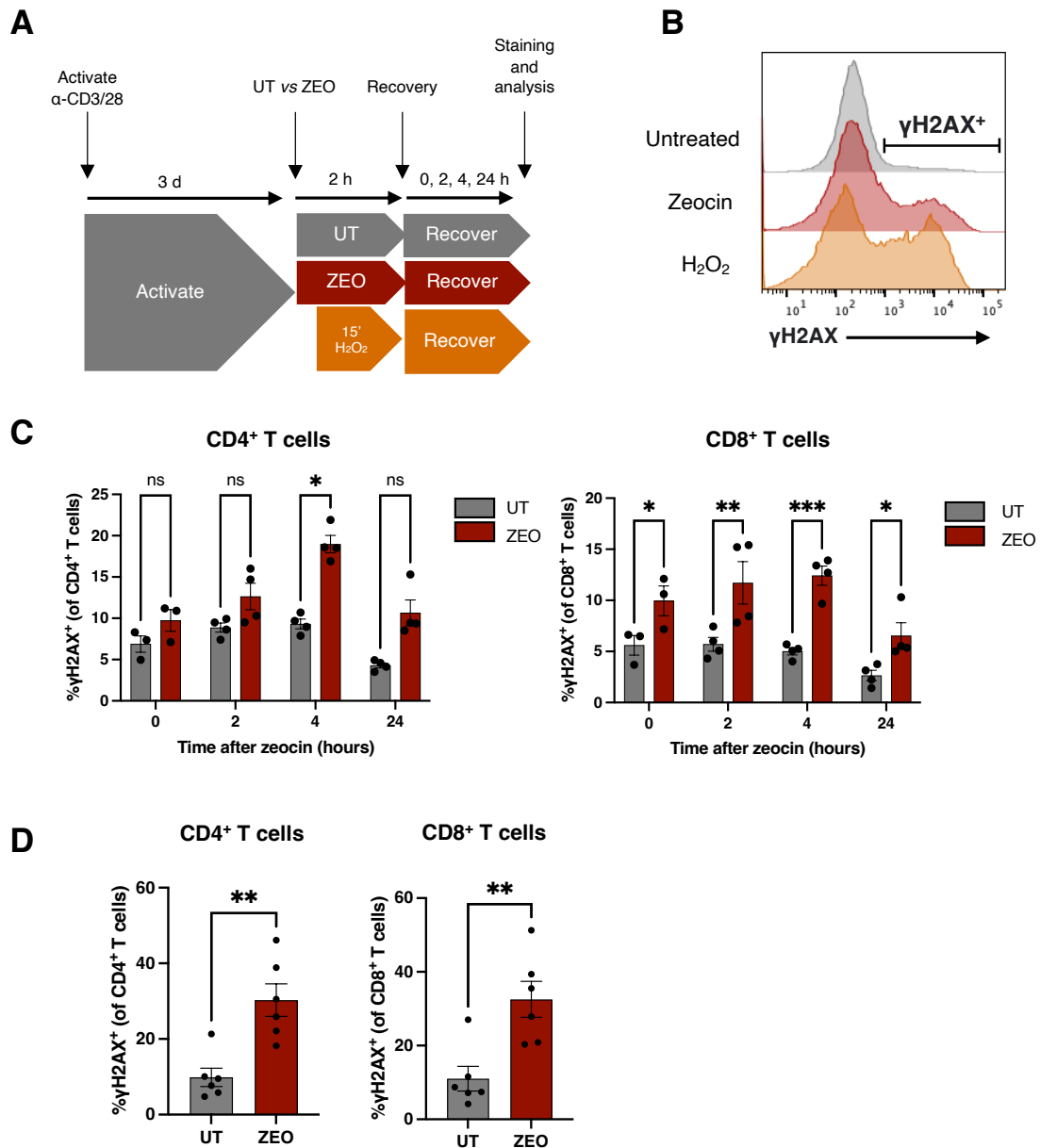


Figure 4.5 Developing a DNA damage assay in human T cells from healthy donors

(A–C) (A) PBMCs from a healthy donor were activated with 1 μ g/ml α -CD3/28 each for 3 days. Afterwards, cells were harvested and treated with zeocin (ZEO, 200 μ g/ml) or untreated (UT) for 2 hours. As a positive control for DNA damage, cells were treated with 25 μ M H_2O_2 for 15 mins. Cells were then left to recover after ending zeocin treatment for 0, 2, 4, or 24 hours, before being collected and stained for analysis by flow cytometry. Data from $n=4$ technical replicates from 1 donor. (B) Representative histograms of γ H2AX levels by flow cytometry in untreated, zeocin-treated, and H_2O_2 -treated PBMCs gated on CD4⁺ T cells. (C) Proportion of γ H2AX⁺ of CD4⁺ (left) and CD8⁺ (right) T cells across different recovery times after zeocin treatment. P -values are derived from a two-way ANOVA with Šídák's multiple comparisons test. (D) Proportion of γ H2AX⁺ of CD4⁺ (left) and CD8⁺ (right) T cells after 4 hours recovery from zeocin treatment with $n=6$ healthy donors. P -values are derived from a Mann-Whitney test.

4.2.5 Relationship between mTOR activity and DNA damage

To explore the relationship between mTOR activity and DNA damage, zeocin-treated cells were gated according to γ H2AX staining (γ H2AX⁺ or γ H2AX⁻, Figure 4.6A) and the phosphorylation levels of mTORC1/2 targets assessed by flow cytometry. Interestingly, both CD4⁺ and CD8⁺ T cells that were positive for γ H2AX displayed significantly higher levels of p-S6 than those negative for γ H2AX (Figure 4.6B). No differences were observed in phosphorylation of mTORC2 target, p-Akt, between γ H2AX⁺ and γ H2AX⁻ cells (Figure 4.6C). This indicates that DNA damage is associated with high activity of mTORC1, but not mTORC2, consistent with my observations that senescent CD4⁺ TEMRA cells expressed both high γ H2AX and p-S6, but not p-Akt (Figure 4.1E—F).

To investigate further cell type-specific differences in the relationship between mTOR activity and DNA damage, I compared p-S6 and p-Akt in γ H2AX⁺ and γ H2AX⁻ cells between CD4⁺ and CD8⁺ T cells (Figure 4.6D—E). Interestingly, p-S6 levels in γ H2AX⁺ cells were significantly higher in CD8⁺ than CD4⁺ T cells, while p-Akt was significantly lower (Figure 4.6D—E). This mirrors the differing activities of mTOR observed during 3-day activation in the previous experiment, in which mTORC1 activity was much higher in CD8⁺ than CD4⁺ T cells (Figure 4.2B—C).

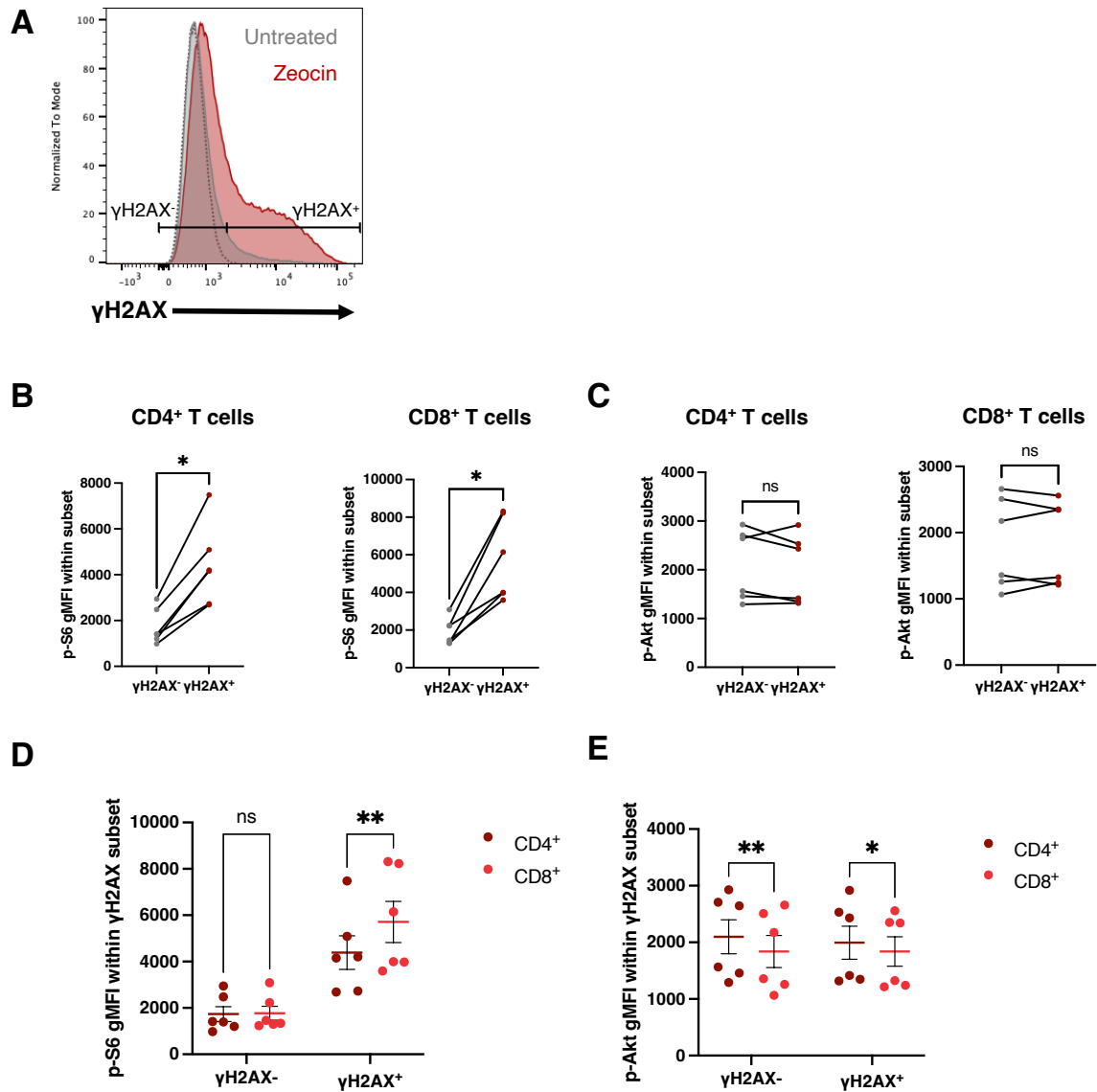


Figure 4.6 Relationship between mTOR activity and γH2AX in T cells

PBMCs from healthy donors were activated for 3 days and treated with zeocin (200 $\mu\text{g/ml}$) or untreated for 2 hours followed by 4-hour recovery, before staining and flow cytometric analysis. **(A)** Representative gating of γH2AX^+ and γH2AX^- cells based on untreated (grey), zeocin-treated (red) cells, and fluorescence minus one (FMO, dotted grey) control. **(B–C)** Geometric mean fluorescence intensity (gMFI) of **(B)** p-S6 and **(C)** p-Akt in γH2AX^+ and γH2AX^- CD4⁺ (left) or CD8⁺ (right) T cells. *P*-values are derived from a Wilcoxon matched-pairs signed rank test, $n=6$ healthy donors. **(D–E)** Comparison of the geometric fluorescence intensity (gMFI) of **(D)** p-S6 and **(E)** p-Akt in zeocin-treated γH2AX^+ and γH2AX^- cells between CD4⁺ and CD8⁺ T cells. *P*-values are derived from a two-way ANOVA with Šídák's multiple comparisons test, $n=6$ healthy donors.

4.2.6 Investigating the effect of mTOR inhibition on DNA damage in human T cells

4.2.6.1 *mTOR inhibition limits the zeocin-induced increase in γ H2AX in T cells*

My data from Figure 4.6 suggest that DNA damage levels (indicated by γ H2AX positivity) correlate with mTORC1 activity. To probe this relationship further, I investigated how mTOR inhibition affected the induction of DNA damage by zeocin. PBMCs from healthy donors were activated with T-cell-specific antibodies and treated with zeocin (or untreated control) in the continuous presence of the mTOR inhibitors, rapamycin (10 nM), AZD8055 (100 nM), or DMSO control, with γ H2AX levels measured by flow cytometry (Figure 4.7A). Representative histograms are shown in Figure 4.7B. Zeocin treatment strongly induced γ H2AX positivity in a subpopulation of both CD4⁺ and CD8⁺ T cells (Figure 4.7C). Continuous treatment with either rapamycin or AZD8055 significantly limited the induction of this γ H2AX⁺ population in CD4⁺ T cells (Figure 4.7C), while in CD8⁺ T cells, 10 nM rapamycin, but not 100 nM AZD8055, also significantly reduced the presence of γ H2AX (Figure 4.7C).

Taken together, this indicates that not only is DNA damage associated with high mTORC1 activity in human T cells (Figure 4.6B), but that continuous inhibition of mTOR limits the presence of DNA damage, as measured by γ H2AX positivity. Moreover, these results are consistent with those from Chapter 3, in which AZD8055 treatment significantly decreased the number of γ H2AX⁺ and 53BP1⁺ DNA damage foci in primary human fibroblasts undergoing DNA damage-induced senescence (Figure 3.11).

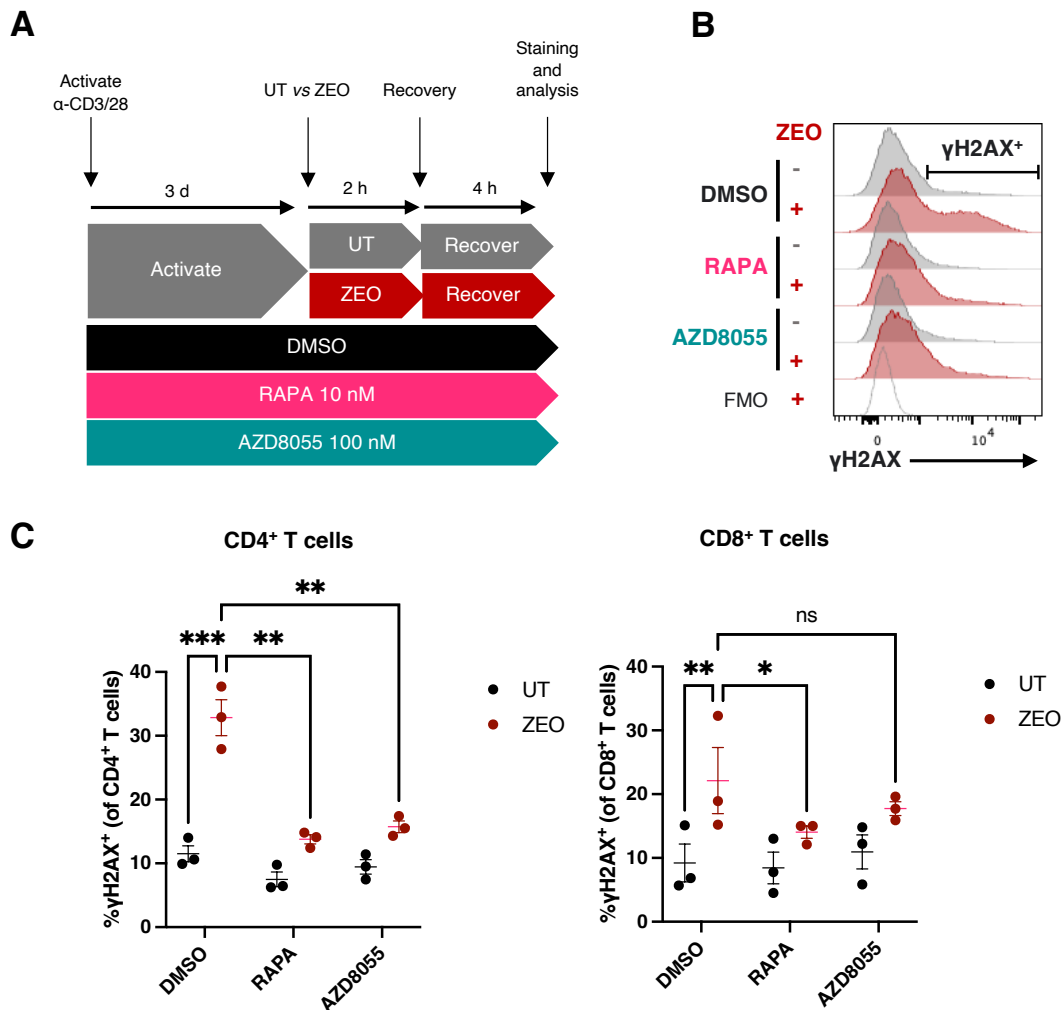


Figure 4.7 mTOR inhibition limits γH2AX⁺ DNA damage in human T cells from healthy donors

(A) PBMCs were activated for 3 days with 1 μg/ml α-CD3/28, then treated for 2 hours with zeocin (ZEO, 200 μg/ml) or untreated, and left to recover for 4 hours, before staining and flow cytometric analysis. Throughout the experiment, cells were either treated continuously with 10 nM rapamycin (RAPA), 100 nM AZD8055, or DMSO control. (B) Representative histograms of γH2AX levels in CD4⁺ T cells across conditions with fluorescence minus one (FMO) control. (C) Proportion of CD4⁺ (left) and CD8⁺ (right) T cells positive for γH2AX across treatment conditions. *P*-values are derived from a two-way ANOVA with Šídák's multiple comparisons test, n=3 healthy donors.

4.2.6.2 mTOR inhibition limits cell death after zeocin treatment

Since persistent, unresolved DNA damage can lead to apoptosis, I tested whether the effect of continuous mTOR inhibition on limiting DNA damage affected the percentage of live cells present after zeocin treatment, measured by their absence of fluorescence for a membrane-

permeable viability dye (Figure 4.8A). Zeocin treatment significantly reduced the proportion of live CD4⁺ and CD8⁺ T cells at 4 hours recovery compared to untreated cells (Figure 4.8B), which was further exacerbated at 24 hours recovery from zeocin (Figure 4.8C). Remarkably, continuous 10 nM rapamycin treatment significantly prevented zeocin-induced cell death compared to DMSO control (~60% vs 25% live cells in rapamycin vs DMSO at 24 hours after zeocin treatment) (Figure 4.8B—C). This suggests that the protection from γ H2AX⁺ DNA damage by rapamycin, either through limiting initial damage or enhancing subsequent DNA repair, may protect cells from DNA damage-induced cell death.

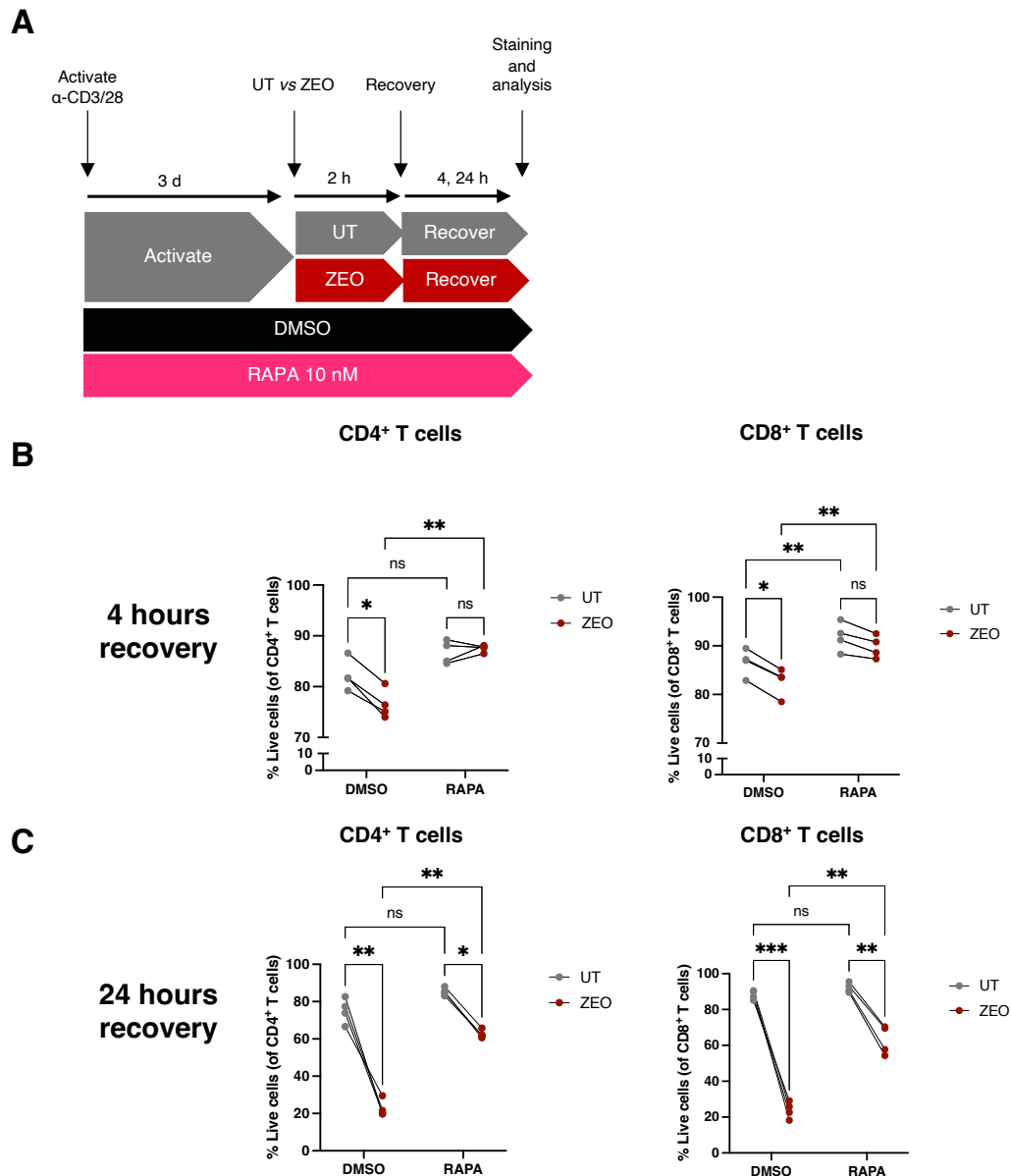


Figure 4.8 mTOR inhibition limits cell death after DNA damage in T cells from healthy donors

(A) PBMCs were activated for 3 days with 1 μ g/ml α -CD3/28 and afterwards treated with zeocin (ZEO, 200 μ g/ml) or untreated (UT), and left to recover for 4 or 24 hours, before staining and flow cytometric analysis. Live cells were determined by their absence of fluorescence for a viability dye. Throughout the experiment, cells were either treated continuously with 10 nM rapamycin (RAPA) or DMSO control. (B—C) Percentage of live cells (B) at 4 hours and (C) 24 hours recovery from zeocin in CD4⁺ (left) and CD8⁺ (right) T cells. *P*-values are derived from a two-way ANOVA with Tukey's multiple comparisons test, *n*=4 healthy donors.

4.2.7 Cell-type-autonomous effects of mTOR inhibition on zeocin-induced DNA damage

The results so far indicated that mTOR inhibition during DNA damage of PBMCs limited the induction of γ H2AX in T cells. Since PBMCs comprise a wide variety of different immune cell subtypes with potential intercellular communication, I investigated whether the effect of mTOR inhibition on T cells was either cell-type-autonomous or required the presence of other immune cells. To determine this, PBMCs or isolated CD4⁺ T cells from the same donor were activated and then treated for 2 hours with zeocin, in the continuous presence of 10 nM rapamycin, 100 nM AZD8055, or DMSO control, and the proportion of γ H2AX⁺ cells was measured by flow cytometry (Figure 4.9A). Rapamycin and AZD8055 treatment limited the induction of γ H2AX in isolated CD4⁺ T cells as efficiently as in total PBMCs (Figure 4.9B), indicating that the effect of mTOR inhibition on limiting DNA damage induction was T cell autonomous. Since performing this assay in the context of all PBMCs represents a more physiological *in vitro* model of DNA damage, as a variety of cell types are present during *in vivo* DNA damage, and requires less experimental processing of the cells, subsequent experiments were performed using total PBMCs rather than isolated T cells.

and p-Chk2 in CD4⁺ and CD8⁺ T cells were then measured by flow cytometry (representative histograms are shown in Figure 4.10B). Zeocin treatment increased p-Chk1 (Figure 4.10C) and p-Chk2 (Figure 4.10D) levels in both T cell types, consistent with induction of DNA damage and initiation of DDR signalling. Similarly to γ H2AX (Figure 4.7C), continuous rapamycin or AZD8055 treatment significantly inhibited the increase in p-Chk1 and p-Chk2 signals (Figure 4.10C—D), suggesting that mTOR inhibition reduced other aspects of the DDR.

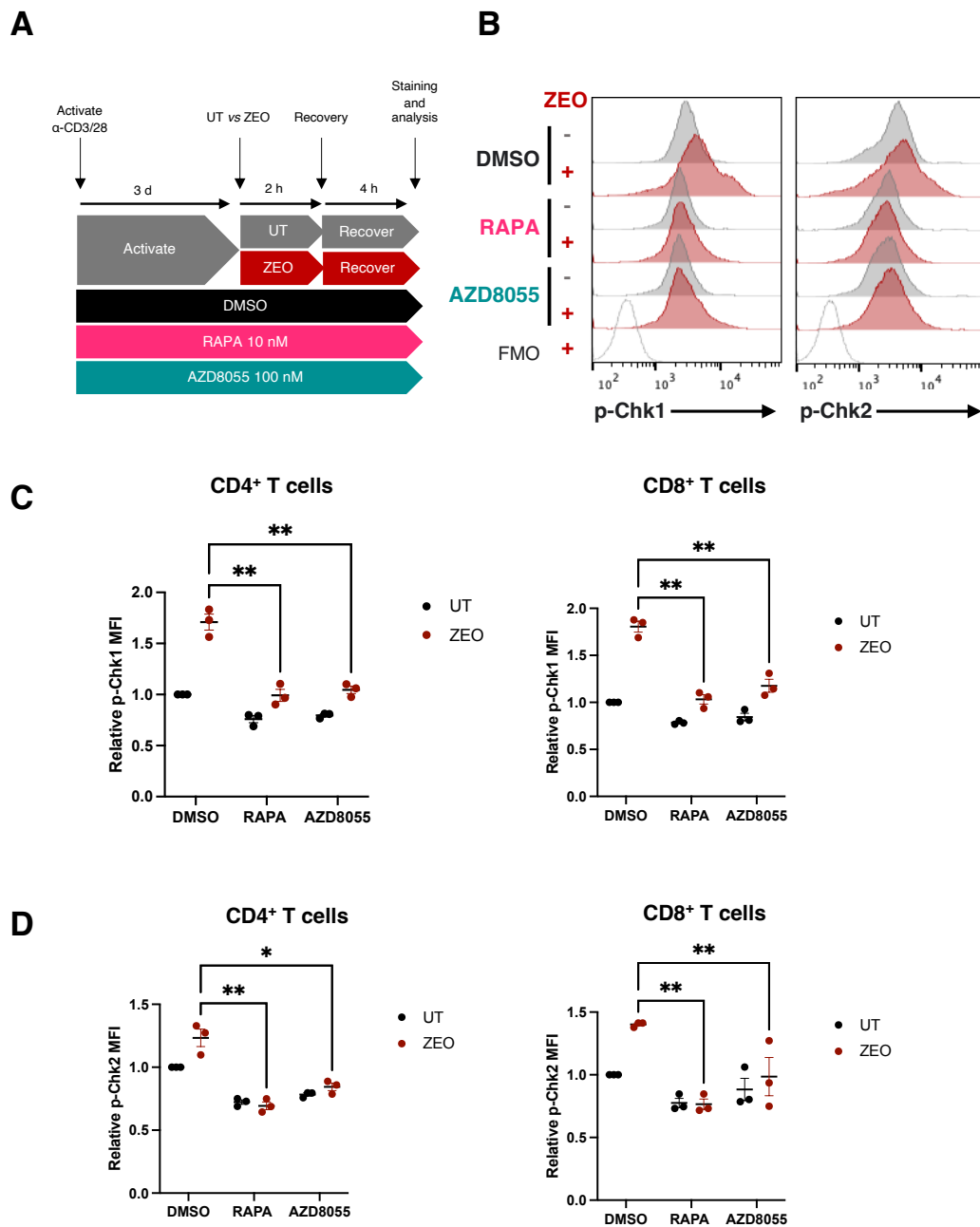


Figure 4.10 Effect of mTOR inhibition on zeocin-induced DDR activation in PBMCs from healthy donors

(A) PBMCs were activated for 3 days with 1 μ g/ml α -CD3/28 and afterwards treated with zeocin (ZEO, 200 μ g/ml) or untreated (UT), and left to recover for 4 hours, before staining and flow cytometric analysis. Throughout the experiment, cells were either treated continuously with 10 nM rapamycin (RAPA), 100 nM AZD8055, or DMSO control. (B) Representative histograms of p-Chk1 (left) and p-Chk2 (right) levels in CD4⁺ T cells across conditions with fluorescence minus one (FMO) control. (C–D) Mean fluorescence intensity (MFI) of (C) p-Chk1 and (D) p-Chk2 of CD4⁺ (left) and CD8⁺ (right) T cells across conditions, relative to DMSO untreated control. *P*-values derived from a two-way ANOVA with Šídák's multiple comparisons test, *n*=3 healthy donors.

4.2.9 mTOR inhibition with rapamycin either before, during, or after zeocin treatment

Together, the results in this chapter so far indicate that continuous mTOR inhibition in T cells limits the induction of the DDR in a cell-type-autonomous manner. To understand the point in the DDR during which mTOR inhibition limited the induction of γ H2AX in T cells, I tested whether rapamycin limited γ H2AX induction when used prior to, during, or after treatment with zeocin (i.e. pre-treatment, co-treatment, or post-treatment (Figure 4.11A)). PBMCs were anti-CD3/28-activated and then treated with rapamycin either before, during, and/or after treatment with zeocin, and the induction of γ H2AX in either CD4⁺ or CD8⁺ T cells was assessed as a fold change from DMSO→DMSO→DMSO (DDD) untreated controls. Intriguingly, rapamycin treatment at any point with respect to zeocin significantly suppressed the induction of γ H2AX in CD4⁺ T cells compared to DDD zeocin-treated cells (Figure 4.11B). In CD8⁺ T cells, however, only rapamycin during (DRD) and/or after zeocin treatment (DRR, DDR) limited the induction of γ H2AX (Figure 4.11C). This indicated that mTOR inhibition limited the DNA damage response after short-term treatment, though this effect differed depending on the T cell subset assessed.

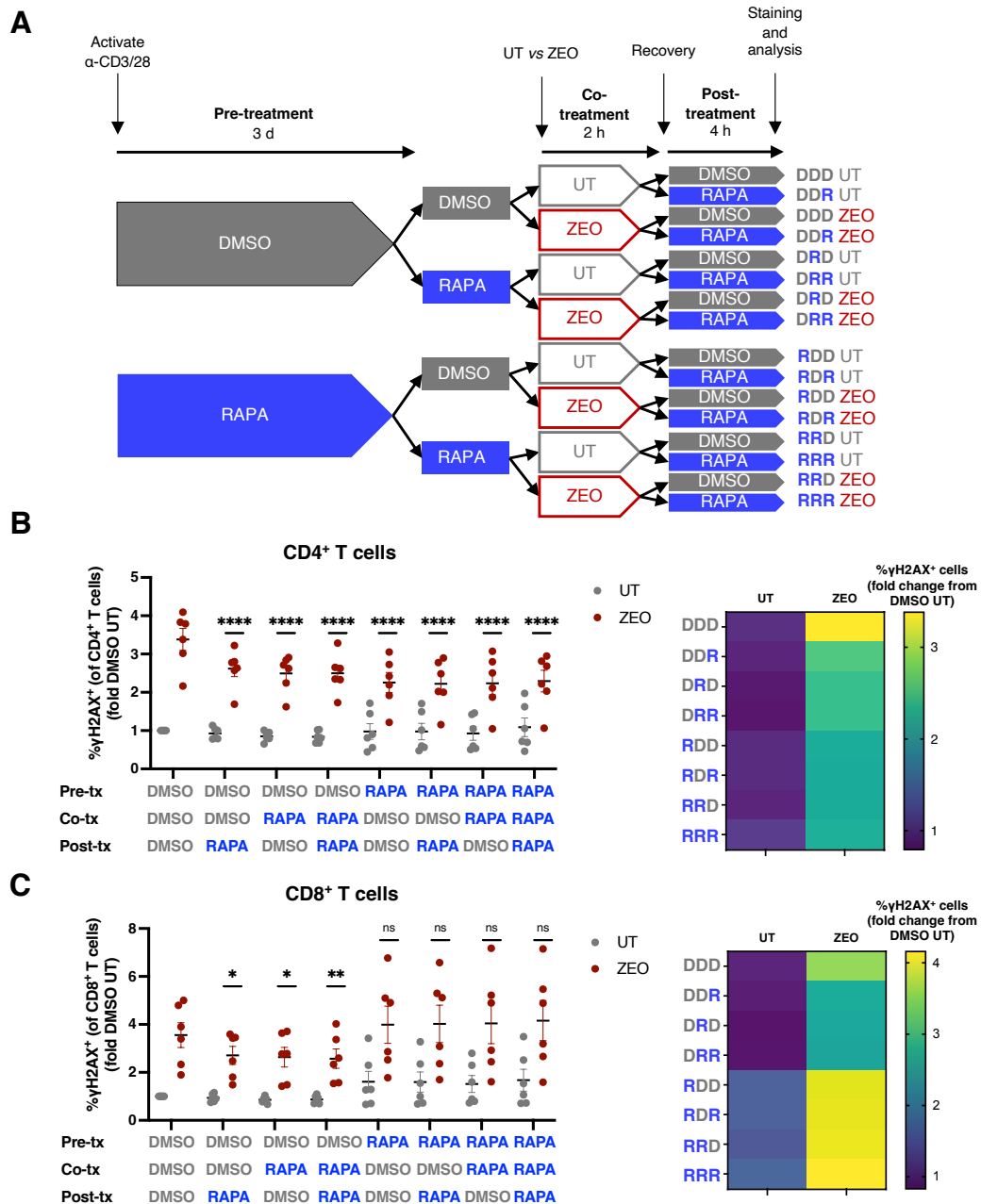


Figure 4.11 mTOR inhibition before, during, or after treatment with zeocin
(A) PBMCs were activated with 1 μ g/ml α -CD3/28 for 3 days, in the presence of 10 nM rapamycin (RAPA) or DMSO (vehicle). Cells from each condition were halved and treated with RAPA or DMSO control, and then either untreated (UT) or treated for 2 hours with 200 μ g/ml zeocin (ZEO). Cells were then collected and further halved, and each condition treated with either RAPA (10 nM) or DMSO control to recover for 4 hours from zeocin, before staining and flow cytometric analysis. **(B–C)** (Left) Proportion of γ H2AX⁺ of **(B)** CD4⁺ and **(C)** CD8⁺ T cells, in PBMCs treated with 10 nM RAPA pre-, co-, and/or post-treatment (tx) with zeocin. Data are expressed as fold change from DMSO→DMSO→DMSO (DDD) UT controls for each donor. (Right) Heatmap of fold change of % γ H2AX⁺ cells from DDD UT across conditions. *P*-values indicated represent comparisons to the DMSO→DMSO→DMSO ZEO condition and are derived from a two-way ANOVA with Šídák's multiple comparisons test, *n*=6 healthy NHS-BT donors.

4.2.10 Extent of mTOR suppression correlates with the level of DNA damage

To explore how mTOR suppression and γ H2AX levels were related, I assessed the extent of mTOR suppression across the various conditions in Figure 4.11 by measuring p-S6 and p-Akt as readouts for mTORC1 and mTORC2 activity. p-S6 was significantly reduced in most rapamycin treatment conditions in CD4⁺ T cells (Figure 4.12A). p-Akt was suppressed only in cells that underwent 3-day pre-treatment with rapamycin (Figure 4.12B), consistent with prior reports that chronic rapamycin treatment (>24 hours) leads to mTORC2 inhibition (Sarbasov et al., 2006). To assess the relationship between mTORC1 and mTORC2 suppression and DNA damage responses, I performed simple linear regression analyses on the induction of γ H2AX and either p-S6 (Figure 4.13A) or p-Akt (Figure 4.13B), which revealed that both p-S6 and p-Akt levels positively correlated with the induction of γ H2AX in CD4⁺ T cells. These findings suggest a significant inverse correlation between mTOR inhibition by rapamycin and induction of γ H2AX by zeocin in CD4⁺ T cells.

Notably, CD8⁺ T cells behaved differently from CD4⁺ T cells with respect to their response to rapamycin. First, p-S6 was suppressed only when CD8⁺ T cells were treated with rapamycin before DNA damage (Figure 4.12C), whereas p-S6 was suppressed in almost all treatment conditions in CD4⁺ T cells (Figure 4.12A). Perhaps this is because p-S6 levels were much higher in activated CD8⁺ T cells compared to CD4⁺ T cells (Figure 4.2B, Figure 4.6D), and therefore CD8⁺ T cells require a longer duration of treatment with rapamycin to achieve significant suppression of S6 phosphorylation. p-S6 levels still strongly correlated with the induction of DNA damage (γ H2AX positivity) in a linear regression analysis (Figure 4.13C). As in CD4⁺ T cells, p-Akt was predominantly suppressed when rapamycin was used prior to zeocin treatment (Figure 4.12D). However, unlike in CD4⁺ T cells, p-Akt levels in CD8⁺ T cells negatively correlated with the induction of γ H2AX in a linear regression analysis (Figure 4.13D), consistent with the result that treatment with rapamycin only during or after zeocin treatment (at times when p-Akt was not suppressed) could minimise γ H2AX

induction (Figure 4.11C). Overall, these data strongly suggest that mTOR inhibition, particularly of mTORC1, inhibits the induction of γ H2AX in T cells after genotoxic treatment.

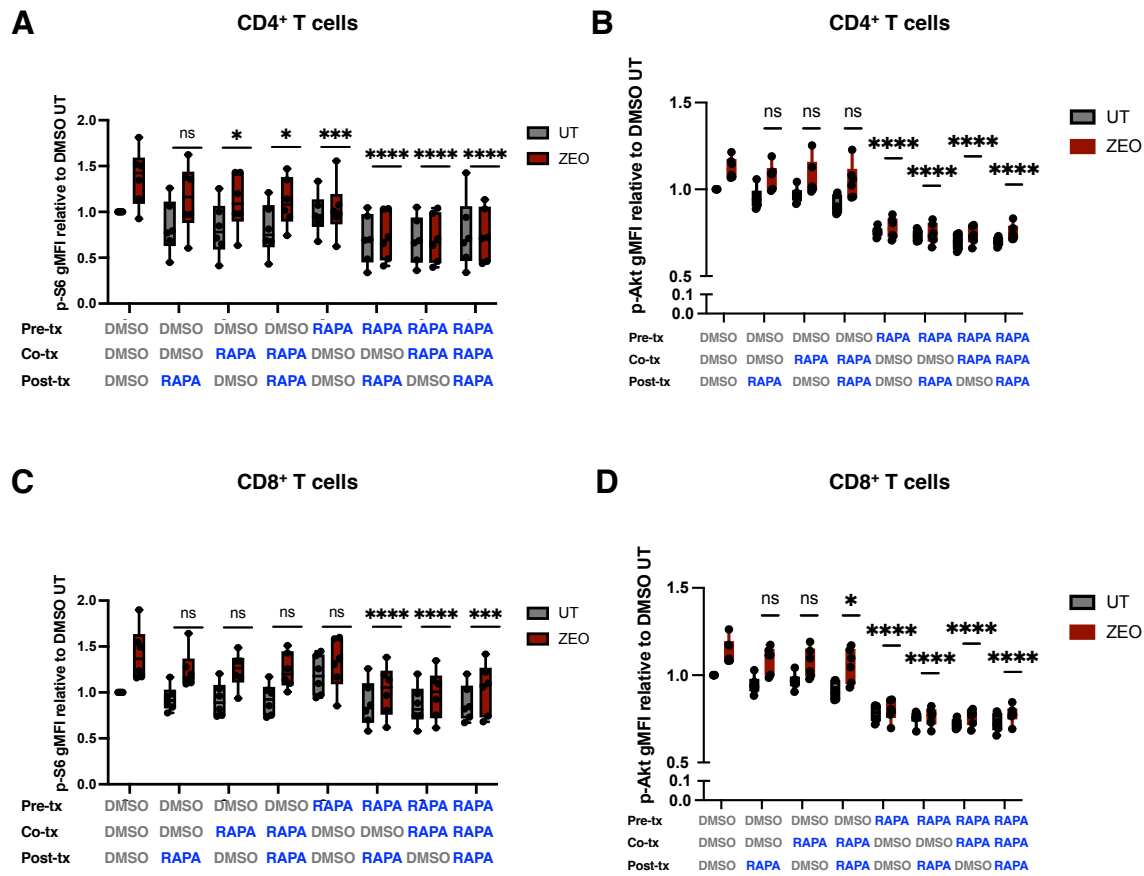


Figure 4.12 p-S6 and p-Akt levels in T cells treated with rapamycin before, during, or after zeocin treatment

Experimental design as per Figure 4.11A Geometric fluorescence intensity (gMFI) of (A, C) p-S6 and (B, D) p-Akt across conditions of (A, B) CD4⁺ and CD8⁺ (C, D) T cells treated with 10 nM rapamycin or DMSO control pre-, co-, and/or post-treatment (tx) with zeocin (ZEO). All data are normalised to the DMSO→DMSO→DMSO UT condition. *P*-values indicated represent comparisons to the DMSO→DMSO→DMSO ZEO condition and are derived from a two-way ANOVA with Šídák's multiple comparisons test, n=6 healthy donors.

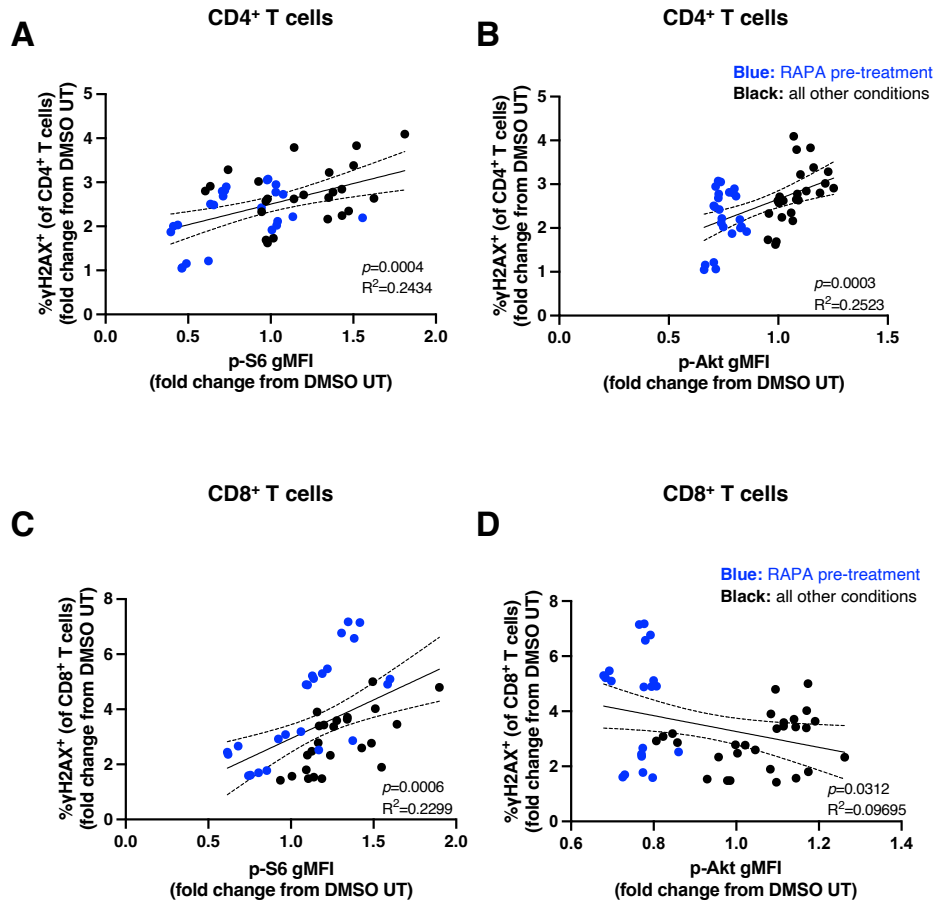


Figure 4.13 Extent of mTOR activity suppression correlates with the level of DNA damage

Simple linear regression analyses from experiment in Figure 4.11A of γH2AX and either (A, C) p-S6 or (B, D) p-Akt in zeocin-treated (A, B) CD4⁺ or (C, D) CD8⁺ T cells. All values are expressed as fold change from DMSO→DMSO→DMSO UT. Blue dots indicate rapamycin pre-treatment conditions, black dots all other conditions. Solid line indicates line of best fit, with dotted lines representing 95% confidence intervals. Data are pooled from 6 independent experiments, n=6 healthy donors.

4.2.11 Effects of DNA damage and mTOR inhibition on the cell cycle

Progression through the cell cycle is halted during the initial stages of the DDR to allow for repair of DNA lesions. Thus, one possible explanation for my observation that mTOR inhibition limits γH2AX induction in T cells is that mTOR inhibition differentially affects the DDR at different phases of the cell cycle. To assess how DNA damage affected cell cycle distribution, PBMCs were T cell-activated and treated with zeocin vs control, and allowed

to recover for 4 hours (Figure 4.14A). Cell cycle phase (G_0/G_1 , S, or G_2/M) was determined by incubating cells with 10 μ M EdU (an alkyne thymidine analogue) during the 4-hour recovery from zeocin release and assessing DNA-incorporated EdU and total DNA content using flow cytometry (Figure 4.14B). As expected, zeocin treatment of both $CD4^+$ and $CD8^+$ T cells greatly reduced the proportion of cells in S-phase (from 52% in UT to 26% in ZEO conditions), but increased cells in G_2/M phase (1.3% to 15%) (Figure 4.14C), suggesting activation of cell cycle checkpoints to prevent cell division.

To test whether mTOR inhibition affected cell cycle progression, cells were treated with rapamycin continuously (RRR), during (DRD), and/or after treatment (DRR, DDR) with zeocin (Figure 4.15A). Under these conditions, rapamycin limited zeocin-induced γ H2AX levels in either or both $CD4^+$ and $CD8^+$ T cells (Figure 4.11). Cell cycle distribution was not affected by rapamycin during and/or after zeocin treatment (DDR, DRD, and DRR), in either untreated or zeocin-treated conditions (Figure 4.15B). However, continuous rapamycin treatment (RRR) increased the proportion of cells in G_0/G_1 phase (Figure 4.15B). To investigate the effect of continuous rapamycin treatment on DNA damage further, I assessed the cell cycle distributions in γ H2AX⁺ and γ H2AX⁻-gated zeocin-treated cells (Figure 4.15C). Though continuous rapamycin treatment (RRR) significantly increased the proportion of γ H2AX⁻ cells that were in G_0/G_1 -phase (Figure 4.15C), mirroring the observation in Figure 4.15B for ungated cells, it did not affect the proportion of G_0/G_1 -phase cells in the γ H2AX⁺ population, indicating that continuous rapamycin treatment did not change cell cycle phase distribution in the context of DNA damage. Additionally, since all of these rapamycin treatments (DDR, DRD, DRR and RRR) effectively limited the induction of γ H2AX in T cells previously (e.g. Figure 4.11), but did not affect cell cycle phase distribution in DNA-damaged γ H2AX⁺ cells, I concluded that the effect of rapamycin on γ H2AX was not due to effects on the cell cycle.

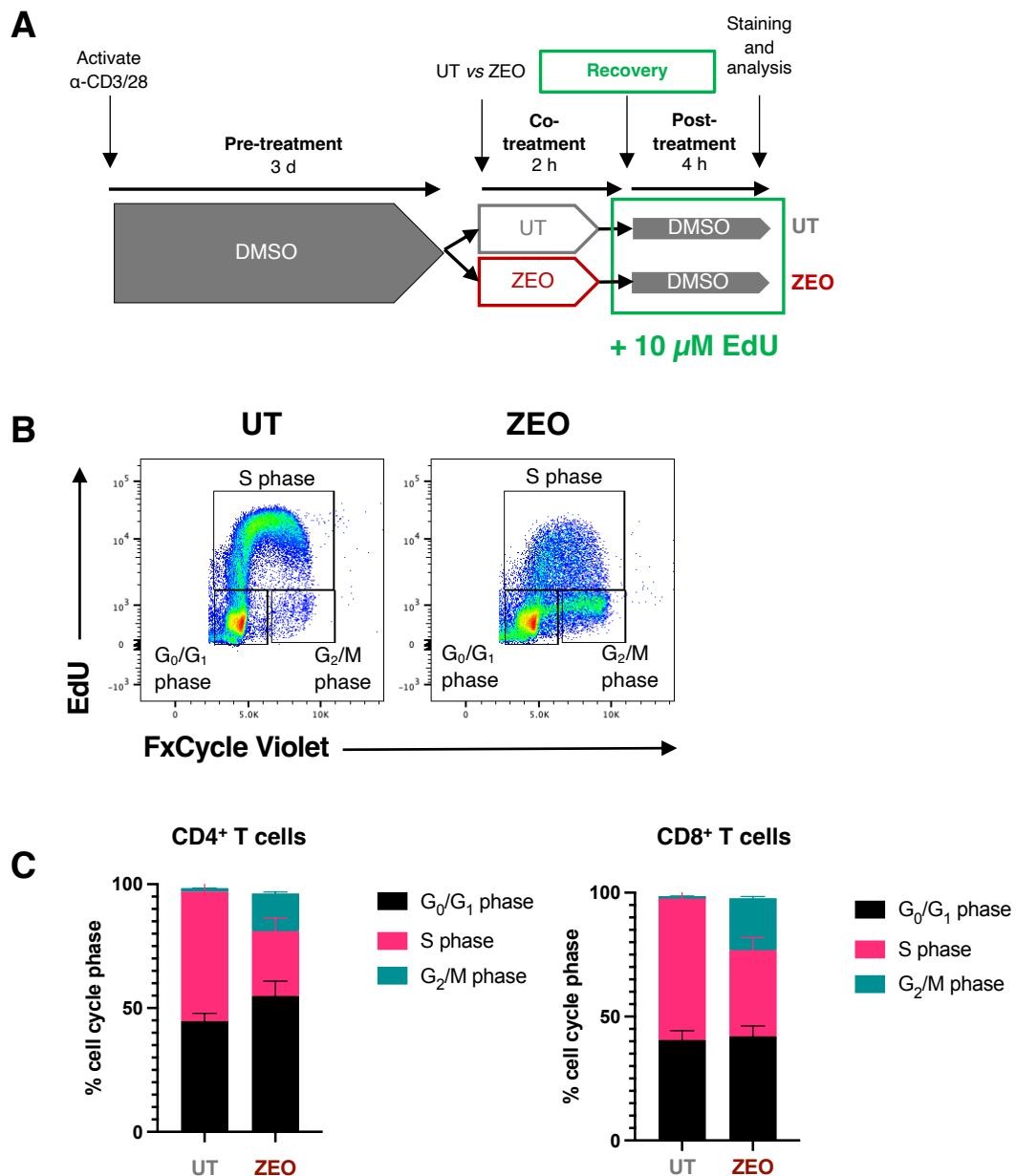


Figure 4.14 Cell cycle effects of DNA damage in T cells from healthy donors

(A) PBMCs from $n=3$ healthy donors were activated for 3 days with 1 μ g/ml α -CD3/28 and treated for 2 hours with zeocin (ZEO, 200 μ g/ml) or untreated (UT), and left to recover for 4 hours with 10 μ M EdU. (B) Representative flow cytograms of untreated and zeocin-treated CD4⁺ T cells displaying DNA-incorporated EdU and total DNA content stained with FxCycle Violet, with cell cycle phases indicated. (C) Quantification of cell cycle phases in untreated and zeocin-treated conditions in CD4⁺ T cells (left) and CD8⁺ T cells (right).

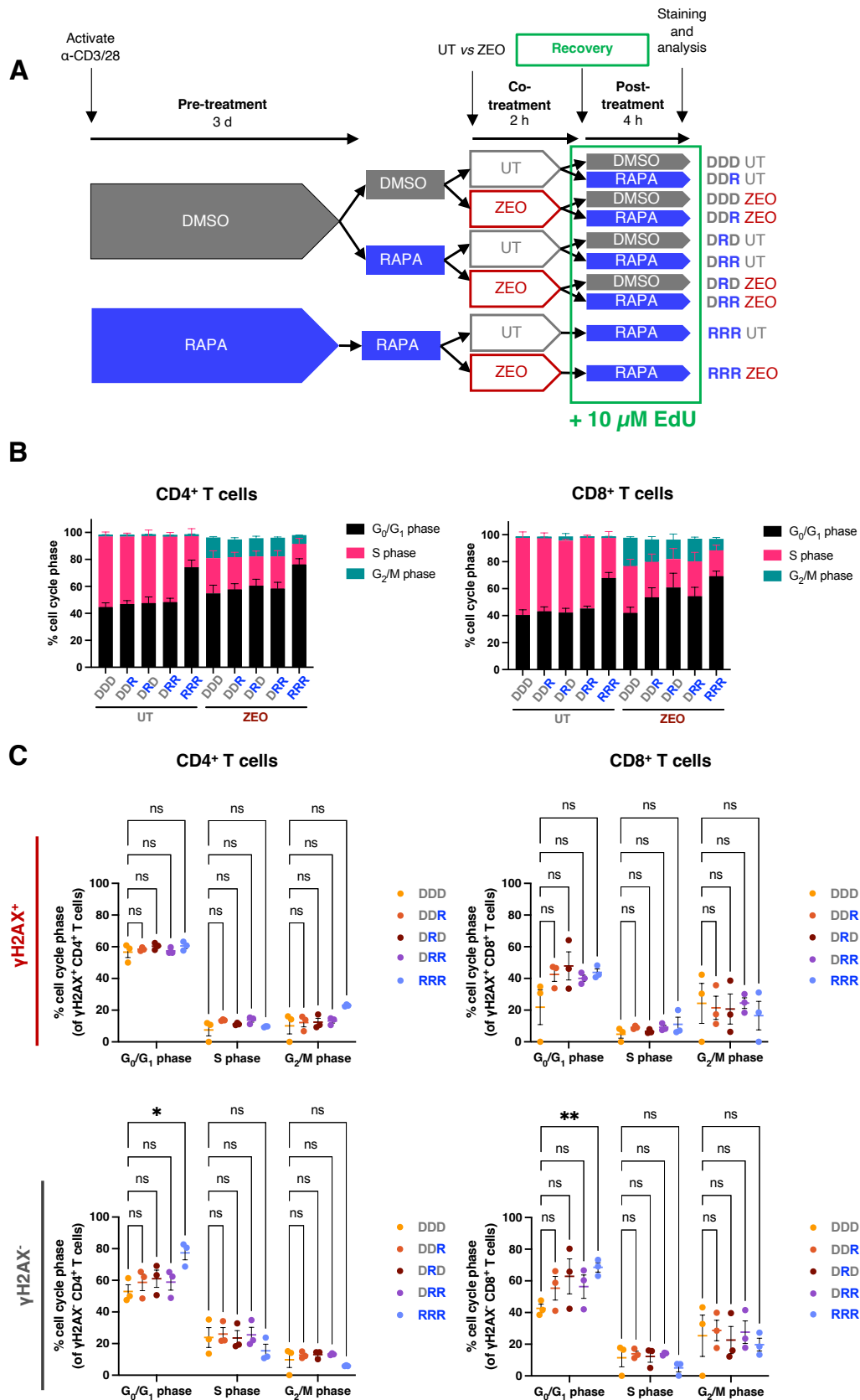


Figure 4.15 Cell cycle effects of rapamycin in γ H2AX-gated cells from healthy donors

(A) PBMCs were activated with α -CD3/28 and treated with 10 nM rapamycin or DMSO control either pre-, co-, or post-zeocin (ZEO, 200 μ g/ml) for 2 hours, and recovered for 4 hours in the presence of 10 μ M EdU. (B) Cell cycle phases in

untreated (UT) and zeocin-treated (ZEO) conditions in CD4⁺ T cells (left) and CD8⁺ T cells (right) across all conditions from n=3 healthy donors. **(C)** Cell cycle phases in γ H2AX⁺ (upper) and γ H2AX⁻ (lower) gated subsets of zeocin-treated CD4⁺ T cells (left) and CD8⁺ T cells (right) across all conditions. *P*-values are derived from a two-way ANOVA with Dunnett's multiple comparisons test, n=3 healthy donors.

4.2.12 Impacts of mTOR inhibition on nascent protein synthesis

The levels of phosphorylated DDR proteins such as H2AX, Chk1, and Chk2, are all dependent on the presence of their unphosphorylated forms. Since mTOR is a master anabolic regulator of protein synthesis, a possible explanation for the effect of mTOR inhibition on limiting the zeocin-induced increase in DDR phospho-protein levels (Figure 4.7, Figure 4.10) was that their pan-protein levels were decreased. To evaluate the effects of rapamycin on nascent protein synthesis after DNA damage, activated PBMCs were treated with 10 nM rapamycin either continuously (RRR) or before and after zeocin treatment (DRR) and allowed to recover for 4 hours (Figure 4.16A). During the final 30 minutes of the 4-hour recovery, cells were treated with 20 μ M O-propargyl-puromycin (OPP), an alkyne analogue of puromycin that inhibits protein translation (Figure 4.16A); measuring the mean fluorescence of labelled OPP in cells could then be used as a read out for total nascent protein synthesis over a short period. Cells treated for 1 hour with 50 μ g/ml cycloheximide (CHX) served as a positive control for inhibition of protein synthesis. As expected, CHX-treated cells incorporated significantly less OPP than the DMSO untreated controls (Figure 4.16B). Treatment of cells with 10 nM rapamycin during and after zeocin (DRR) did not significantly affect OPP incorporation, though continuous rapamycin treatment (RRR) showed a non-significant trend towards lower OPP incorporation (Figure 4.16B). Since there was no consistent effect of rapamycin in decreasing OPP levels, this suggests that the effect of rapamycin on limiting zeocin-induced DDR signalling levels was not due to decreasing total protein expression.

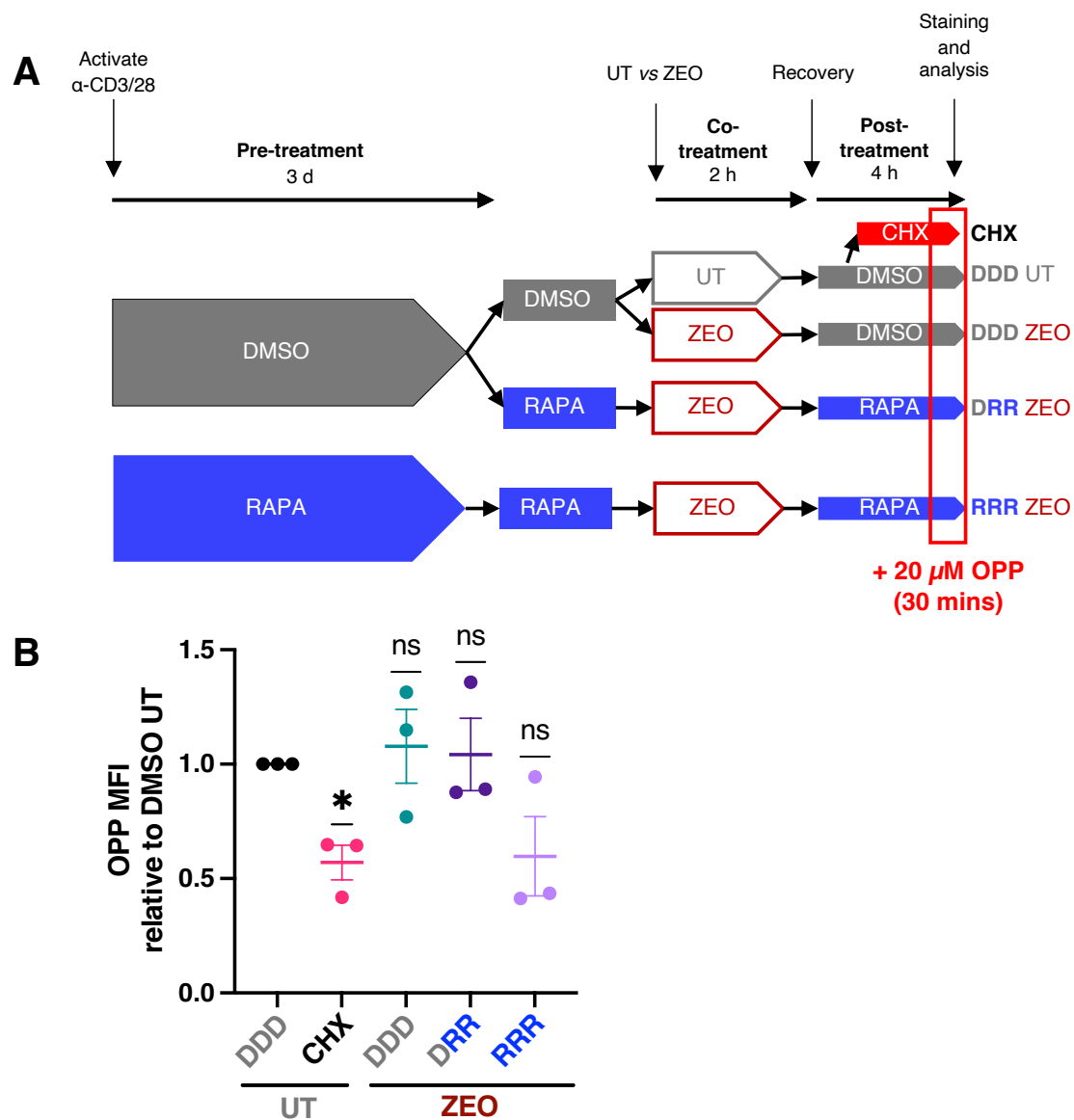


Figure 4.16 Impact of rapamycin and zeocin on nascent protein synthesis in PBMCs from healthy donors

(A) PBMCs were activated for 3 days with 1 μ g/ml α -CD3/28 and afterwards treated with zeocin (ZEO, 200 μ g/ml) or untreated (UT), and left to recover for 4 hours. Cells were treated with either 10 nM rapamycin (RAPA) or DMSO control either continuously (RRR) or during and after zeocin treatment (DRR). For protein synthesis measurement, cells were treated with 20 μ M O-propargyl-puromycin (OPP) for the final 30 mins of recovery from zeocin. As a positive control for inhibition of protein synthesis, DMSO-treated cells were treated with 50 μ g/ml cycloheximide (CHX) for 1 hour, with the addition of OPP for the final 30 mins. (B) OPP mean fluorescence intensity (MFI) of CD4⁺ T cells across conditions, relative to the DMSO untreated condition. *P*-values are derived from a one-sample t-test (theoretical mean = 1), *n*=3 healthy donors.

4.2.13 mTOR inhibition and DNA damage induction on DNA lesions in the comet assay

The impact of mTOR inhibition on diminishing the zeocin-induced upregulation of DDR signalling was therefore independent of effects on the cell cycle and global protein synthesis. To test the hypothesis that mTOR inhibition may limit DNA lesion burden, I used the comet assay, in which electrophoresis of gel-embedded single cells is used to extrude broken DNA (Moller et al., 2020); the migration length of fragmented and extruded DNA can then be used as a measure for the extent of DNA damage per cell. Since zeocin treatment increased both p-Chk1 and p-Chk2 levels (Figure 4.10), suggesting that both single-strand breaks (SSBs) and double-strand breaks (DSBs) were generated, the alkaline form of this assay was necessary (Ribas-Maynou et al., 2012). As expected, treatment of isolated and activated CD4⁺ T cells with either zeocin (2 hours, 200 μ g/ml) or H₂O₂ (25 μ M, 15 minutes) strongly induced comets with a high level of tail migration (Figure 4.17).

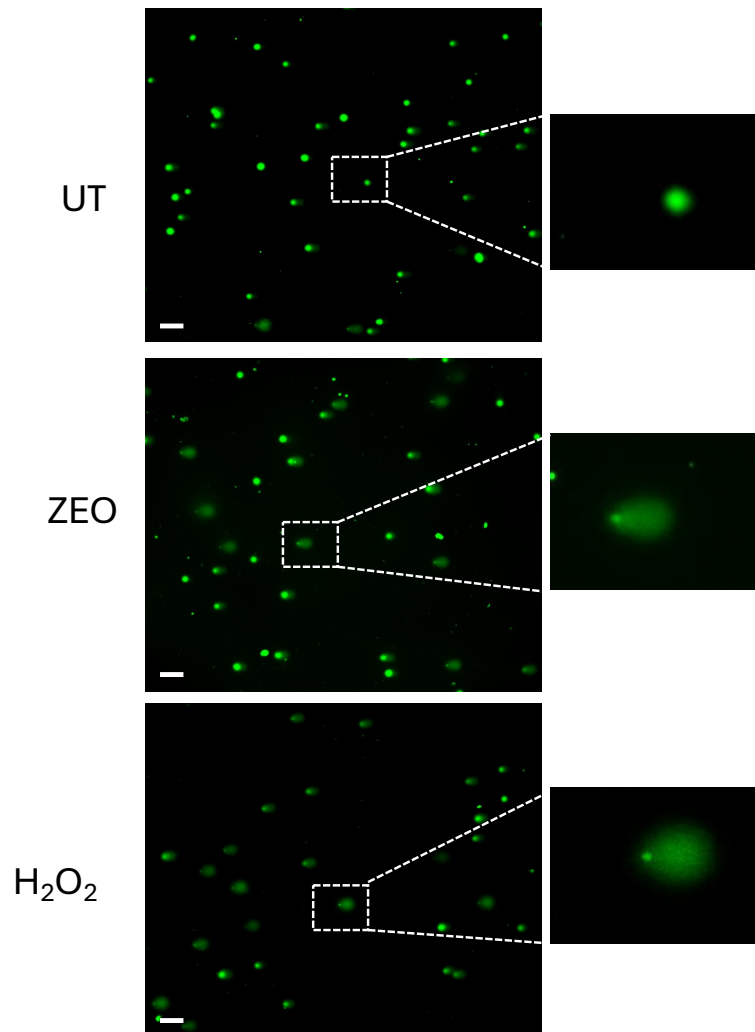


Figure 4.17 Images of CD4⁺ T cells in the alkaline comet assay

Representative images of 3-day activated CD4⁺ T cells from a healthy donor treated with zeocin (ZEO, 2 hours, 200 μ g/ml), H₂O₂ (15 minutes, 25 μ M), or untreated (UT). Scale bars represent 100 μ m. Data are representative of two independent experiments.

To investigate whether mTOR inhibition affected DNA lesion burden, isolated CD4⁺ T cells from one healthy donor were treated either with rapamycin continuously (RRR), or co- and post-treatment (DRR) with respect to DNA-damaging zeocin treatment (Figure 4.18). Short-term H₂O₂ treatment served as a positive control for robust DNA damage induction. After DNA-damaging treatment, cells were allowed to recover for either 0, 4, 8, or 24 hours, and analysed using the comet assay (representative images at 0 hour recovery, Figure 4.19) based on three features: % Tail DNA (Figure 4.20A), Tail Moment (Figure 4.20B), and

Olive Moment (Figure 4.20C) (Moller et al., 2020). At each recovery time point, both zeocin and H_2O_2 strongly induced all three of these features (Figure 4.20A–C). DNA lesions peaked at 4 hours of recovery (Figure 4.21A–C), consistent with my previous time-course data on γH2AX levels after zeocin treatment as measured by flow cytometry (Figure 4.5C). Strikingly, continuous rapamycin treatment (RRR) significantly reduced all three measures of the comet DNA lesions at every time point, including directly after the zeocin treatment (0 hours) (Figure 4.19, Figure 4.20, Figure 4.21). However, co-and-post-treatment of zeocin with rapamycin (DRR) did not affect DNA lesions. Overall, these preliminary data from a single donor suggest that continuous mTOR inhibition may protect human T cells from DNA lesions induced by acute genotoxic treatment.

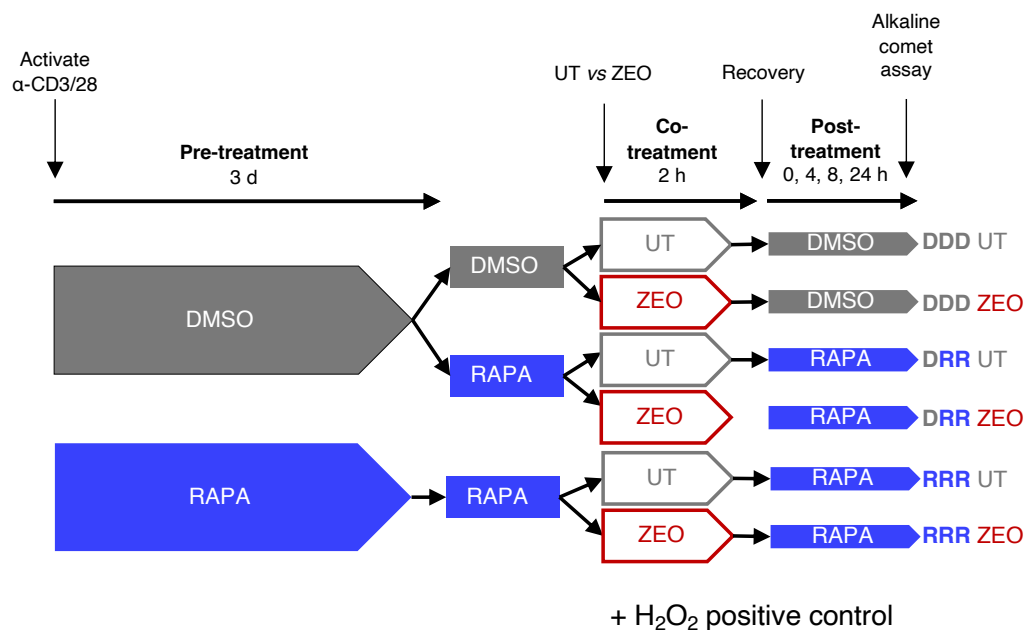


Figure 4.18 Experimental design for alkaline comet assay with CD4⁺ T cells from a healthy donor

Isolated CD4⁺ T cells from one healthy donor were activated for 3 days with 1 $\mu\text{g/ml}$ $\alpha\text{-CD3/28}$ and afterwards treated with zeocin (ZEO, 200 $\mu\text{g/ml}$) or untreated (UT), and allowed to recover for 0, 4, 8, or 24 hours as indicated. Throughout the experiment, cells were treated with 10 nM rapamycin (RAPA) or DMSO control either continuously (RRR), or co- and post-treatment (DRR) with zeocin. As a positive control for DNA damage, some cells were treated with 25 μM H_2O_2 for 15 mins and assessed at the same time points.

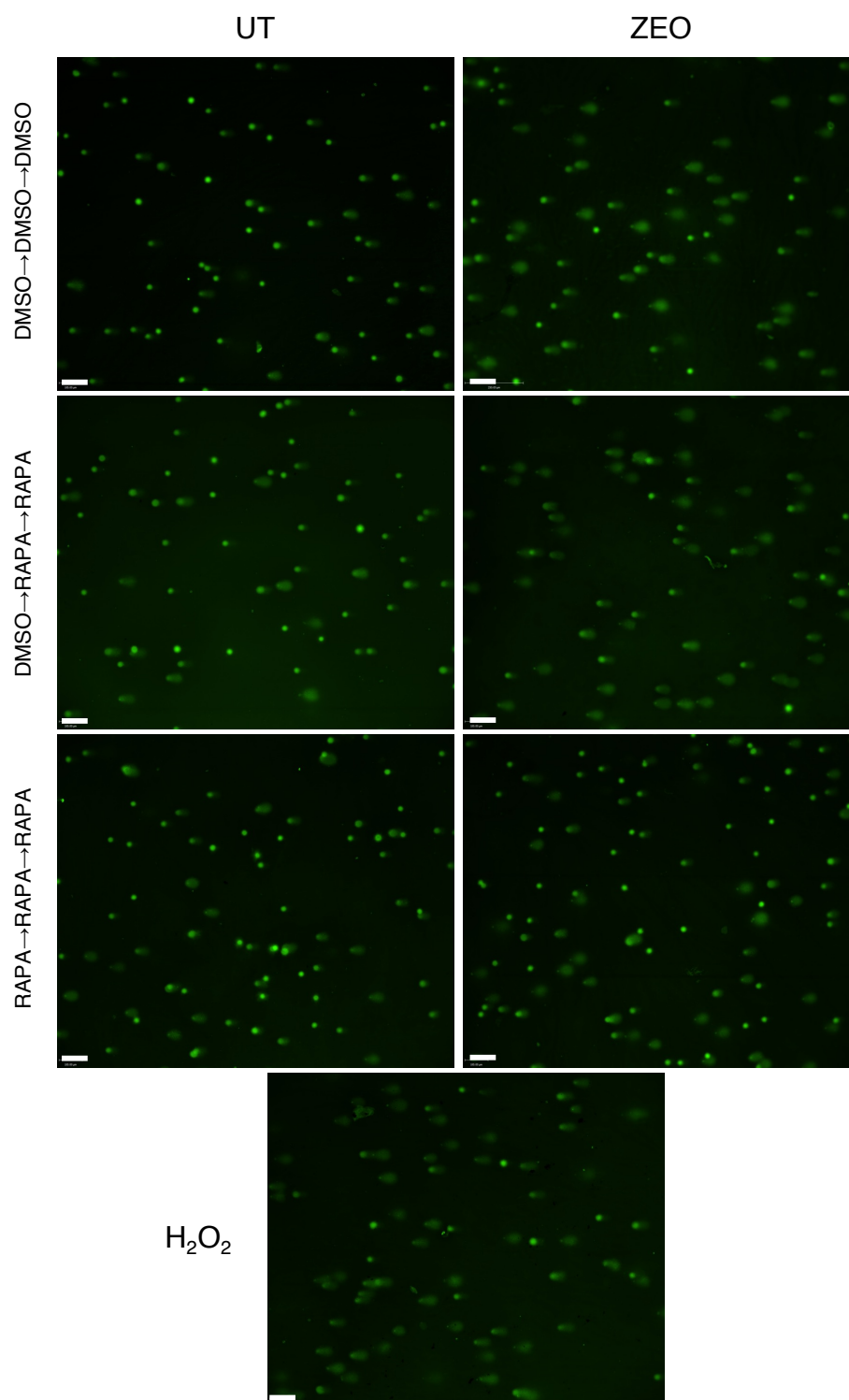


Figure 4.19 Representative images for the effect of mTOR inhibition on zeocin-induced DNA lesions in the comet assay

Representative images of CD4⁺ T cells comet assay from the experiment in Figure 4.18 at 0-hour recovery from zeocin release across conditions. Scale bars represent 100 μ m.

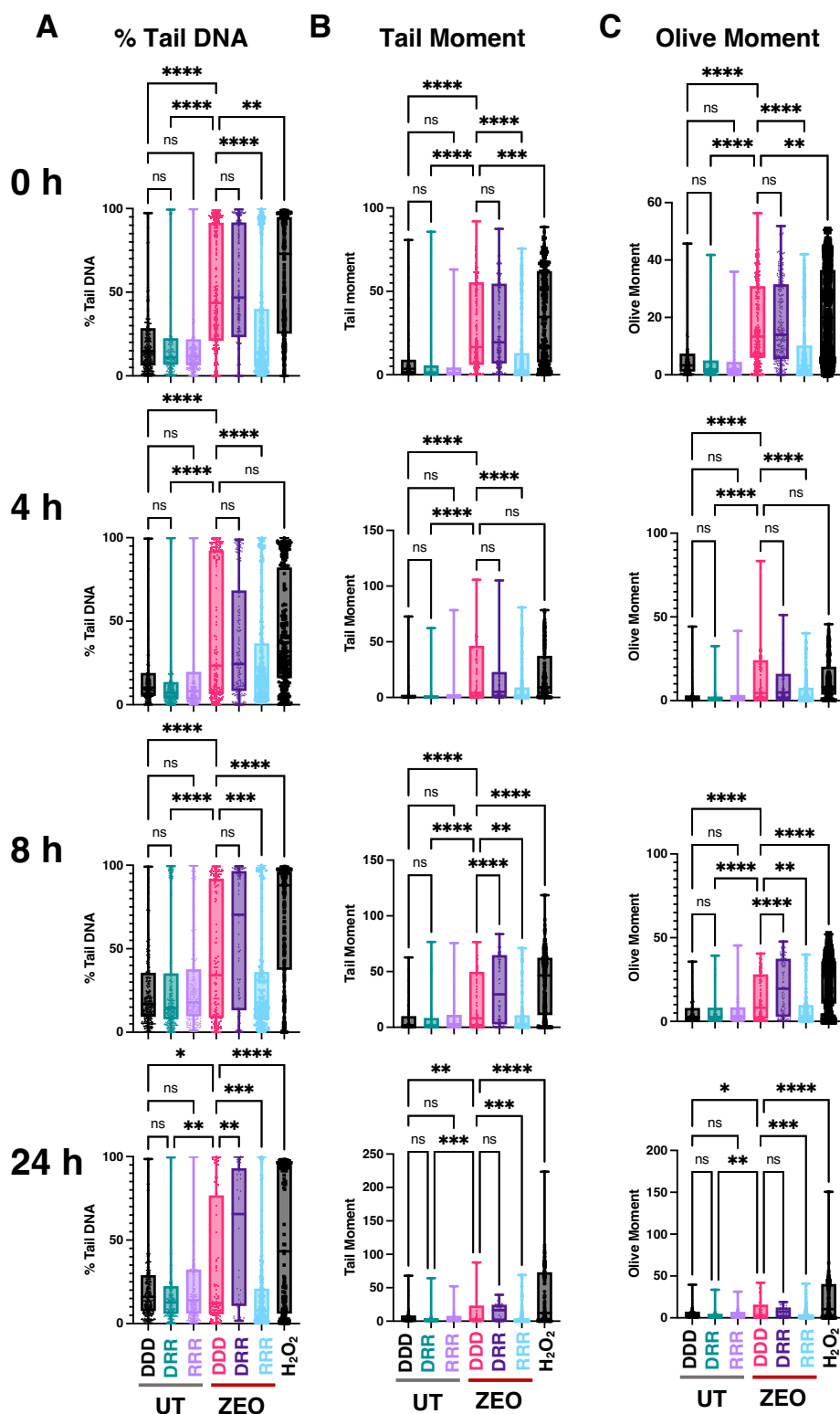


Figure 4.20 Comet assay readouts per time point

Experiment from Figure 4.18. (A–C) (A) % Tail DNA, (B) Tail moments, and (C) Olive moments of comets across conditions and timepoints of recovery. Condition labels represent treatment with DMSO (D) or rapamycin (R) pre→co→post with respect to zeocin. *P*-values are derived from a one-way ANOVA with Tukey's multiple comparisons tests, analysing 100-250 nuclei per condition, from CD4⁺ T cells from n=1 healthy donor.

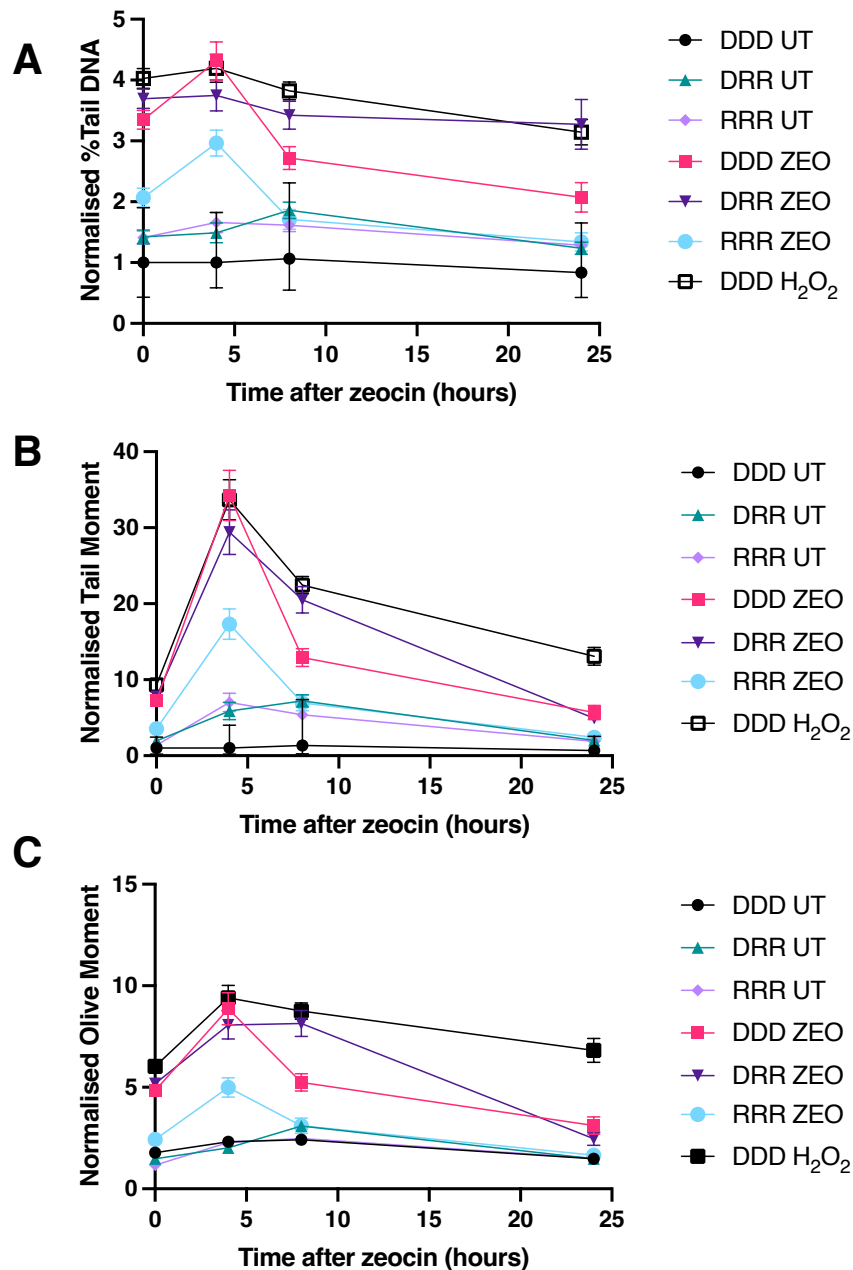


Figure 4.21 Normalised comet assay readouts over time

Experiment from Figure 4.18. **(A–C)** Normalised **(A)** % Tail DNA, **(B)** Tail moments, and **(C)** Olive moments of comets across conditions and timepoints of recovery from zeocin. Each comet value is normalised to the median value of the DMSO→DMSO→DMSO untreated (UT) condition at each time point. Data are the median $\pm$ SEM of 100-250 nuclei analysed per condition, from CD4⁺ T cells from one healthy donor.

4.2.14 Effect of DNA damage and mTOR inhibition on autophagic flux

Next, I aimed to understand the mechanism behind how mTOR affects DNA damage lesions. mTOR increases translation, which I previously found was not changed by rapamycin in activated T cells (Figure 4.16). Another major role of mTOR is the suppression of autophagy (Figure 1.5). Autophagy is one of the major cellular degradation pathways and plays an important role in the DDR (Vessoni et al., 2013). Furthermore, autophagy decreases in immune cells from older donors, including in T cells (Alsaleh et al., 2020). To measure autophagy by flow cytometry, cells were treated with either vehicle (Veh) or 10 nM bafilomycin A₁ (BafA), an inhibitor of the lysosomal V-ATPase and autophagosome-lysosome fusion (Figure 4.22A); calculating the difference in autophagosome-bound LC3 between these conditions provides a quantitative measure of autophagic flux (Alsaleh et al., 2020). Using this assay (Figure 4.22A), and as expected (Arnold et al., 2014), autophagy increased between 6 hours and 3 days of activation of CD4⁺ T cells isolated from a healthy NHS-BT donor (Figure 4.22B).

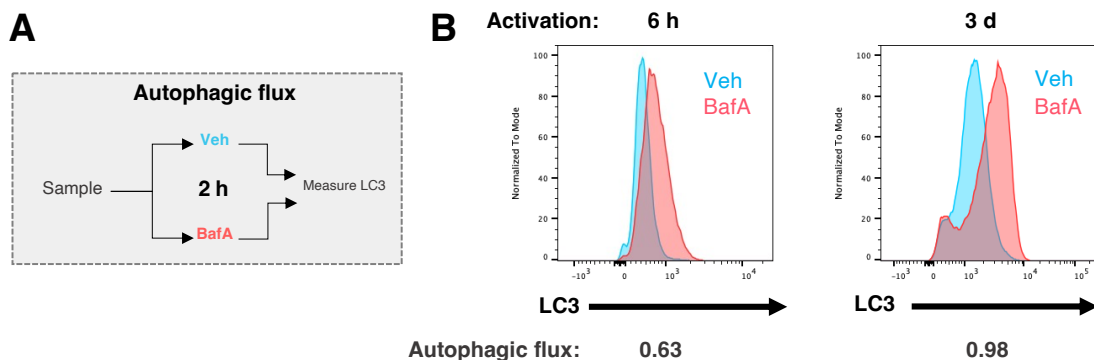
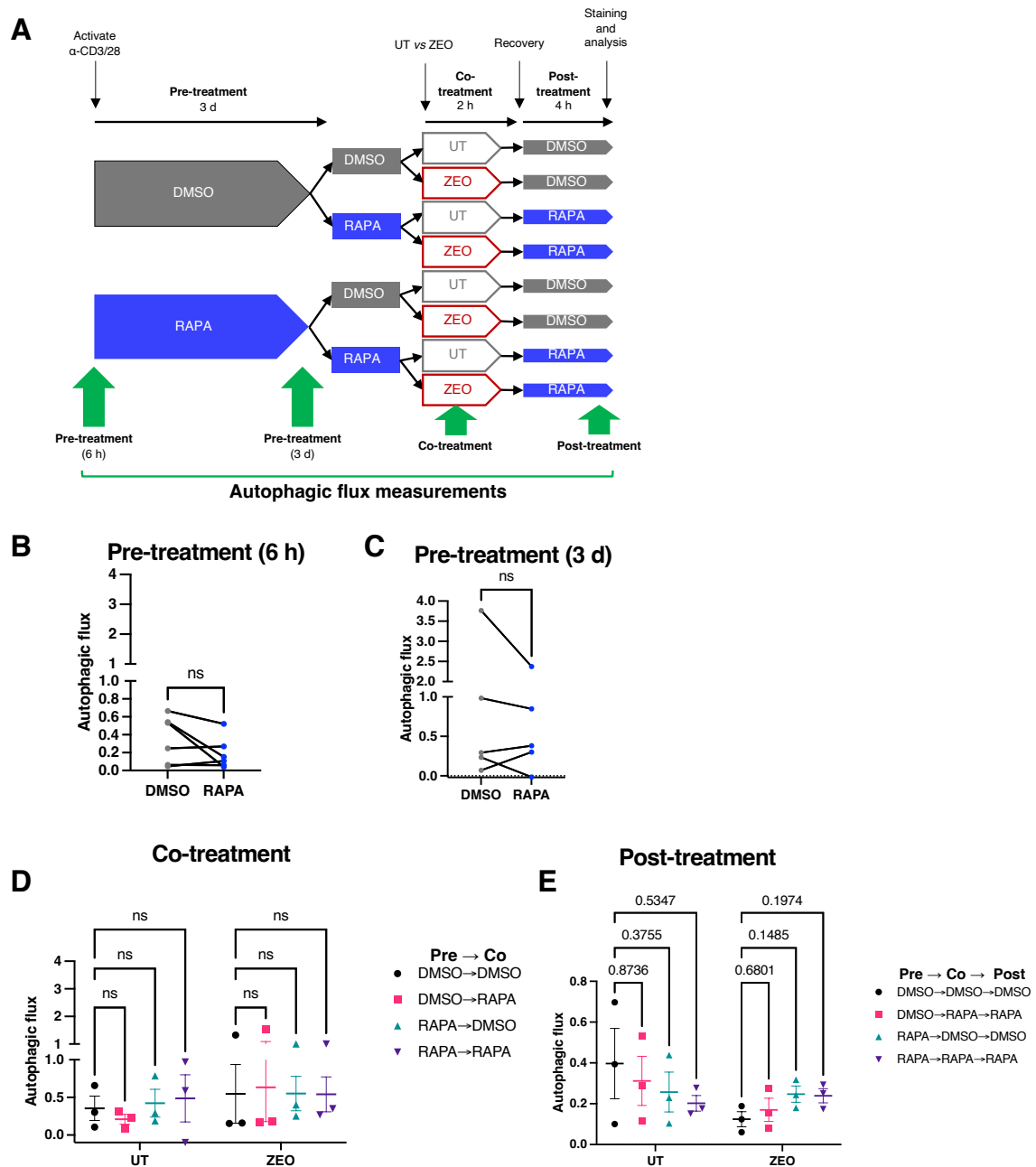


Figure 4.22 Measuring autophagic flux using flow cytometry

(A) Assay for measuring autophagic flux with flow cytometry by comparing autophagosome-bound LC3-II levels between cells treated with vehicle (Veh, 0.1% DMSO) or 10 nM bafilomycin A₁ (BafA) for 2 hours. (B) LC3-II fluorescence histograms of CD4⁺ T cells that were activated for 6 hours and 3 days with 1 μ g/ml α -CD3/28. Data are representative of three independent experiments.

To evaluate whether mTOR inhibition affected autophagy during DNA damage, cells were treated with rapamycin either continuously (RRR), before (RDD), or during and after

treatment (DRR) with zeocin (Figure 4.23A). Autophagic flux was measured at several points throughout the experiment with respect to zeocin: either 6 hours into the pre-treatment activation (Figure 4.23B), 3 days into the pre-treatment activation (Figure 4.23C), during (co-)treatment with zeocin (Figure 4.23D), and after (post-)treatment, in the final 2 hours of the recovery period (Figure 4.23E). Rapamycin did not significantly change the autophagic flux of CD4⁺ T cells at any time point of measurement (Figure 4.23A—E). However, there appeared to be a non-significant increase in autophagic flux when cells were measured during recovery from zeocin (post-treatment) (Figure 4.23E). Since I had previously observed that the proportion of γ H2AX⁺ cells after zeocin treatment ranged between 18-50% (Figure 4.5D), I reasoned that any changes in autophagic flux could be hidden when analysing bulk zeocin-treated cells. To measure the autophagic flux in γ H2AX⁺ and γ H2AX⁻ cells, I therefore optimised a combined protocol for measuring autophagic flux and DNA damage by flow cytometry.



4.2.15 Optimising a combined autophagic flux and DNA damage assay in human T cells

To optimise both autophagic flux (LC3) and DNA damage (γ H2AX) flow cytometry staining protocols, PBMCs were activated with T-cell-specific antibodies and afterwards treated with zeocin and allowed to recover for 4 hours; cells were then harvested and treated with either 10 nM bafilomycin A₁ or DMSO vehicle control. Cells were then stained with 1 of 5 different protocols: either 1) LC3 in its optimal conditions, 2) LC3 in its optimal conditions followed by 30 mins with γ H2AX in its optimal conditions, 3) LC3 in its optimal conditions, followed by overnight γ H2AX staining in its optimal conditions, 4) LC3 in its optimal conditions with γ H2AX co-staining, or 5) γ H2AX in its optimal conditions only (Figure 4.24A). First, I evaluated whether measurement of autophagic flux was altered by the different staining protocols (Figure 4.24B, upper). The shift in bafilomycin-treated cells was consistent and unchanged by any of the γ H2AX staining protocols (Figure 4.24B). However, γ H2AX staining after the LC3 protocols led to a large level of background fluorescence (Figure 4.24B, lower). To minimise background γ H2AX staining, future experiments were performed using the staining protocol where LC3 and γ H2AX were co-stained (protocol 4) with the staining period from 30 minutes to 1 hour, to improve the γ H2AX signal.

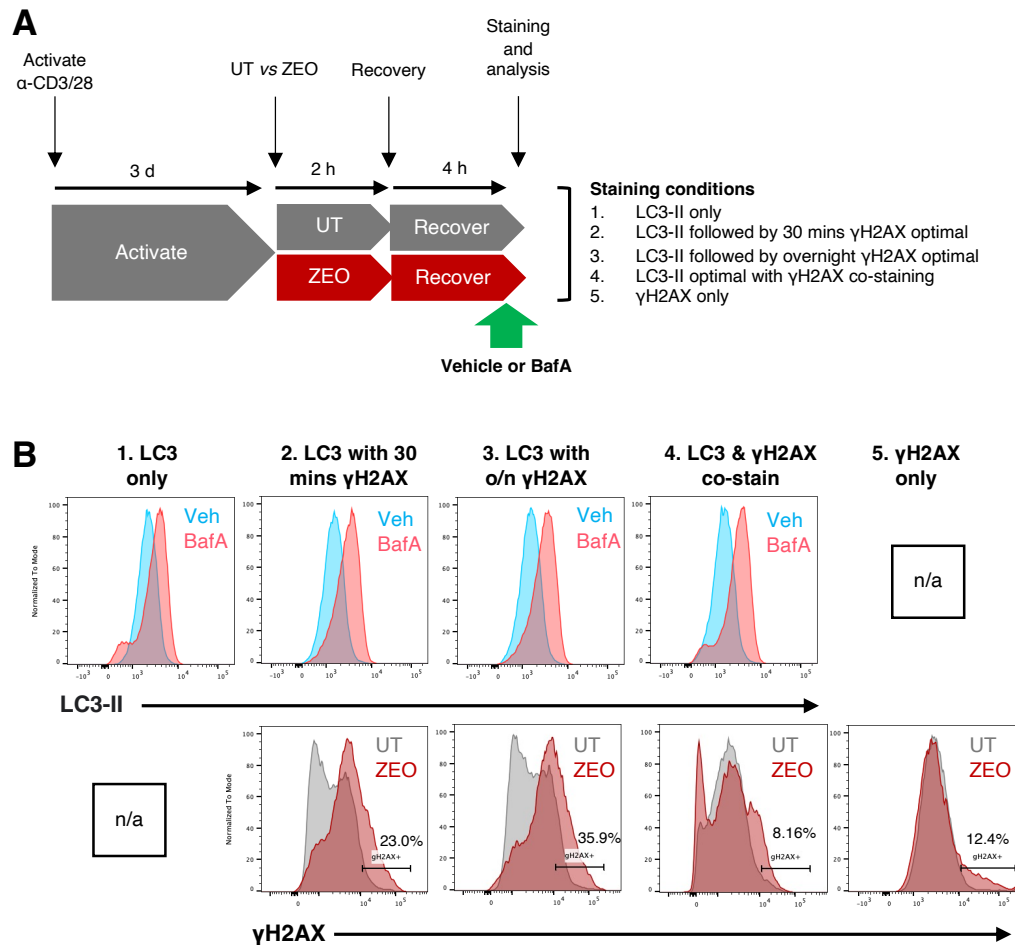


Figure 4.24 Optimising a combined autophagic flux and DNA damage assay in PBMCs from healthy donors

(A) PBMCs were activated for 3 days with 1 μ g/ml α -CD3/28 and afterwards treated with zeocin (ZEO, 200 μ g/ml) or untreated (UT), and left to recover for 4 hours. Cells were stained using 5 separate protocols to identify an optimal staining protocol for combined LC3-II and γ H2AX detection by flow cytometry. (B) Histograms of the levels of LC3-II (top) and γ H2AX (bottom) in CD4⁺ T cells. Graphs are overlays of cells treated without zeocin (untreated) that had been treated with 10 nM Bafilomycin A₁ (BafA) or Vehicle ('veh', 0.1% DMSO). n/a = not applicable. For γ H2AX staining, graphs are overlays of UT and ZEO (veh conditions), and the % γ H2AX⁺ (of CD4⁺ T cells) in the zeocin-treated conditions are indicated.

4.2.16 Measuring autophagic flux in γ H2AX⁺ and γ H2AX⁻ T cells

Using this optimised LC3 and γ H2AX staining protocol, I assessed whether autophagy was affected by rapamycin in γ H2AX-gated cells after DNA damage. PBMCs were treated with rapamycin either continuously (RRR), before (RDD), or during and after zeocin (DRR), and allowed to recover for 4 hours as in Figure 4.23A. In the final 2 hours of recovery, cells were

treated either with 10 nM bafilomycin A₁ or DMSO control and subsequently stained for LC3 and γ H2AX for analysis by flow cytometry. Cells positive for γ H2AX exhibited significantly lower autophagic flux than those negative for γ H2AX, in both CD4⁺ and CD8⁺ T subsets (Figure 4.25A). This suggests that DNA damage is associated with low autophagy, consistent with my data that γ H2AX⁺ cells displayed high activity of mTORC1, a negative regulator of autophagy (Figure 4.6B). Rapamycin significantly increased autophagic flux in both γ H2AX⁺ and γ H2AX⁻ CD4⁺ T cells (Figure 4.25B) and in γ H2AX⁺ cells CD8⁺ T cells (Figure 4.25B), especially when treated before zeocin (RRR and RDD conditions). Co-and-post-treatment of zeocin with rapamycin (DRR) mildly increased autophagic flux. Together, these data suggest that rapamycin may increase autophagic flux after DNA damage in γ H2AX⁺ T cells to a level comparable to that of γ H2AX⁻ cells (Figure 4.25A). Since autophagy plays a myriad of roles in the DDR (Vessoni et al., 2013), the induction of autophagy by mTOR inhibition may explain the effects of rapamycin in limiting DNA lesion burden, γ H2AX, and global DDR activation after zeocin treatment.

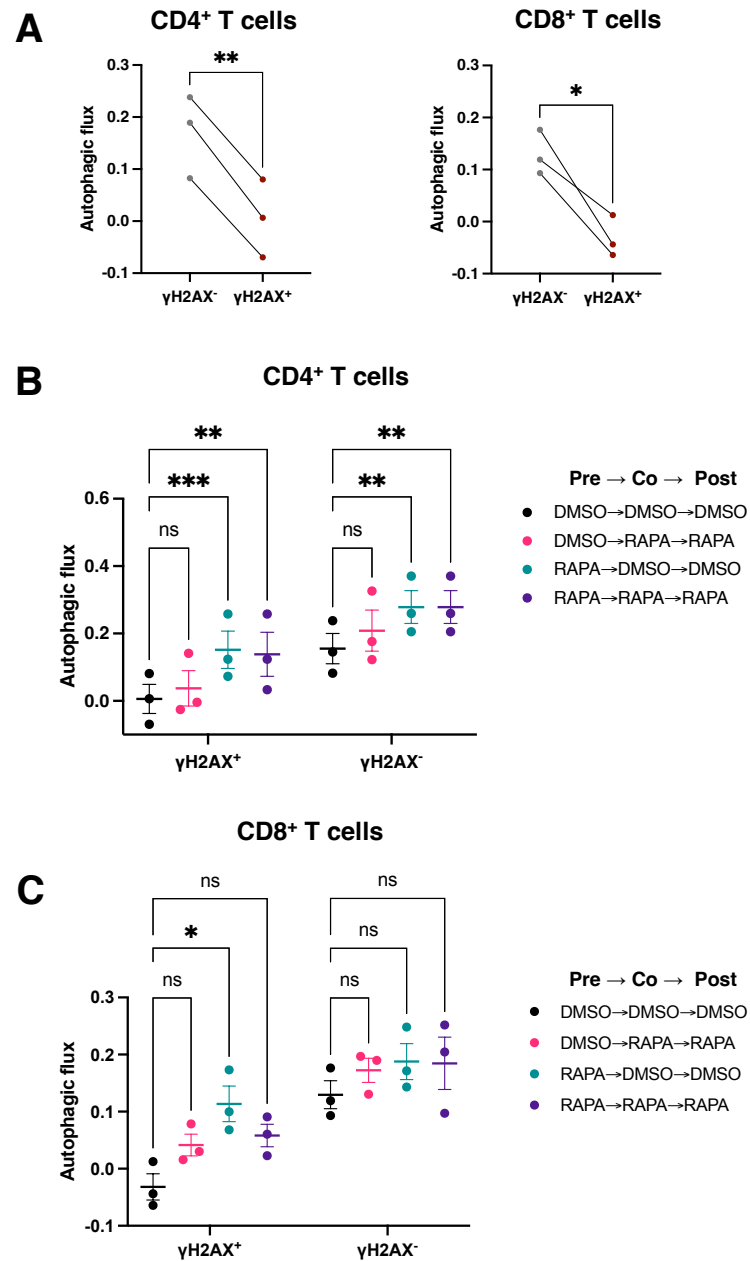


Figure 4.25 Autophagic flux in γH2AX -gated T cells

Experimental design as in Figure 4.23A measuring autophagic flux in γH2AX -gated subsets during the final 2 hours of recovery from zeocin treatment in (A) CD4⁺ T cells (left) or CD8⁺ T cells or (B) CD4⁺ and (C) CD8⁺ T cells across different rapamycin treatment conditions as in Figure 4.23E using a flow cytometry-based LC3 and γH2AX assay. *P*-values were derived from a paired t-test (A) or a two-way ANOVA with Šidák's multiple comparisons test (B–C), all with *n*=3 healthy NHS-BT donors.

4.3 Discussion

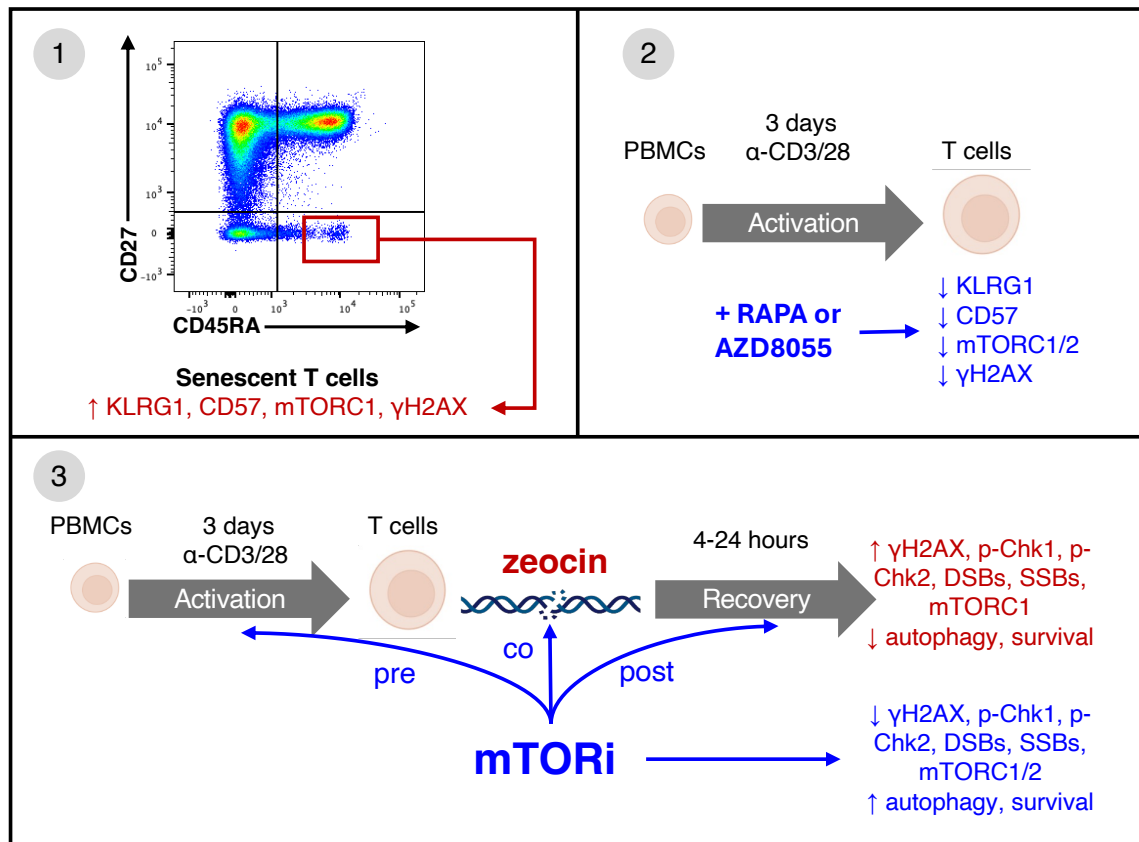


Figure 4.26 Summary of major findings from Chapter 4

1) CD27-CD45RA⁺ TEMRA cells displayed surface markers associated with T cell senescence, high mTOR activity, and DNA damage marked by γH2AX. **2)** The activation of T cells in the presence of the mTOR inhibitors, rapamycin (RAPA) or AZD8055, decreased levels of T cell senescence markers, mTOR activity, and DDR signalling. **3)** Zeocin treatment of activated T cells caused DNA damage, increased mTORC1 activity, and decreased autophagy and survival. mTOR inhibition (mTORi) before, during, and/or after treatment with zeocin limited the induction of γH2AX; continuous mTORi treatment limited the DDR, increased autophagy, and cell survival after acute DNA damage.

While both senescent T cells (defined as CD27-CD45RA⁺, or TEMRA cells) and total T cells from older donors acquire characteristic features of cell senescence (section 1.3.1.3), it was not fully understood whether they also featured mTOR hyperactivity, as I had observed in fibroblast DDIS in Chapter 3 (Figure 3.7). Furthermore, it was unclear whether the senescence phenotype of persistent DNA damage was functionally related to mTOR activity. In this chapter, I explored the relationships between mTOR activity, acute DNA

damage, and human T cell senescence using *in vitro* models. A summary of the major findings in this chapter is presented in Figure 4.26.

CD4⁺ and CD8⁺ TEMRA cells displayed cell-surface markers consistent with T cell senescence, such as CD57 and KLRG1 expression (Figure 4.1C—D). However, only CD4⁺ TEMRA cells displayed significantly higher levels of the DNA damage marker, γ H2AX, than their naïve counterparts (Figure 4.1E). CD8⁺ TEMRA cells, however, did not show increased γ H2AX levels compared to naïve cells, contrary to previous studies; however, these studies often analysed CD8⁺ TEMRA cells after short-term stimulation (8 hours to overnight) with anti-CD3, whereas I examined cells directly *ex vivo* (Henson et al., 2014, Henson et al., 2015). CD4⁺, but not CD8⁺, TEMRA cells also displayed significantly higher levels of the downstream mTORC1 signalling target, p-S6, but there were no differences in p-Akt or p-S6 in CD8⁺ T cell subsets (Figure 4.1F—G). Few studies have directly measured levels of mTOR downstream phospho-proteins in resting senescent T cell subsets. Some have reported that total mTOR and S6K1 protein levels were lower in CD27⁻CD28⁻ CD4⁺ T cells but did not measure phospho-protein targets (Lanna et al., 2017). Others have observed that CD27⁻CD28⁻CD8⁺ T cells display low levels of p-Akt after 2-hour stimulation, compared to naïve CD8⁺ T cells (Plunkett et al., 2007). Generally, the results from these studies investigating T cell senescence phenotypes vary due to both the range of definitions for senescent T cells (either CD27⁻CD45RA⁺ or CD27⁻CD28⁻) and how mTOR activity was measured (at rest or after short-term activation). Measuring mTOR activity after short-term activation likely provides greater insight into TCR-dependent mTOR activation (which is known to be impaired in senescent T cells) rather than assessing endogenous mTOR activity levels. Furthermore, studies tend to evaluate mTOR activity at early time points of activation, whereas my data suggest greatly differing activation dynamics of p-S6 between T cell memory subsets across 72 hours (Figure 4.2).

While mTOR inhibitors did not affect CD25 positivity in activated T cells (Figure 4.4A), they limited the expression of the senescence markers CD57, KLRG1, and γ H2AX⁺ DNA damage (Figure 4.4B—D). To identify whether mTOR activity plays a role in DNA damage in human T cells, I developed an assay for measuring acute DNA damage using flow cytometry (Figure 4.5) and the alkaline comet assay (Figure 4.17). γ H2AX⁺ cells displayed high levels of mTORC1 activity (Figure 4.6), and low-dose mTOR inhibition strongly limited DNA lesion burden, DDR activation, and cell death after genotoxic treatment (Figure 4.7, Figure 4.8). Furthermore, treating cells with the mTOR inhibitor rapamycin either before, during, or after the DNA-damaging agent successfully inhibited the induction of the γ H2AX⁺ population of cells (Figure 4.11).

There are reports that mTOR inhibition improves genome stability and thus immune resilience. In the *Vav-iCre^{+/+};Ercc1^{-/-}* DNA damage-induced immunosenescence model, rapamycin improved the immune response to a model antigen, and reduced the expression of the cyclin kinase inhibitors, *CDKN2A* and *CDKN1A* in T cells and several other tissues, though the effect of rapamycin treatment on markers of DNA damage in the immune cells was not reported (Yousefzadeh et al., 2021b). Direct evidence that mTOR inhibition is genoprotective comes from the observation that long-term culture with rapamycin reduces the number of 53BP1⁺ DNA damage foci in fibroblasts deficient in the DNA repair factor, WRN (Saha et al., 2014). In addition to this, dietary restriction (DR), a well-known life extension strategy that converges on the inhibition of the mTOR signalling pathway (Cox and Mattison, 2009), elicited a 3-fold increase in median lifespan in both DNA repair-deficient *Ercc1^{Δ/-}* and *Xpg^{-/-}* progeroid mice compared to *ad libitum*-fed mutant mice (Vermeij et al., 2016). Importantly, genome damage, as measured by the fraction of γ H2AX⁺ neuronal cells, was reduced in DR-fed mice, suggesting that DR could alleviate genome instability in the context of DNA repair deficiency (Vermeij et al., 2016). Since the mTOR pathway is inhibited during nutrient deprivation (particularly via reduced phosphorylation of

ones of its downstream targets, p-Akt (at Ser⁴⁷³), this indirectly suggests that DR-induced genome stability may be through mTOR inhibition (Vermeij et al., 2016).

In pursuit of a mechanism behind the effects of mTOR inhibition in the DNA damage assay, I found that autophagic flux was lower in γ H2AX⁺ than γ H2AX⁻ cells, suggesting that DNA damage persistence was associated with low autophagy (Figure 4.25A). Furthermore, rapamycin treatment increased autophagic flux in both γ H2AX⁺ and γ H2AX⁻ cells (Figure 4.25B—C). Of note, autophagic flux was measured using a flow cytometry-based assay comparing LC3 levels in γ H2AX⁺-gated cells treated with either vehicle or bafilomycin A₁, an autophagy inhibitor (Alsaleh et al., 2020). A limitation here is that bafilomycin A₁ treatment may have affected γ H2AX levels, and thus possibly skewed the measurement of autophagic flux between γ H2AX⁺-gated cells between vehicle and BafA conditions. Future work could employ immunofluorescence-based measurements of autophagic flux, such as by using pH-sensitive dyes which indicate autophagosome-lysosomal fusion events, and co-staining for γ H2AX (Maulucci et al., 2015). Furthermore, the data that rapamycin both increases autophagic flux and limits γ H2AX induction in DNA-damaged cells is only correlative. To test robustly whether the protection of DNA damage by mTOR inhibition is dependent on the induction of autophagy, the effect of mTOR inhibition on γ H2AX in autophagy-deficient *ATG7*^{-/-} T cells could be measured.

Boosting autophagy is an attractive strategy for attenuating immunosenescence. Impaired macroautophagy is a hallmark of ageing (Schmauck-Medina et al., 2022, Lopez-Otin et al., 2023). Additionally, autophagic flux decreases in immune cells with ageing and is correlated with inadequate antigen-specific responses, which can be improved by treatment with autophagy-enhancing supplements (Alsaleh et al., 2020, Zhang et al., 2019, Puleston et al., 2014). There is evidence that autophagy is associated with low DNA damage. For example, senescent CD57⁺ CD8⁺ T cells with low autophagy display the highest levels of γ H2AX⁺ DNA damage, compared to CD57⁻ cells, or those with high autophagy (Phadwal et

al., 2012). Furthermore, cellular senescence and DNA damage cause the phosphorylation and activation of ATM (at Ser¹⁹⁸¹), which phosphorylates and inhibits assembly of lysosomal ATPase V₁ subunits, preventing lysosomal acidification and effective autophagy; thus, ATM activation links DNA damage and autophagy (Kang et al., 2017).

Boosting autophagy may attenuate nuclear DNA damage through both direct and indirect means. First, autophagy is the main pathway for the degradation of ROS-producing dysfunctional mitochondria (termed mitophagy) and is induced by acute DNA damage in human cells (Dan et al., 2020, Bektas et al., 2019). As discussed earlier (section 1.3.2.1), immune cells from old donors accumulate dysfunctional, ROS-producing mitochondria, which may be a consequence of their decline in autophagy. Thus it is possible that rapamycin may enhance autophagy and therefore limit zeocin-induced mitochondrial DNA damage, dysfunction, and ROS release, preventing further oxidative damage to nuclear DNA (Vessoni et al., 2013). To test this, future experiments could evaluate whether rapamycin affects ROS production in zeocin-induced γ H2AX⁺ T cells. Interestingly, rapamycin boosts autophagic flux in all CD8⁺ T cell subsets apart from the TEMRA population (Henson et al., 2014); this contrasts with my observation that rapamycin increased autophagic flux in γ H2AX⁺ CD8⁺ T cells, perhaps highlighting the limits of this DNA damage assay in modelling physiological T cell senescence phenotypes.

Autophagy plays many roles in the DNA damage response, independent of its effects on the removal of DNA-damaging mitochondria. For example, DDR enzymes require a pool of dNTPs and ATP as substrates for DNA repair, which may be provided by autophagy. In support of this is the observation that autophagy-deficient cells experience a profound nucleotide depletion under starved conditions (Guo et al., 2016). Furthermore, the induction of DNA replication stress by hydroxyurea strongly induces autophagy; indeed, autophagy-deficient cells displayed high levels of γ H2AX⁺ and 53BP1⁺ DNA damage, which could be reversed by experimentally providing deoxynucleosides to aid DNA synthesis (Vanzo et al.,

2020). To test whether these effects of autophagy explain the results from this chapter, nucleotide concentration in DNA-damaged cells with and without rapamycin treatment could be quantified using a novel click-chemistry assay (Huang et al., 2020a). Furthermore, the effects of adding dNTPs into the media of cells recovering from zeocin treatment, and whether this recapitulated the effects of rapamycin treatment, could be tested.

Autophagy regulates the DDR pathway by selective degradation of proteins involved in DNA metabolism. Studies in yeast have identified the autophagy-dependent degradation of Ribonucleotide reductase 1 (Rnr1), without which cells become vulnerable to MMS-induced DNA damage (Dyavaiah et al., 2011). On the other hand, induction of autophagy by either HDAC inhibition or rapamycin promoted the degradation of Sae2 (orthologue of human CtIP), leading to impaired activation of Rad53 in response to DSBs in budding yeast (Robert et al., 2011). Finally, autophagy can directly remove DNA-protein crosslinks (e.g. TOP1cc) induced by topoisomerase inhibitors, through selective nucleophagy (Lascaux et al., 2024). This demonstrates the diverse roles of autophagy in the regulation of DNA repair pathways. To test whether autophagy selectively degrades DNA repair proteins in the DNA damage assay developed in this chapter, the autophagosome cargo could be mapped using the LC3B-AP2 mouse model, where LC3-proximal proteins in the autophagosome can be biotinylated, purified, and identified by mass spectrometry (Zhou et al., 2022).

This work is limited by the fact that it is all performed *in vitro*, using PBMCs from young and healthy donors from the NHS Blood Transfusion service (NHS-BT). It would be particularly interesting to test whether the response to acute DNA damage differed between young and old donor PBMCs, and whether donor sex plays a role in this phenotype. Additionally, in these assays, DNA damage induction was performed using the radiomimetic drug, zeocin; using a broader range of genotoxic agents that produce other forms of DNA lesions, such as interstrand crosslinks, would expand the relevance of this research. Additionally, the comet assay experiments in Figure 4.19 were performed using CD4⁺ T cells from only one

donor, and so require validation with cells from more donors. In my experiments, *in vitro* T cell activation was achieved using soluble anti-CD3/28 antibodies, which may not replicate physiological antigen stimulation in the context of MHC-restricted peptides on antigen-presenting cells. Finally, this chapter focused on the acute effects of DNA damage (on γ H2AX and cell death) rather than T cell DNA damage-induced senescence; future research could investigate the effect of mTOR inhibition during long-term culture of PBMCs, in which cells display telomere attrition and increased mitochondrial ROS (Sanderson and Simon, 2017). In summary, the wider implications of this *in vitro* work are that mTOR inhibitors may be effective 'genoprotectors', and that this may partially explain their beneficial impact in attenuating immunosenescence in older people (Kell et al., 2023).

5

Results

Low-dose rapamycin modulates features of
immunosenescence and immune exhaustion
in a small human trial

5.1 Introduction

5.1.1 Immunosenescence in humans

Immunosenescence is partially underpinned by the age-related changes in immune cells, such as the accumulation of age-associated, end-stage memory immune subsets across different immune cell types which display features characteristic of senescence (Zhou et al., 2021) (Table 1.1). Age-associated immune subsets are associated with poor immune responses with age: for example, the proportion of circulating senescent T cells negatively correlates with effective responses to the mRNA COVID-19 vaccine (Palacios-Pedrero et al., 2022). However, the extent to which cellular senescence contributes to the induction of age-associated immune subsets *in vivo*, and how they are affected by anti-ageing interventions (such as mTOR inhibition), is not fully understood.

5.1.2 Interventions to boost immune resilience

Generally, studies evaluate the effects of interventions on immunosenescence in humans by assessing their impacts on responses to an immune challenge. Several anti-ageing interventions have shown promise in attenuating immunosenescence (Table 5.1). For example, a cutaneous antigen recall challenge model using varicella zoster virus (VZV) showed that oral administration of either a p38 MAPK inhibitor (losmapimod) or vitamin D₃ improved adaptive memory responses in older people (Vukmanovic-Stejic et al., 2018, Chambers et al., 2021b). Further *in vitro* work suggested that both interventions may act by inhibiting p38 MAPK and the subsequent SASP of senescent stromal cells, which otherwise suppresses immune responses (Sayegh et al., 2024) (section 1.3.4.2). p38 MAPK inhibition also improves innate immunity in older people, for example by increasing monocyte efferocytosis in a model of cutaneous acute inflammation induced by cantharidin (De Maeyer et al., 2020).

The efficacy of immunosenescence interventions can also be evaluated by their ability to prevent mortality from environmentally acquired infections to which old people are particularly susceptible. One example of this is community-acquired pneumonia with sepsis (CAP +S). The increased mortality from CAP +S with age is associated with impaired neutrophil functions, such as NETosis and chemotaxis, which can be restored using statins (e.g. simvastatin) (Sapey et al., 2014). In a trial of patients older than 55 years who were admitted to hospital with CAP +S, 7-day simvastatin treatment improved neutrophil chemotaxis and NETosis, and dramatically increased hospitalisation-free survival over 1 year compared to placebo (Table 5.1) (Sapey et al., 2019). Another anti-ageing intervention, metformin, was found significantly to improve survival from COVID-19 infection in older and obese diabetic women in a retrospective analysis (Table 5.1) (Bramante et al., 2021). Therefore, anti-ageing interventions show efficacy in boosting immune resilience against infections in an ageing context.

5.1.3 mTOR inhibitors as a promising strategy targeting immunosenescence

One of the most promising anti-ageing interventions to boost immune resilience in older people is inhibition of mTOR (Table 5.1). At high doses (e.g. 5 mg/day), mTOR inhibitors, such as rapamycin, are immunosuppressive (Dominguez et al., 2000). However, low-dose rapamycin is immunoprotective; a recent meta-analysis of 23 studies in mice (Phillips and Simons, 2022) showed that low-dose rapamycin promoted survival from acute pathogenic insults, including influenza infection (Keating et al., 2013). In addition to mice, low-dose mTOR inhibition strongly promotes immune resilience in humans, as demonstrated by clinical trials in older people. First, mTOR inhibition by either RAD001 (also named everolimus, a first-generation rapalogue), or dual therapy with RAD001 and BEZ235, a second-generation ATP-competitive PI3K and mTOR inhibitor, improved influenza vaccination responses and decreased subsequent respiratory tract infection rates 1 year

after treatment (Table 5.1) (Mannick et al., 2014, Mannick et al., 2018). Treatment with BEZ235 alone (also named RTB101) showed trends towards immune improvement (Mannick et al., 2021). There is also promising recent evidence that mTOR inhibition improves survival from COVID-19 and its associated respiratory complications, though larger studies are needed (Table 5.1). First, in a small trial of hospitalised patients with pneumonia from COVID-19, low-dose rapamycin for 14 days showed improvements in oxygenation, reduced hospital stays, and a decreased incidence of thromboembolisms (Singla et al., 2024). Furthermore, prophylactic treatment with RTB101 in nursing homes potentially improved survival from COVID-19 infection compared to placebo (mortality 0/18 RTB101 group, 2/17 placebo group, trial NCT04409327) (Table 5.1).

The mechanism for immunoprotection by mTOR inhibition is not fully understood. mTOR inhibition has been observed to decrease %PD-1⁺ T cells, increase vaccine-specific B cell antibody titres, and increase type I interferon gene expression in peripheral blood (Mannick et al., 2014, Mannick et al., 2018, Mannick et al., 2021). Crucial to understanding its mechanism, the low nanomolar doses used in *in vitro* studies are achieved *in vivo* (e.g. in one study, 1 mg/day of rapamycin raised the average blood concentrations of the drug to 7.88 nM (range 5.14 – 12.91) (Kraig et al., 2018)). Though low-dose rapamycin improves immune responses to challenge, it is less understood how mTOR inhibition affects phenotypes of immunosenescence in an immune-unchallenged context (i.e., in steady state). Recently, the development of spectral flow cytometry has greatly expanded the number of features that can be analysed within one heterogeneous cell sample (Park et al., 2020b). Hence, multiple panels from conventional flow cytometry can be combined, saving precious samples, time, and increasing immunophenotyping depth. This novel technology allows us to interrogate more deeply the effects of existing anti-ageing interventions, such as mTOR inhibition, on peripheral immune phenotypes. In Chapters 3 and 4, I explored how low doses of mTOR inhibitors, namely rapamycin and AZD8055, limited fibroblast

senescence phenotypes induced by DNA damage, and protected T cells from acute DNA-damaging treatment. In this chapter, I aim to test the hypothesis that mTOR inhibition *in vivo* may act, at least in part, by limiting DNA damaged-induced cell senescence phenotypes in immune cells.

5.1.4 Chapter aim and objectives

In this chapter, I aim to evaluate the impact of *in vivo* rapamycin treatment on senescence in human age-associated peripheral immune subsets.

Objectives:

1. To evaluate whether biomarkers of senescence increase in age-associated immune cell subsets from healthy donors.
2. To identify whether biomarkers of senescence increase in immune cells from donors of advanced age or an age-related disease (osteoarthritis).
3. To assess the impact of *in vivo* low-dose rapamycin, by:
 - i. Assessing markers of cellular senescence and immunosenescence in peripheral immune cells from the UK's first single-blind, placebo-controlled rapamycin trial.
 - ii. Validating the effects of rapamycin using an *in vitro* model.

Table 5.1 Studies demonstrating pharmacological efficacy in attenuating immunosenescence

Type of treatment	Treatment regimen	Challenge	Outcome	Reference
p38 MAPK inhibition	Losmapimod 15 mg/day twice daily for 4 days	VZV antigen recall challenge	Improved clinical adaptive immune response to cutaneous challenge	(Vukmanovic-Stejc et al., 2018)
p38 MAPK inhibition	Losmapimod 15 mg twice daily for 4 days	Cantharidin blisters for acute inflammation	Improved resolution of acute inflammation by efferocytosis	(De Maeyer et al., 2020)
Vitamin D ₃	Vitamin D ₃ 6400 IU/day for 14 weeks	VZV antigen recall challenge	Improved clinical adaptive immune response to cutaneous challenge	(Chambers et al., 2021b)
Metformin	Metformin use (pharmacy prescription)	Retrospective cohort analysis of older obese people with diabetes admitted to hospital with COVID-19	Metformin associated with reduced mortality from COVID-19 in women only	(Bramante et al., 2021)
Statins	Simvastatin 80 mg/day for 7 days vs placebo	Community-acquired pneumonia with sepsis	Improved neutrophil function and hospitalisation-free survival	(Sapey et al., 2019)
mTOR inhibition	RAD001, RTB101, or combined for 6 weeks, prior to vaccine challenge vs placebo	Influenza vaccine challenge	Higher antibody titre, antiviral gene expression, and reduced respiratory tract infection rate after 1 year	(Mannick et al., 2014, Mannick et al., 2021, Mannick et al., 2018)
mTOR inhibition	Sirolimus 6 mg on day 1 followed by 2 mg daily for 14 days vs placebo	Hypoxemic respiratory failure due to COVID-19	Trends toward benefits in oxygenation, decreased stay in hospital, and risk of developing thromboembolism	SCOPE trial (NCT04341675) (Singla et al., 2024)
mTOR inhibition	RTB101 10 mg/day for 4 weeks vs placebo	Environmental exposure to COVID-19 in a nursing home	0/18 deaths (RTB101 group) vs 2/17 deaths (placebo group) due to COVID-19	Trial NCT04409327

5.2 Results

5.2.1 Features of senescence in age-associated subsets from healthy donors

To identify and evaluate features of immune cell senescence in peripheral immune cells, PBMCs obtained from healthy donors from the NHS Blood Transfusion (NHS-BT) service were analysed using 27-colour spectral flow cytometry (see section 2.6.2). The function of each marker, and the cell types on which it is predominantly expressed, is summarised in Table 5.2. Using this panel, T cells, B cells, natural killer (NK) cells, monocytes, and dendritic cells (DCs) were identified based on a published gating strategy (Figure 5.1 and Table 5.3) (Jensen and Wnek, 2020). The proportion of each peripheral immune subset retrieved from healthy NHS-BT donors is indicated in Figure 5.2.

Immune cell subsets that accumulate during ageing (age-associated immune subsets) can be identified by their expression of cell surface markers and are indicated (Figure 5.1 and Table 5.3). It is unclear whether these age-associated subsets also acquire features of cellular senescence. To test the hypothesis that end-differentiated, age-associated immune subsets display higher levels of senescence biomarkers, PBMCs from healthy NHS-BT donors were stained with antibodies detecting p16^{Ink4a}, p21^{Cip1/Waf1}, p53, γH2AX, p-S6, and p-Akt, and the signals for these biomarkers compared across the different immune cell subsets.

Table 5.2 Cell surface markers used in 27-colour spectral flow cytometry of human PBMCs

Marker	Name	Function	Cell types
CD3	Cluster of differentiation 3	Signalling component of the TCR	T cells
CD19	Cluster of differentiation 19	Type-I transmembrane glycoprotein immunoglobulin	B cells
CD4	Cluster of differentiation 4	Co-receptor for the TCR	CD4 ⁺ T cells, monocytes, dendritic cells
CD8	Cluster of differentiation 8	Co-receptor for the TCR	CD8 ⁺ T cells, NK cells
IgD	Immunoglobulin D	Antibody isotype for the naïve BCR	Unswitched B cells
CD27	Cluster of differentiation 27	Co-stimulatory receptor	Naïve and EM T cells, memory B cells
CD28	Cluster of differentiation 28	Co-stimulatory receptor	Naïve/early-differentiated T cells
CD45RA	Cluster of differentiation 45 RA	Splice variant of the tyrosine phosphatase CD45	Naïve and EMRA T cells, B cells, monocytes
CD57	Cluster of differentiation 57	Terminal differentiation marker	T cells, NK cells
KLRG1	Killer cell lectin-like receptor G1	Co-inhibitory receptor, terminal differentiation marker	T cells, NK cells
CD38	Cluster of differentiation 38	Cyclic ADP-ribose hydrolase	T cells, B cells, NK cells
NKG2A	NKG2 type A	Inhibitory receptor signalling	T cells, NK cells
NKG2D	NKG2 type D	Activating receptor signalling	T cells, NK cells
PD-1	Programmed cell death protein 1	Co-inhibitory receptor	T cells, B cells
LAG3	Lymphocyte Activation Gene 3	Co-inhibitory receptor	T cells
CD56	Cluster of differentiation 56	Activator of cytotoxicity	NK cells, T cells
HLA-DR	Human Leukocyte Antigen DR Isotype	MHC class II cell surface receptor	B cells, monocytes, dendritic cells
CD14	Cluster of differentiation 14	Co-receptor with TLR4 for LPS detection	Classical and intermediate monocytes
CD16	Cluster of differentiation 16	Fc receptor (FcγRIIIa)	NK cells, non-classical monocytes, T cells
CD11b	Cluster of differentiation 11b	Cell-cell adhesion	Monocytes, NK cells, T cells

Table 5.3 Immunophenotyping major peripheral cell subsets using spectral flow cytometry

Cell type	Cell surface markers (gated on Cells/Live cells/Single cells)
CD4 ⁺ T Naïve (CD4 TN)	CD3 ⁺ CD19 ⁻ CD4 ⁺ CD27 ⁺ CD45RA ⁺
CD4 ⁺ T Central Memory (CD4 TCM)	CD3 ⁺ CD19 ⁻ CD4 ⁺ CD27 ⁺ CD45RA ⁻
CD4 ⁺ T Effector Memory (CD4 TEM)	CD3 ⁺ CD19 ⁻ CD4 ⁺ CD27 ⁻ CD45RA ⁻
CD4⁺ T Effector Memory Re-expressing CD45RA (CD4 TEMRA)	CD3 ⁺ CD19 ⁻ CD4 ⁺ CD27 ⁻ CD45RA ⁺
CD8 ⁺ T Naïve (CD8 TN)	CD3 ⁺ CD19 ⁻ CD8 ⁺ CD27 ⁺ CD45RA ⁺
CD8 ⁺ T Central Memory (CD8 TCM)	CD3 ⁺ CD19 ⁻ CD8 ⁺ CD27 ⁺ CD45RA ⁻
CD8 ⁺ T Effector Memory (CD8 TEM)	CD3 ⁺ CD19 ⁻ CD8 ⁺ CD27 ⁻ CD45RA ⁻
CD8⁺ T Effector Memory Re-expressing CD45RA (CD8 TEMRA)	CD3 ⁺ CD19 ⁻ CD8 ⁺ CD27 ⁻ CD45RA ⁺
B Naïve Unswitched	CD19 ⁺ CD3 ⁻ IgD ⁺ CD27 ⁻
B Memory Unswitched	CD19 ⁺ CD3 ⁻ IgD ⁺ CD27 ⁺
B Memory Switched	CD19 ⁺ CD3 ⁻ IgD ⁻ CD27 ⁺
B Double Negative (DN)	CD19 ⁺ CD3 ⁻ IgD ⁻ CD27 ⁻
NK cells CD57 ⁻ CD16 ⁻	CD3 ⁻ CD19 ⁻ CD56 ⁺ CD56 ^{bright or dim} CD57 ⁻ CD16 ⁻
NK cells CD57 ⁺ CD16 ⁻	CD3 ⁻ CD19 ⁻ CD56 ⁺ CD56 ^{bright or dim} CD57 ⁺ CD16 ⁻
NK cells CD57 ⁻ CD16 ⁺	CD3 ⁻ CD19 ⁻ CD56 ⁺ CD56 ^{bright or dim} CD57 ⁻ CD16 ⁺
NK cells CD57⁺CD16⁺	CD3 ⁻ CD19 ⁻ CD56 ⁺ CD56 ^{bright or dim} CD57 ⁺ CD16 ⁺
Classical monocytes	CD3 ⁻ CD19 ⁻ CD56 ⁻ HLA-DR ⁺ CD14 ⁺ CD16 ⁻
Intermediate monocytes	CD3 ⁻ CD19 ⁻ CD56 ⁻ HLA-DR ⁺ CD14 ⁺ CD16 ⁺
Non-classical monocytes	CD3 ⁻ CD19 ⁻ CD56 ⁻ HLA-DR ⁺ CD14 ⁻ CD16 ⁺
Dendritic cells	CD3 ⁻ CD19 ⁻ CD56 ⁻ HLA-DR ⁺ CD14 ⁻ CD16 ⁻

Bold subsets represent age-associated peripheral immune subsets.

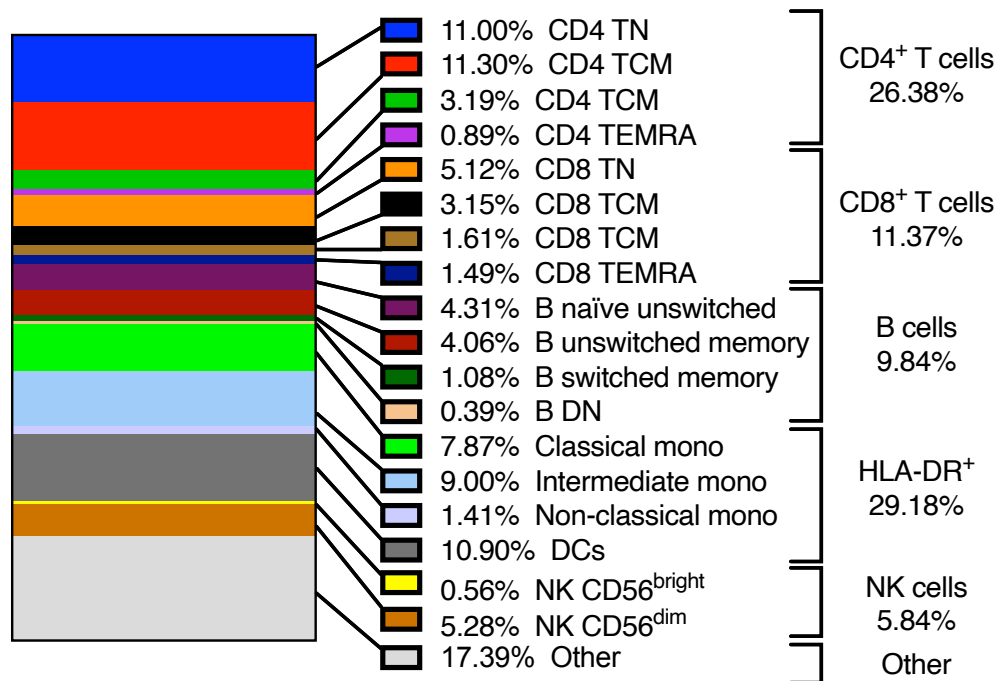


Figure 5.2 Distribution of peripheral immune subsets in healthy NHS-BT human blood

Proportion of peripheral immune cells expressed as a percentage of live cells as an average of n=8 NHS-BT healthy donors. Subsets are defined as in Table 5.3.

5.2.1.1 T cells

CD4⁺ and CD8⁺ T cell memory subsets can be characterised as naïve (TN, CD27⁺CD45RA⁺), central memory (TCM, CD27⁺CD45RA⁻), effector memory (TEM, CD27⁻CD45RA⁻), and effector memory re-expressing CD45RA (TEMRA, CD27⁻CD45RA⁺). Using the gating strategy above (Figure 5.1), I assessed whether the CD4⁺ and CD8⁺ T cell age-associated subsets, the TEMRA cells, differentially expressed markers associated with T cell ageing in healthy NHS-BT donors.

CD28⁻ T cells expand with ageing (Fagnoni et al., 1996). Supporting the hypothesis that this expansion of CD28⁻ T cells occurs in the TEMRA subset, analysis of blood from healthy NHS-BT donors showed that the percentage of CD28⁺ cells was significantly lower in the TEMRA compared to naïve subset, in both CD4⁺ and CD8⁺ T cells (Figure 5.3A). CD38 is an NAD-dependent ecto-enzyme that has been reported to be induced on macrophages by surrounding senescent cells, leading to age-related tissue NAD⁺ deficiency (Chini et al., 2020, Covarrubias et al., 2020). Examination of T cell subsets in healthy NHS-BT blood revealed that CD38 positivity appeared to decline in central and effector memory cells, but returned in TEMRA to similar levels as in naïve T cells (Figure 5.3B). Consistent with the literature and my earlier findings (Figure 4.1C—D), TEMRA cells displayed higher proportions of CD57⁺ and KLRG1⁺ T cells compared to naïve cells, especially in the CD8⁺ subset (Figure 5.3C—D). PD-1 and LAG3 are T cell exhaustion markers (Table 5.2). PD-1 positivity was significantly enriched in TEMRA compared to naïve T cells (Figure 5.3E); while LAG3 positivity decreased in both central and effector memory T cells, but returned to the same levels as naïve cells in TEMRA cells (Figure 5.3F).

Senescent CD8⁺ T cells acquire an NK-like phenotype whereby cells express high levels of NK receptors (NKR) such as NKG2D and NKG2A (Pereira et al., 2020). Combined with high expression of the adaptor protein, DAP12, this gain of NKR expression drives an MHC-unrestricted cytotoxic phenotype in senescent T cells with age (section 1.3.3) (Pereira et

al., 2020). Both NKG2D⁺ and NKG2A⁺ cells were significantly less enriched in TEMRA compared to naïve T cells, but higher than central and effector memory cells, consistent with an induction of an NK-like phenotype with T cell differentiation (Figure 5.3G—H). Overall, age-associated TEMRA cells displayed several cell-surface markers consistent with T cell ageing.

To determine the cellular senescence phenotype of each T cell memory subset, I measured p16^{Ink4a}, p21^{Cip1/Waf1}, p53, γH2AX, p-S6, and p-Akt fluorescence signal intensities in CD27/45RA-gated subpopulations from healthy NHS-BT donors. Both CD4⁺ and CD8⁺ TEMRA cells displayed significantly increased levels of p16, p21, and p53, compared to naïve cells (Figure 5.4A—C). As observed in Chapter 4 (Figure 4.1E), CD4⁺ TEMRA cells also displayed significantly higher levels of the DNA damage marker, γH2AX, compared to naïve cells, whereas CD8⁺ TEMRA cells did not (Figure 5.4D). As a measure of mTORC1 downstream activity, p-S6 levels were higher in TEMRA cells compared to naïve cells, in both CD4⁺ and CD8⁺ T cells (Figure 5.4E). Levels of p-Akt, a readout for mTORC2 activity, were also higher in CD4⁺ TEMRA cells, but lower in CD8⁺ TEMRA cells, compared to their naïve counterparts (Figure 5.4F). Altogether, these data suggest that both CD4⁺ and CD8⁺ senescent T cells displayed classic biomarkers of senescence, such as high cyclin kinase inhibitor expression, DNA damage response activation, and mTOR hyperactivity.

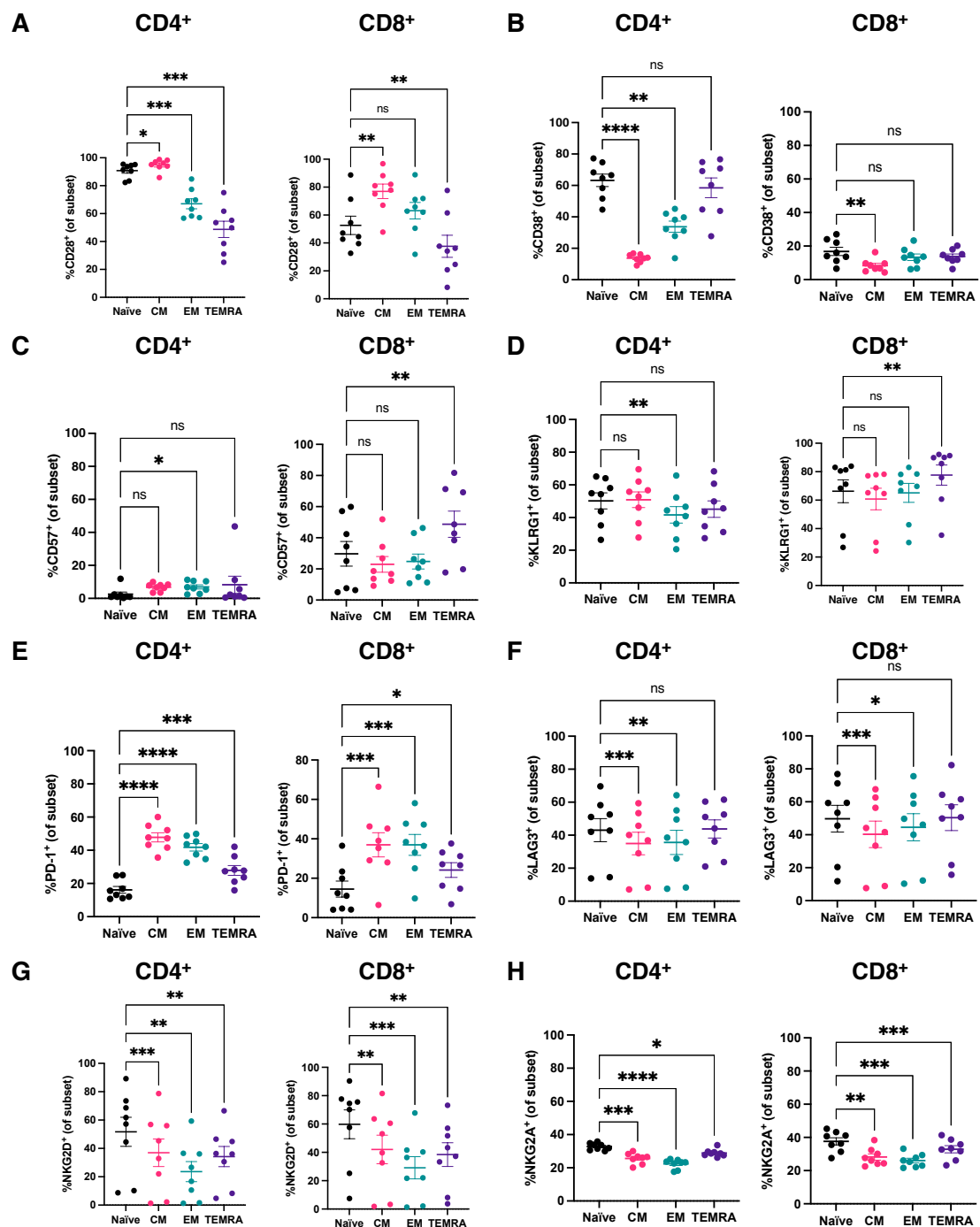


Figure 5.3 Cell-surface marker expression on age-associated T cell subsets from healthy NHS-BT donors

(A–H) Surface marker positivity in CD4⁺ (left) and CD8⁺ (right) T cell memory subsets from healthy donors, assessed by spectral flow cytometry: (A) CD28, (B) CD38, (C) CD57, (D) KLRG1, (E) PD-1, (F) LAG3, (G) NKG2D, and (H) NKG2A. T cell subsets are defined by CD27 and CD45RA as: naïve, CD27⁺CD45RA⁺; CM, central memory, CD27⁺CD45RA⁺; EM, effector memory, CD27⁺CD45RA⁺; TEMRA, effector memory re-expressing CD45RA, CD27⁺CD45RA⁺. *P*-values are derived from a one-way ANOVA with comparisons between each subset and its naïve counterpart calculated using Dunnett's multiple comparisons test, *n*=8 healthy donors.

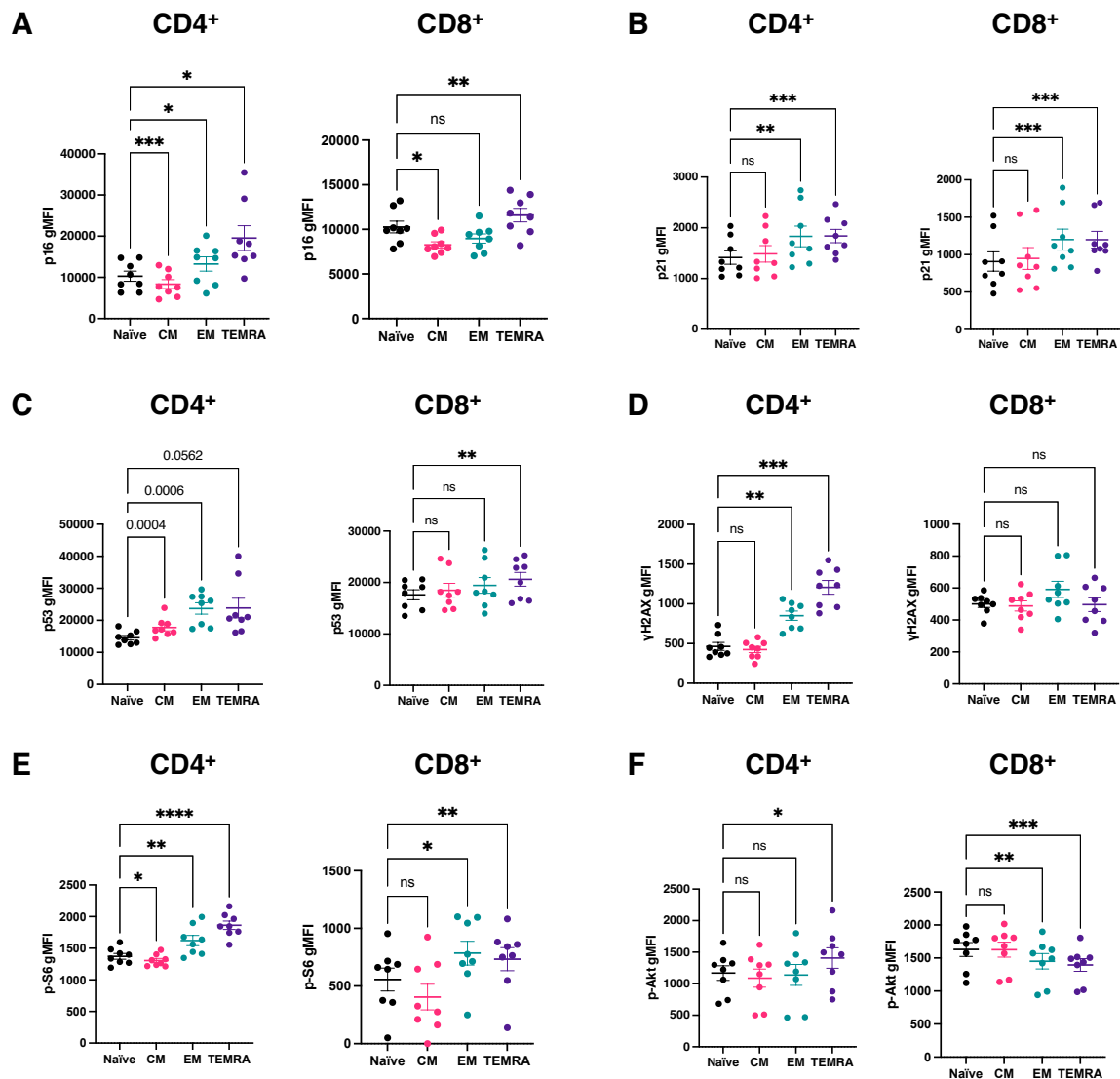


Figure 5.4 Senescence and mTOR activity in age-associated T cell subsets from healthy NHS-BT donors

(A–F) The geometric mean fluorescence intensity (gMFI) of senescence and mTOR activity markers in CD4⁺ (left) and CD8⁺ (right) T cell memory subsets in healthy donors, assessed by spectral flow cytometry: (A) p16, (B) p21, (C) p53, (D) γH2AX, (E) p-S6, (F) p-Akt. T cell subsets are defined by CD27 and CD45RA as following: naïve, CD27⁺CD45RA⁺; CM, central memory, CD27⁺CD45RA⁺; EM, effector memory, CD27⁺CD45RA⁺; TEMRA, effector memory re-expressing CD45RA, CD27⁺CD45RA⁺. *P*-values are derived from a one-way ANOVA with comparisons between each subset and its naïve counterpart calculated using Dunnett's multiple comparisons test, n=8 healthy donors.

5.2.1.2 B cells

Aged B cells secrete autoantibodies, are pro-inflammatory, and are less able to elicit an adequate humoral response to immune challenge (Frasca, 2018). The major subsets of CD19⁺ B cells in peripheral blood can be determined by their cell-surface expression of CD27 and IgD (the naïve B cell receptor) (Figure 5.1) as: CD27⁺IgD⁺ (naïve unswitched), CD27⁺IgD⁺ (memory unswitched), CD27⁺IgD⁻ (memory switched), and CD27⁻IgD⁻ (double negative, DN, also termed 'late memory' B cells) (Frasca et al., 2017, Frasca, 2018). Ageing is accompanied by a decrease in the proportion of switched memory B cells and concomitant increases in both naïve and DN proportions increase in peripheral blood (Frasca et al., 2017). Since DN B cells express high levels of *CDKN2A* mRNA (encoding p16^{Ink4a}) and inflammatory cytokines, indicating a senescence-like phenotype, they may be considered an age-associated, senescent B cell subset (Frasca et al., 2017). In healthy NHS-BT donors, flow cytometry-gated DN B cells (Figure 5.1) showed higher fluorescence intensity signals of the cyclin kinase inhibitor, p21 (Figure 5.5B), but not p16 (Figure 5.5A), compared to naïve unswitched B cells. Furthermore, DN B cells exhibited higher levels of p53 and γH2AX than naïve B cells, indicating high DNA damage levels and activation of the DDR (Figure 5.5C—D). However, unlike senescent CD4⁺ T cells, these senescent B cells did not show any differences in p-S6 and p-Akt levels, suggesting that mTOR activity may not be dysregulated in B cell senescence (Figure 5.5E—F).

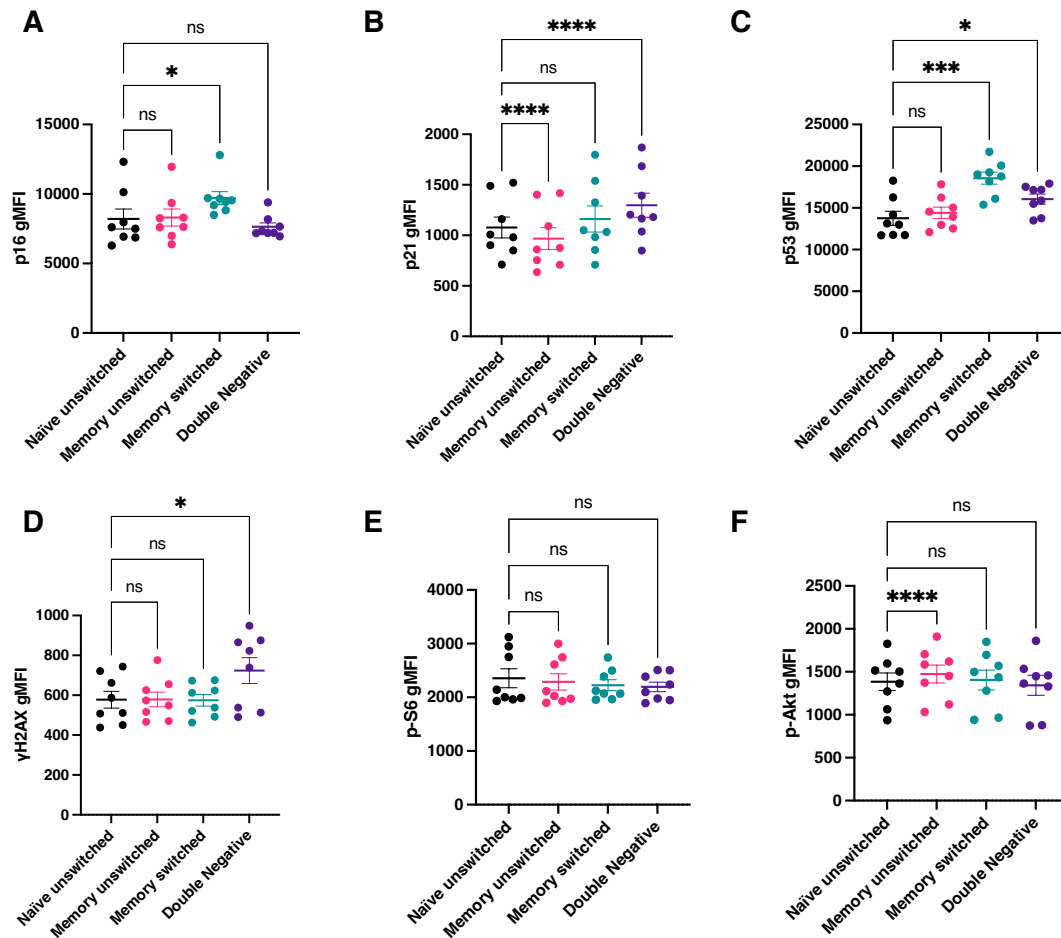


Figure 5.5 Features of senescence in age-associated B cell subsets from healthy NHS-BT donors

(A–F) The geometric mean fluorescence intensity (gMFI) of senescence and mTOR activity markers in B cell memory subsets from healthy NHS-BT donors, assessed by spectral flow cytometry: (A) p16, (B) p21, (C) p53, (D) γH2AX, (E) p-S6, (F) p-Akt. B cell subsets are defined by CD27 and IgD as the following: CD27⁺IgD⁺ (naïve unswitched), CD27⁺IgD⁺ (memory unswitched), CD27⁺IgD[−] (memory switched), and CD27[−]IgD[−] (double negative, DN). *P*-values are derived from a one-way ANOVA with comparisons between each subset and its naïve counterpart calculated using Dunnett's multiple comparisons test, *n*=8 healthy donors.

5.2.1.3 Natural Killer cells

Natural Killer (NK) cells are innate lymphoid cells contributing to the first lines of defence against virus-infected and transformed cells. NK cells, defined as CD3⁺CD56⁺ lymphocytes, are further classed as CD56^{bright} or CD56^{dim} cells which have distinct effector functions (Figure 5.1) (Hazeldine and Lord, 2013). Whereas CD56^{bright} NK cells predominantly serve

roles in cytokine secretion, CD56^{dim} cells tend to express CD16 (FcγRIII) and CD57, a terminal differentiation marker, and perform antibody-dependent cellular cytotoxicity (ADCC) (Brauning et al., 2022). With age, both the absolute number and proportion of CD56^{dim} NK cells increase, with a concomitant decrease in CD56^{bright} NK cells (Hazeldine and Lord, 2013). Thus, CD56^{dim}CD57⁺CD16⁺ (double positive) cells may represent an age-associated NK cell subset, though CD16 and CD57 can also be expressed in CD56^{bright} NK cells (Figure 5.1). In healthy NHS-BT donors, the fluorescence signal intensities of p16, p21, and p53 were all significantly higher in CD57⁺CD16⁺ (double positive) NK cell populations compared to their CD57⁻CD16⁻ (double negative) counterparts (Figure 5.6A—C), supporting the hypothesis that these age-associated subsets display features of cellular senescence. Interestingly, γH2AX decreased in the CD57⁺CD16⁺ NK cells, both in the CD56^{bright} and CD56^{dim} subsets, and this co-varied with decreased p-S6 and p-Akt in CD56^{dim} cells (Figure 5.6D—F). No differences in p-S6 were observed between the CD56^{bright} subsets, though p-Akt was increased in CD57⁻CD16⁺ CD56^{bright} cells compared to their double negative counterparts. Overall, these data suggest that age-associated NK cell subsets display classical senescence biomarkers, though, unlike other age-associated subsets, display low levels of DNA damage and mTOR activity.

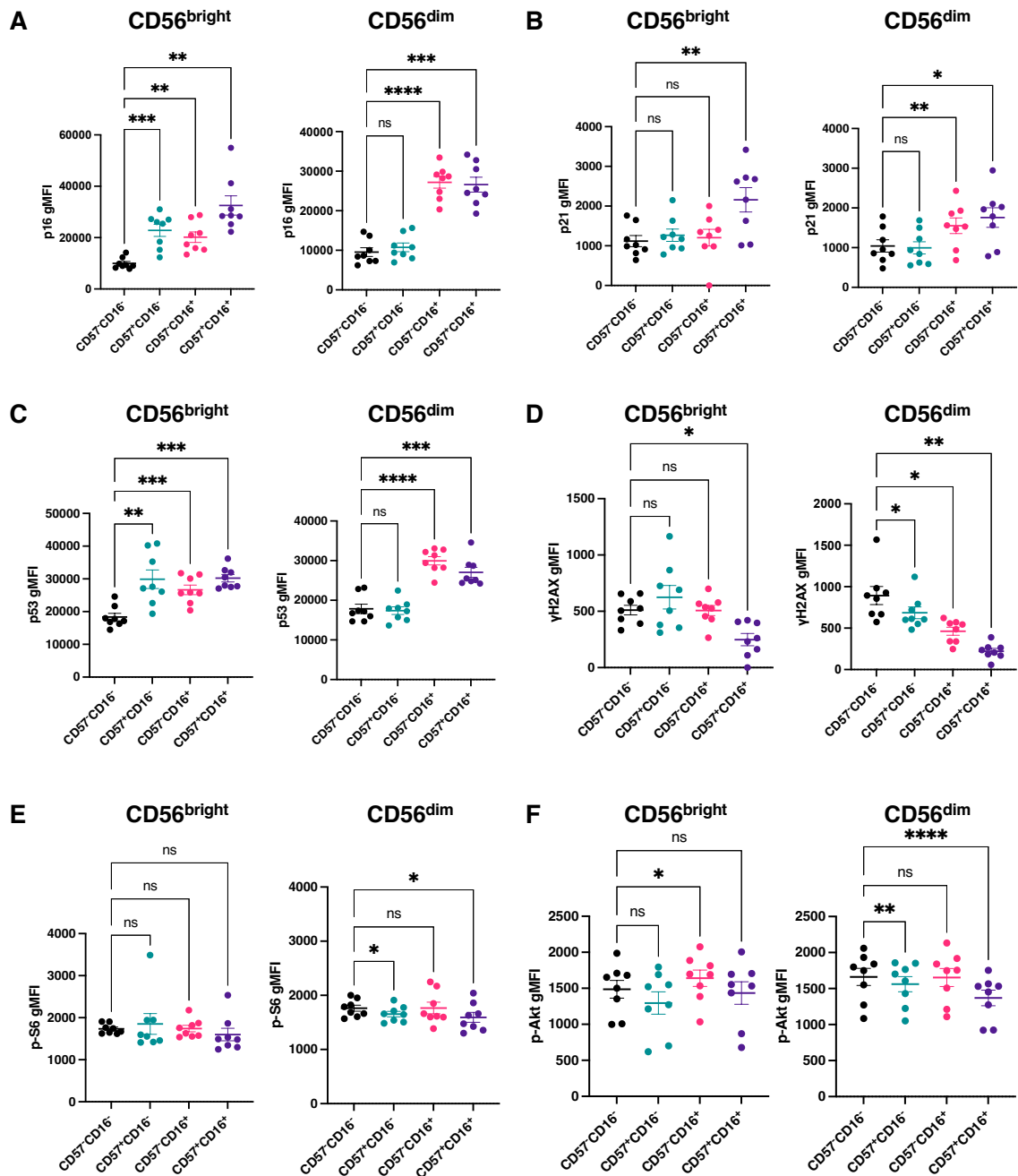


Figure 5.6 Features of senescence in age-associated NK cell subsets from healthy NHS-BT donors

(A–F) The geometric mean fluorescence intensity (gMFI) of senescence and mTOR activity markers in NK cell subsets in healthy donors, assessed by spectral flow cytometry: (A) p16, (B) p21, (C) p53, (D) γ H2AX, (E) p-S6, (F) p-Akt. NK cell subsets are defined first as CD56^{bright} (left) and CD56^{dim} (right), and then based on CD57 and CD16 expression. *P*-values are derived from a one-way ANOVA with comparisons between each subset and its CD57-CD16 counterpart calculated using Dunnett's multiple comparisons test, *n*=8 healthy donors.

5.2.1.4 Monocytes

Human monocytes are innate myeloid cells that patrol the body's vasculature, extravasating towards sites of inflammation. Defined as HLA-DR⁺ cells, monocyte subsets are further characterised by their differential cell-surface expression of CD14 and CD16 as classical (CD14⁺CD16⁻), intermediate (CD14⁺CD16⁺), and non-classical (CD14⁻CD16⁺) (Figure 5.1) (De Maeyer and Chambers, 2021). Human ageing is accompanied by increases in the proportion both of circulating intermediate and non-classical monocytes, and a decline in the proportion of classical monocytes (Nyugen et al., 2010, Hearps et al., 2012). Non-classical monocytes may therefore be considered an age-associated immune population, though it is not known whether they also display features of cellular senescence (De Maeyer and Chambers, 2021).

Gating on monocytes from healthy NHS-BT donors based on CD14/16 marker expression (Figure 5.1) and measuring the signal intensities of senescence biomarkers revealed that age-associated non-classical monocytes expressed significantly higher levels of p16, p21, and p53, than classical monocytes (Figure 5.7A—C). However, like NK cells (Figure 5.6D), their high p53 expression was not accompanied by high DNA damage as detected by γ H2AX (Figure 5.7D). Like senescent CD4⁺ and CD8⁺ TEMRA cells (Figure 5.4E), non-classical monocytes displayed high levels of p-S6 and p-Akt, indicating mTORC1/2 hyperactivity (Figure 5.7E—F). Thus, age-associated non-classical monocytes from healthy NHS-BT exhibited several features of senescence and mTOR dysregulation.

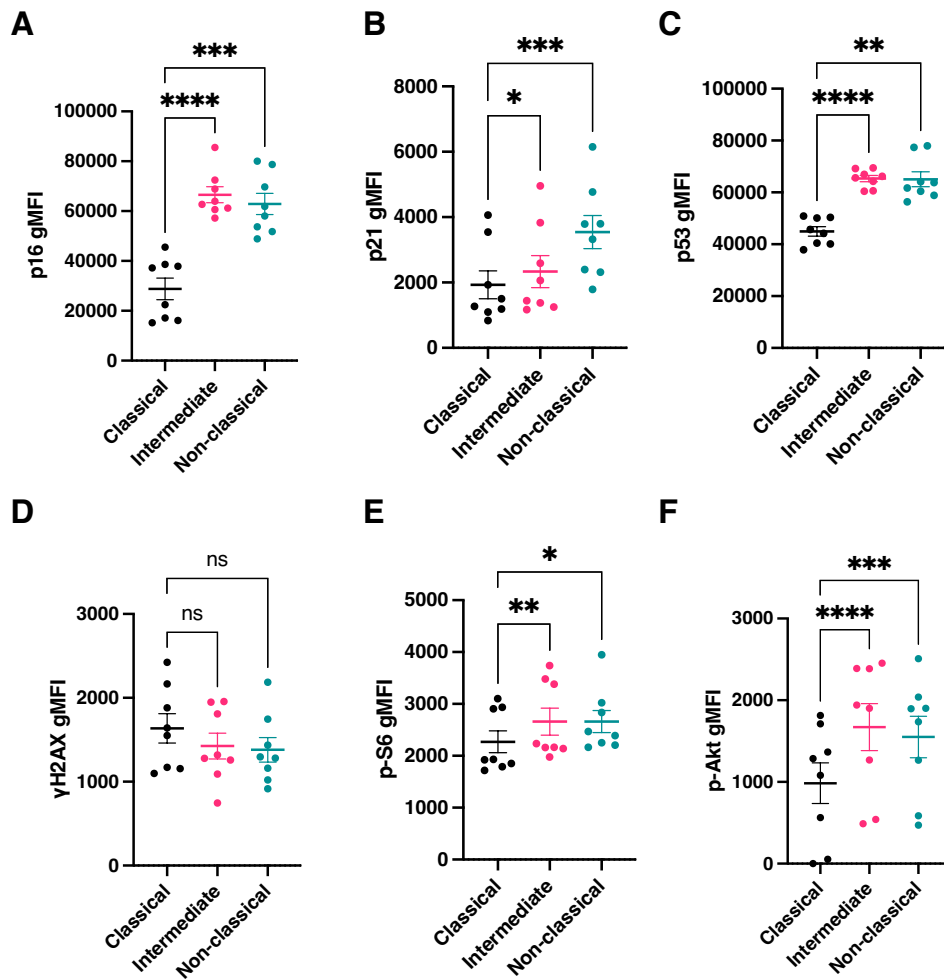


Figure 5.7 Features of senescence in age-associated monocyte subsets from healthy NHS-BT donors

(A–F) The geometric mean fluorescence intensity (gMFI) of senescence and mTOR activity markers in monocyte subsets from healthy NHS-BT donors, assessed by spectral flow cytometry: (A) p16, (B) p21, (C) p53, (D) γH2AX, (E) p-S6, (F) p-Akt. Monocyte subsets are defined by CD14 and CD16 expression as following: CD14⁺CD16⁻ (classical), CD14⁺CD16⁺ (intermediate), and CD14⁻CD16⁺ (non-classical). *P*-values are derived from a one-way ANOVA with comparisons between each subset and classical monocytes, calculated using Dunnett's multiple comparisons test, n=8 healthy donors.

5.2.1.5 Age-associated immune cell subsets are enlarged

Cellular enlargement is a reliable feature of replicative and DNA damage-induced fibroblast senescence (Figure 3.1 and Figure 3.3). Given the presence of senescence biomarkers in the age-associated immune subsets observed so far, I assessed whether age-associated immune cells were also enlarged, using flow cytometry to measure forward scatter (FSC-A), a feature directly proportional to cell size (Stern et al., 2017). As was the case with many of the senescence markers, the age-associated subsets (CD4⁺ TEMRAs, CD8⁺ TEMRAs, IgD⁻CD27⁻ (DN) B cells, CD57⁺CD16⁺CD56^{bright} (but not CD56^{dim}) NK cells, and CD14⁻CD16⁺ non-classical monocytes) all exhibited a significant increase in mean FSC-A, compared to their naïve, early-stage-differentiated counterparts (Figure 5.8). Taken together, this indicates that cellular enlargement is also a feature of most age-associated immune cell subsets from healthy NHS-BT donors.

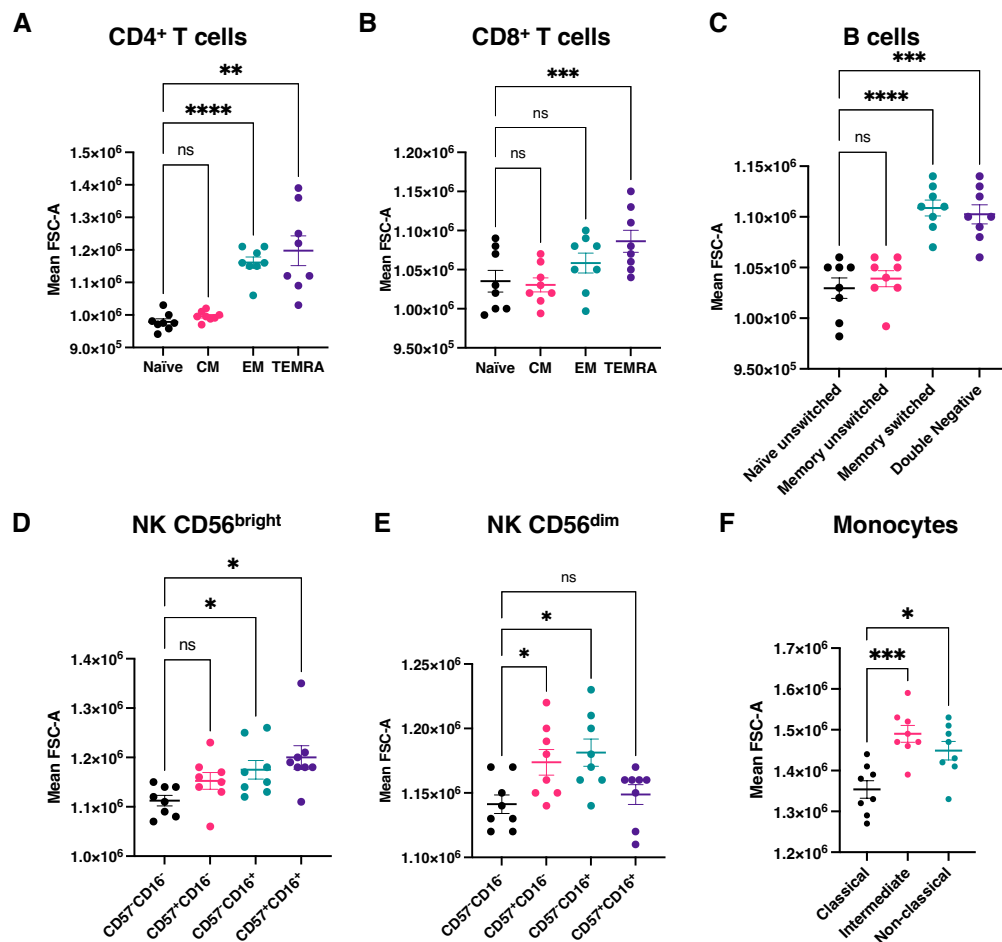


Figure 5.8 Age-associated immune subsets from healthy NHS-BT donors display cellular enlargement

Forward scatter area (FSC-A) mean fluorescence intensities in immune cell subsets, assessed by spectral flow cytometry. *P*-values are derived from a one-way ANOVA with comparisons between each subset and their early-differentiated counterparts, calculated using Dunnett's multiple comparisons test, *n*=8 healthy donors.

Overall, age-associated peripheral immune cell subsets exhibited increased levels (as measured by mean fluorescence intensity) of many senescence biomarkers compared to their naïve counterparts, encompassing increased cyclin kinase inhibitor expression, DNA damage, mTOR hyperactivation, and cellular enlargement. These results are summarised in Table 5.4. In addition to increased fluorescence intensities of these senescence biomarkers (γH2AX, p16, p53, and p21) in age-associated immune subsets (Table 5.4), these subsets also possessed an increased percentage of cells positive for senescence markers in both the adaptive and innate cell types (Supplementary Figures 7.1 and 7.2). All

age-associated subsets possessed at least 3/7 markers of senescence and/or mTOR activity when compared to their early-differentiated counterparts (Table 5.4), though the exact set of biomarkers expressed was unique to each age-associated subset. For example, whereas DN B cells showed high p21, p53, γ H2AX, and FSC, age-associated CD57⁺CD16⁺ NK cells expressed high p21 and p53, and p16, but not γ H2AX and FSC (Table 5.4). Notably, CD4⁺ TEMRAs and non-classical monocytes displayed the greatest number of senescence and mTOR activity biomarkers (6/7). Notably, heightened expression of cyclin kinase inhibitor p21 was a senescence marker shared by 6/6 of the age-associated immune subsets assessed, with p53, p16, and enlargement (measured by high FSC) shared by 5/6 subsets (Table 5.4). The high co-expression of both p53 and p21 in age-associated immune subsets, and the fact that p53 acts to induce p21 in response to DNA damage to induce senescence (Figure 1.2), provides evidence that the p53-p21 pathway could be a universal immune cell senescence mechanism.

Table 5.4 Senescence biomarkers in age-associated immune subsets from healthy NHS-BT donors

Marker expression higher in age-associated subset?	CD4 ⁺ TEMRA vs CD4 ⁺ Naïve	CD8 ⁺ TEMRA vs CD8 ⁺ Naïve	B DN vs B naïve unswitched	NK CD56 ^{bright} CD57 ⁺ CD16 ⁺ vs NK CD56 ^{bright} CD57 ⁻ CD16 ⁻	NK CD56 ^{dim} CD57 ⁺ CD16 ⁺ vs NK CD56 ^{dim} CD57 ⁻ CD16 ⁻	Non-classical monocytes vs Classical monocytes
↑ p16	✓	✓	✗	✓	✓	✓
↑ p21	✓	✓	✓	✓	✓	✓
↑ p53	✗	✓	✓	✓	✓	✓
↑ γ H2AX	✓	✗	✓	✗	✗	✗
↑ p-S6	✓	✓	✗	✗	✗	✓
↑ p-Akt	✓	✗	✗	✗	✗	✓
↑ FSC-A	✓	✓	✓	✓	✗	✓

Shaded cell indicates that senescence biomarker is significantly increased in age-associated subset

5.2.2 Identifying immune cell senescence in healthy older adults and patients with osteoarthritis (age-related disease)

So far, immune cell senescence was analysed in age-associated immune subsets from healthy NHS-BT donors. However, it is unclear how these senescence features are altered

during ageing or an age-related disease. To test this, I used 27-colour spectral flow cytometry to assess senescence features in PBMCs both from old individuals, and individuals with osteoarthritis (OA) undergoing joint replacement surgery, compared to healthy subjects. The donor information of this pooled cohort is summarised in Table 5.5. OA is an age-related disease characterised by progressive articular cartilage destruction and is strongly associated with heightened inflammageing (Loeser et al., 2016). Increased cellular senescence in the joint is thought to play a role in disease pathogenesis, though the role of the peripheral immune system in the disease is less well understood (Jeon et al., 2017). In the cohort of individuals in Table 5.5, OA was also strongly associated with obesity (BMI > 30), which is also reported to drive immunosenescence (Shirakawa et al., 2016).

Table 5.5 Summary information of old and/or osteoarthritic cohort

Donor	Sex	Age (years) (mean $\pm$ SD)	BMI (kg/m²) (mean $\pm$ SD)	Total
Old OA	Male	63.2 $\pm$ 14.5	33.9 $\pm$ 13.6	5
	Female	57.3 $\pm$ 1.15	34.0 $\pm$ 6.70	3
Old no OA	Male	75	22.4	1
	Female	78	25.9	1
Total		64.1 $\pm$ 12.0	32.0 $\pm$ 10.5	10

BMI, body mass index; SD, standard deviation.

First, I tested whether cell-surface features of T cell ageing were altered in this combined older and OA cohort. Compared to a control population of healthy subjects, this cohort had significantly lower proportions of CD28⁺ CD8⁺ T cells (Figure 5.9A), CD38⁺ CD4⁺ T cells (Figure 5.9B), and NKG2A⁺ CD4⁺ and CD8⁺ T cells (Figure 5.9H), and a significantly higher proportion of terminally differentiated CD57⁺ CD8⁺ T cells (Figure 5.9C). There were no differences in the proportions of KLRG1⁺, PD-1⁺, LAG3⁺, or NKG2D⁺ T cells (Figure 5.9D—G). Overall, this older and OA cohort displayed a number of features of T cell ageing reminiscent of features of age-associated TEMRA cells from healthy NHS-BT donors studied earlier (Figure 5.3).

To assess whether cellular senescence and mTOR activity were changed between this older and/or osteoarthritic cohort, I measured the mean fluorescence intensities of senescence biomarkers in the pan-immune cell subsets and assessed the fold change from healthy controls (Figure 5.10). Both p16 and p53 were significantly elevated in CD8⁺ T cells of old and OA patients compared to controls, and γ H2AX, a marker for DNA damage, was higher in B cells (Figure 5.10). p-S6, a readout for mTORC1 activity, was increased in 3 out of 4 cell populations (though p-S6 in CD8⁺ T cells was almost significantly higher, $p=0.0577$). Taken together, these data suggest that this cohort of older and OA individuals exhibit features of cellular senescence, particularly in CD8⁺ T cells and B cells. Furthermore, mTORC1 hyperactivity, as measured by p-S6, was apparent in most immune cells in this immunosenescent population.

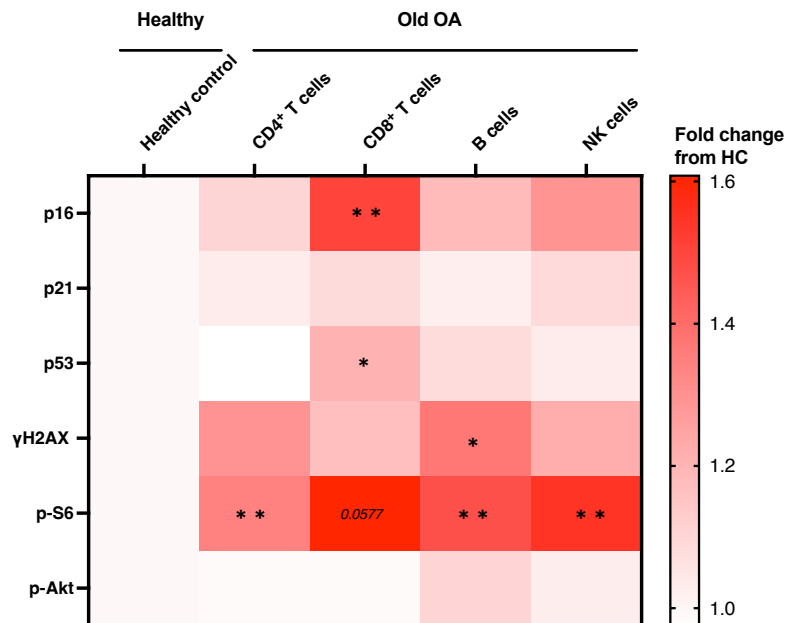


Figure 5.10 Assessing immune cell senescence in older and osteoarthritic patients

The geometric mean fluorescence intensity (gMFI) of senescence and mTOR activity markers in defined immune cell types in old and osteoarthritic donors (n=10), assessed by spectral flow cytometry, presented as fold change from the average value in healthy controls (HC) including those from the NHS blood transfusion (NHS-BT) service (n=10). *P*-values for statistical significance are indicated and are derived from an unpaired t-test between old & OA and HC for each cell type.

5.2.3 Impact of *in vivo* rapamycin treatment in older men on peripheral immunosenescence features in the Rapamune Trial

In Chapter 3, I investigated how mTOR inhibitors attenuated phenotypes of senescence in DNA damage-induced senescence in fibroblasts. In Chapter 4, I developed an assay to induce and measure DNA damage in human immune cells, and found that mTOR inhibition limited the induction of DNA lesions and the DDR. In this chapter so far, I have found that age-associated immune subsets exhibit a number of senescence biomarkers and increased levels of mTORC1 and mTORC2 activity, and that several of these were exacerbated in both healthy older individuals and patients with the age-related disease, osteoarthritis – i.e., a cohort of individuals who are likely to experience immunosenescence.

In the next section, I investigated the effects of *in vivo* rapamycin treatment of older men in a small clinical study named the Rapamune Trial (NCT05414292), undertaken at the University of Nottingham. In this ongoing single-blind, placebo-controlled trial, older men were randomised and received either 1 mg/day rapamycin ($n=4$) or placebo ($n=5$) for 4 months. The primary outcome of the trial was assessment of aged muscle in response to unilateral lower limb resistance exercise training (RET). Additionally, venous blood was drawn at five time points (weeks 0, 2, 4, 8, 16), and PBMCs were isolated (Figure 5.11). Baseline characteristics of the study participants are summarised in Table 5.6.

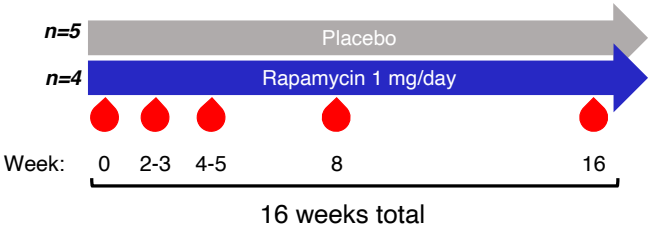


Figure 5.11 The Rapamune Trial: a small human trial for rapamycin treatment in older males

Older male adults were randomised to two groups and received either rapamycin (1 mg/day) or placebo, and peripheral blood was drawn at five timepoints (including baseline) across the 4-month intervention. PBMCs were isolated and analysed for features of immunosenescence using 27-colour spectral flow cytometry.

Table 5.6 Baseline information of participants in the Rapamune trial

	Placebo ($n=5$)	Rapamycin ($n=4$)	p-value
Age (years)	64.2 ± 5.34	60.0 ± 4.62	0.1667 (ns)
BMI (kg/m ²)	26.4 ± 3.31	27.1 ± 1.24	>0.9999 (ns)

Data are presented as mean ± SD. *P*-values are derived from Mann-Whitney tests. ns = not significant.

5.2.3.1 Increased immunosenescence and *mTOR* activity in older men at baseline

To evaluate whether features of immunosenescence were apparent in this older male cohort at baseline as compared to healthy NHS-BT controls, PBMCs were assessed using

27-colour spectral flow cytometry. Like the older and osteoarthritic cohort (Figure 5.9A,H), trial participants at baseline had a significantly lower percentage of CD28⁺ CD8⁺ T cells (Figure 5.12A), consistent with advanced age, and NKG2A⁺ CD4⁺ T cells (Figure 5.12H), compared to the healthy controls. Furthermore, the participants had a higher proportion of terminally differentiated CD57⁺ and KLRG1⁺ CD8⁺ T cells (Figure 5.12C—D). However, participants had lower baseline levels of PD-1⁺ CD4⁺ T cells, and higher LAG3⁺ CD8⁺ T cells (Figure 5.12E—F), than healthy controls, indicating changes in immune exhaustion that were not observed in the older and osteoarthritic donors (Figure 5.9E—F).

To test whether the participants of the Rapamune trial at baseline displayed increased levels of immune cell senescence compared to healthy NHS-BT donors, I measured the mean fluorescence intensities of senescence biomarkers across the immune subsets. Consistent with increased senescence, p16 mean fluorescence intensity was higher in total CD8⁺ T cells (like the older/OA cohort, Figure 5.10) and also in NK cells, compared to healthy NHS-BT controls (Figure 5.13). Additionally, like the older and osteoarthritic donors (Figure 5.10), p-S6 was significantly increased in all immune cell types compared to healthy NHS-BT controls, suggesting mTORC1 hyperactivity (Figure 5.13). Thus, like the older/OA cohort (Figure 5.10), these older male trial participants exhibited a wide range of immunosenescence features at baseline, both in terms of cell surface markers, cell senescence biomarkers, and mTORC1 hyperactivation (Figure 5.13).

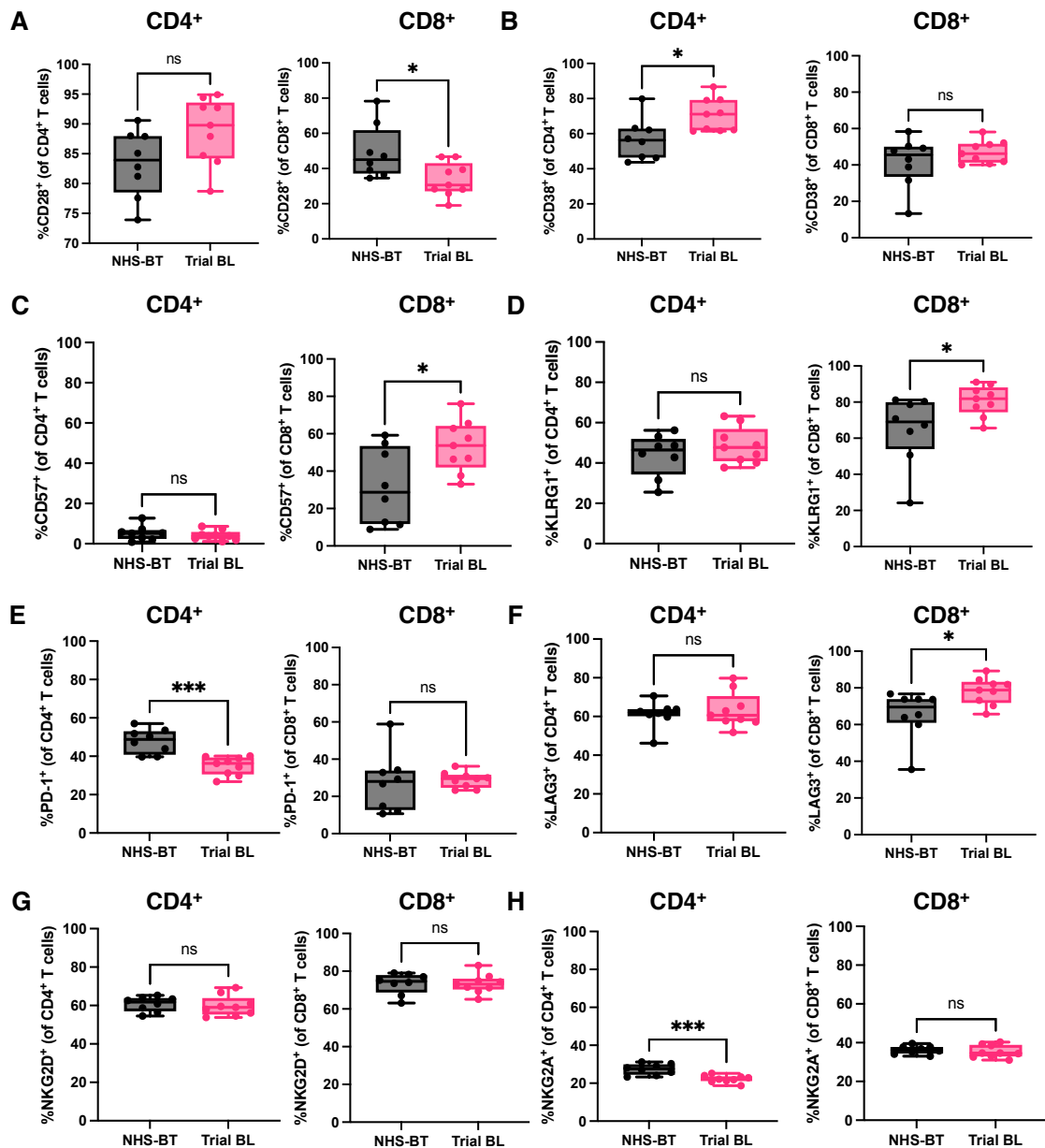


Figure 5.12 Cell-surface features of immunosenescence at Rapamune trial baseline compared to healthy NHS-BT donors

(A–H) The expression of cell-surface markers in CD4⁺ (left) and CD8⁺ (right) T cells between healthy controls from NHS blood transfusion service (NHS-BT) (n=8) and older males at baseline (BL) in the Rapamune trial (n=9), assessed by spectral flow cytometry: (A) CD28, (B) CD38, (C) CD57, (D) KLRG1, (E) PD-1, (F) LAG3, (G) NKG2D, (H) NKG2A. *P*-values are derived from an unpaired *t*-test.

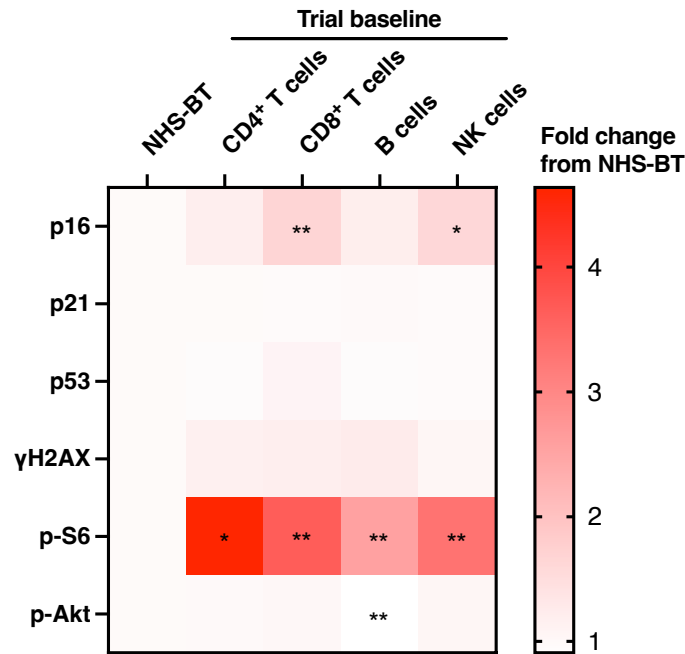


Figure 5.13 Features of senescence in immune cells at trial baseline compared to healthy NHS-BT donors

The geometric mean fluorescence intensity (gMFI) of senescence and mTOR activity markers in defined immune cell types in participants of the Rapamune trial at baseline (n=9) presented as fold change from the average value from healthy NHS blood transfusion (NHS-BT) donors (n=8), assessed by spectral flow cytometry. *P*-values for statistical significance are indicated and are derived from an unpaired t-test between trial baseline and NHS-BT controls.

5.2.3.2 Concentration of rapamycin and leukocytes in blood of participants

After 8 weeks of intervention, the concentration of rapamycin in the blood of participants was assessed. The mean blood concentration of rapamycin was 3.2 ± 1.8 nM (range 1.5 – 5.8) in the treatment group but undetectable in the blood of placebo-treated participants (Figure 5.14A). A similar study reported that 1 mg/day rapamycin led to a slightly higher mean blood rapamycin concentrations of 7.88 nM (range 5.14 – 12.91) (Kraig et al., 2018). To address concerns of immunosuppression by rapamycin, the white blood cell count was assessed at 8 weeks; like in the placebo group, there were no differences in the concentration of leukocytes in the blood after 8-week rapamycin treatment, suggesting that this low-dose rapamycin treatment regimen was not immunosuppressive (Figure 5.14B).

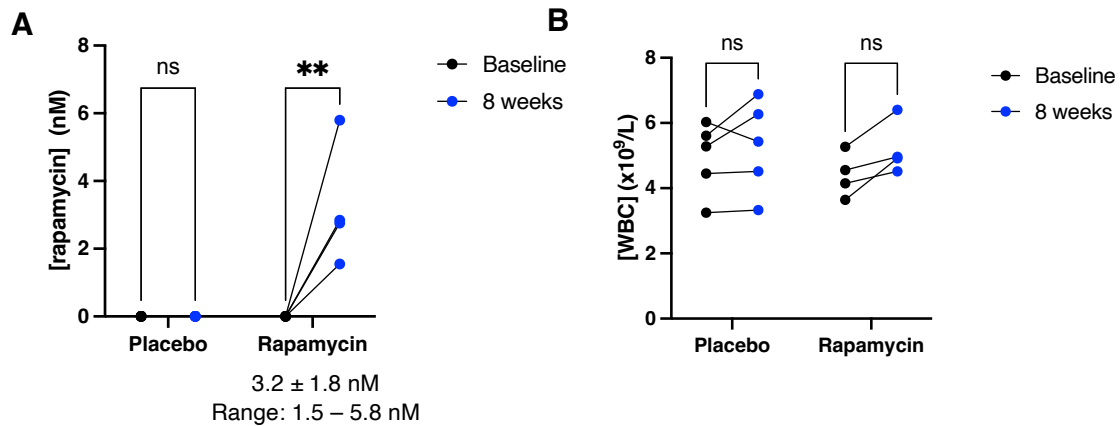


Figure 5.14 Concentration of rapamycin and leukocytes in the blood of trial participants after 8 weeks of intervention

Blood concentrations of (A) rapamycin (mean ± standard deviation and range indicated) and (B) white blood cells (WBC) in participants in the rapamycin (n=4) and placebo (n=5) groups at baseline and 8 weeks of intervention. Data are provided by Dr Eleanor Jones, Prof. Daniel Wilkinson, and Prof. Philip Atherton. *P*-values are derived from a two-way ANOVA with Šídák's multiple comparisons test.

5.2.3.3 Effects of rapamycin on features of immunosenescence

5.2.3.3.1 mTOR activity in the Rapamune trial

To evaluate whether *in vivo* rapamycin treatment affected mTOR activity in peripheral immune cells, I used spectral flow cytometry to measure the mean fluorescence intensities of phosphorylation targets for mTORC1 (p-S6) and mTORC2 activity (p-Akt) in immune subsets from PBMCs isolated across the four timepoints, testing for changes between treatment groups (placebo vs rapamycin) relative to their baseline values at each time point. Data from weeks 2-3 and weeks 4-5 were pooled together to improve statistical power, since some blood donations for the intermediate timepoints were provided 1 week late (e.g. on week 3 rather than week 2, or week 5 rather than week 4). Additionally, some donors only provided blood on 2 or 3 out of the total 5 timepoints, but these participants always provided blood at baseline and at the final time point (16 weeks). There was a significant fold decrease of p-S6 levels in most subsets in the rapamycin group compared to placebo at 4-5 weeks, suggesting successful inhibition of mTORC1, though no changes were

observed after this (Figure 5.15A). Interestingly, p-Akt levels were generally unchanged between placebo and rapamycin groups across the trial (Figure 5.15B). The exception to this was in CD8⁺ effector memory T cells, where there was a small but significant fold increase in p-Akt at weeks 2-3 and 8 weeks in the rapamycin group compared to placebo, which lost significance at 16 weeks (Figure 5.15B). Taken together, low-dose rapamycin treatment led to detectable increases in blood rapamycin concentration (Figure 5.14A), and changes in mTORC1 activity (p-S6 levels) in peripheral immune cells after 4-5 weeks (Figure 5.15A).

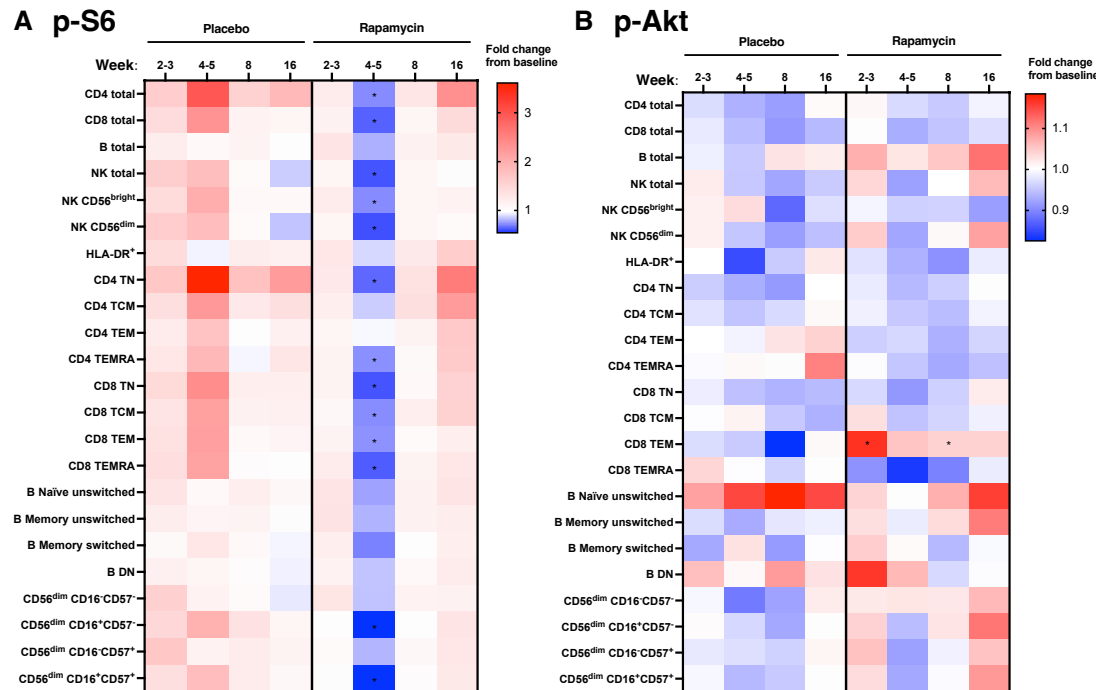


Figure 5.15 mTOR activity in immune cells in the Rapamune trial
(A–B) Geometric mean fluorescence intensity of mTOR activity markers **(A)** p-S6 and **(B)** p-Akt, across defined immune subsets in participants in rapamycin (n=4) and placebo (n=5) groups across four time points, assessed by spectral flow cytometry. Each value is expressed as fold change from baseline. *P*-values are derived from an unpaired t-test between placebo and rapamycin at each time point. Statistically significant *p*-values are indicated.

5.2.3.3.2 Features of immune cell senescence in the Rapamune trial

Features of cellular senescence are elevated in age-associated immune subsets in healthy donors (Table 5.4) and in total immune cells from donors of advanced age or with an age-

related disease (Figure 5.10). To assess whether *in vivo* rapamycin treatment impacted on features of immune cell senescence, I used spectral flow cytometry to measure the mean fluorescence intensities of p21, p16, p53, and γ H2AX in peripheral immune subsets from PBMCs isolated across the four timepoints, and assessed changes between placebo vs rapamycin groups at each time point relative to their baseline values (Figure 5.16). p21 is a cyclin kinase inhibitor and senescence biomarker I had found earlier to be consistently upregulated in age-associated immune subsets from healthy donors (Table 5.4). Interestingly, p21 expression was significantly lower in rapamycin-treated individuals compared to placebo at 16 weeks in almost all the immune subsets analysed (Figure 5.16A), suggesting that cells expressing this senescence marker may be sensitive to mTOR inhibition. By contrast to p21, the fold change in p53 expression was significantly higher in the rapamycin group compared to placebo at 16 weeks, in all cell types measured but one (Figure 5.16B). p16 levels were largely unchanged, except for in total CD8⁺ T cells (predominantly naïve), where it was higher than placebo at 16 weeks, and in CD16⁺ CD56^{dim} NK cells (both CD57⁺ and CD57⁻), where the fold increase in p16 expression was significantly lower in rapamycin than placebo groups at 4-5 weeks (Figure 5.16C). There was no change in γ H2AX levels between placebo and rapamycin arms of the study, though there appeared a mild but insignificant decrease in γ H2AX in CD4⁺ TEMRA cells from weeks 4 until the end of the trial (Figure 5.16D). Overall, *in vivo* rapamycin treatment decreased p21 but increased p53 expression, suggesting modulation of cyclin kinase signalling and potentially cellular senescence.

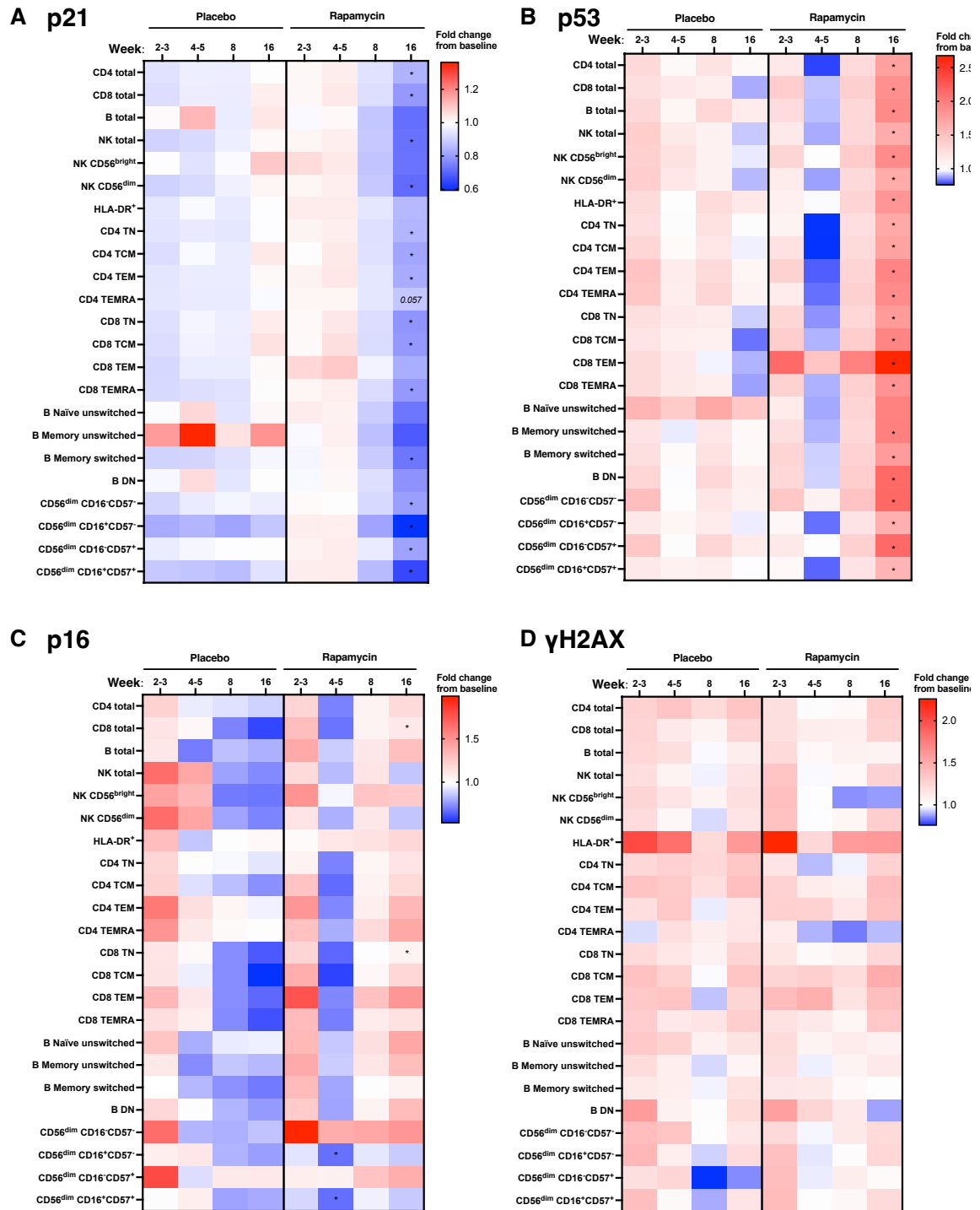


Figure 5.16 Features of immune cell senescence in the Rapamune trial between placebo and rapamycin groups

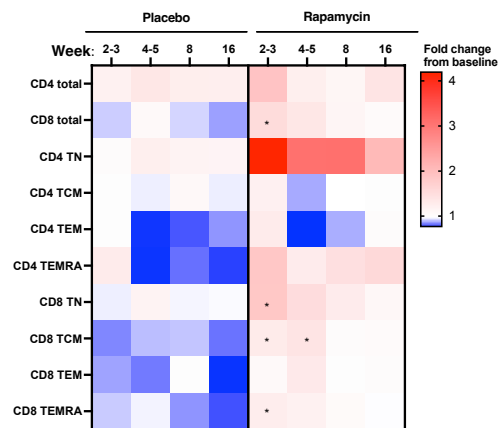
(A–D) Geometric mean fluorescence intensity of senescence markers across defined immune subsets in participants in rapamycin (n=4) and placebo (n=5) groups across four time points, assessed by spectral flow cytometry, for (A) p21, (B) p53, (C) p16, and (D) γH2AX. Each value is expressed as fold change from baseline for each participant. *P*-values are derived from an unpaired t-test between placebo and rapamycin at each time point. Statistically significant *p*-values are indicated.

5.2.3.3.3 Effect of *in vivo* rapamycin on T cell surface markers of immune ageing

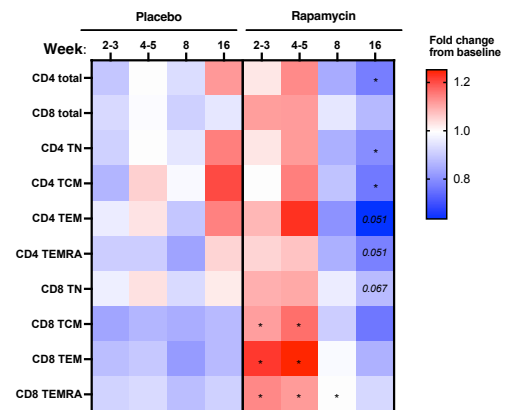
T cells from donors with advanced age or osteoarthritis (Figure 5.9), or at Rapamune trial baseline (Figure 5.12), exhibited changes in age-related cell surface markers. To assess whether cell-surface markers of T cell ageing were altered by rapamycin treatment in the Rapamune trial, I used spectral flow cytometry to measure the percentage of T cells expressing these markers associated with ageing (Figure 5.17). Interestingly, KLRG1⁺ and NKG2A⁺ CD4⁺ T cell subsets were decreased compared to baseline at 16 weeks in the rapamycin group compared to placebo, and there was a trend towards a lower proportion of LAG3⁺ CD4⁺ T cell subsets also at this time point (Figure 5.17B,D,E), suggesting effects on T cell exhaustion markers. There were no changes in PD-1⁺, NKG2D⁺, or CD28⁺ expressing T cells between placebo and rapamycin groups (Figure 5.17C,F,G). Furthermore, while the proportion of CD57⁺, KLRG1⁺, and LAG3⁺ CD8⁺ T cell subsets was increased in the rapamycin group compared to placebo early in the trial, these differences were all lost at 16 weeks (Figure 5.17A,B,D).

Overall, even in this small pilot study, *in vivo* rapamycin elicited significant changes in several features of cellular senescence and immunosenescence across multiple peripheral immune cell types. The most striking of these changes was a consistent decrease in p21 expression across most cell types (Figure 5.16A). Paradoxically, however, this was accompanied by an increase in p53 levels at this time point (Figure 5.16B). Furthermore, several changes were observed in cell-surface markers of T cell exhaustion at 16 weeks of intervention with rapamycin compared to placebo, including reduced proportions of CD4⁺ T cells positive for KLRG1 and NKG2A, and a trend towards decreased LAG3-positive cells.

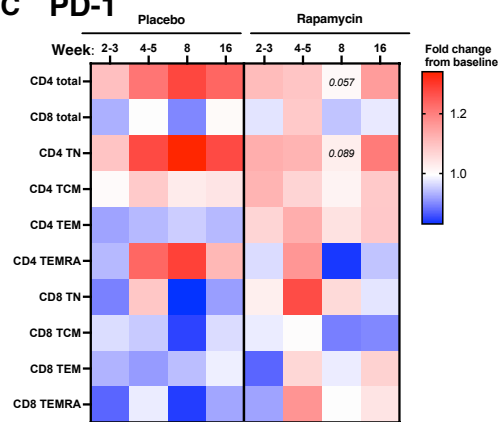
A CD57



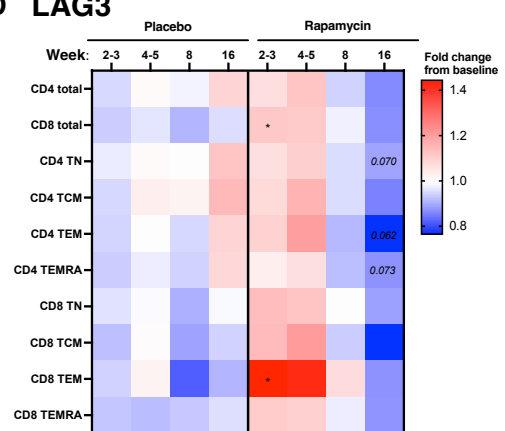
B KLRG1



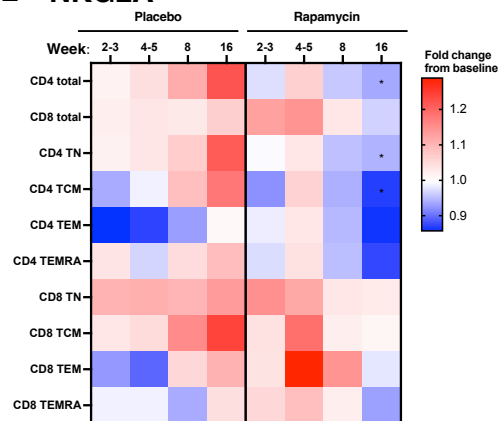
C PD-1



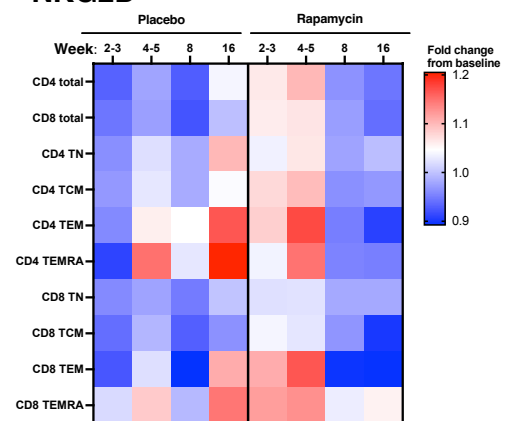
D LAG3



E NKG2A



F NKG2D



G CD28

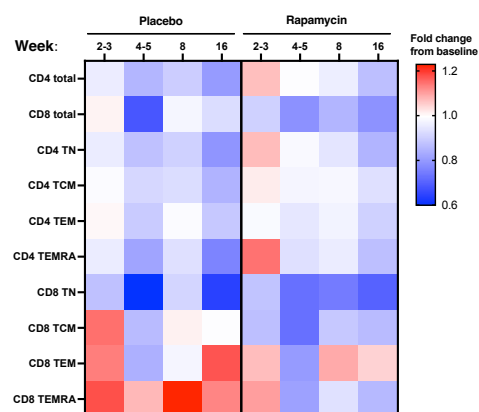


Figure 5.17 Cell-surface markers of T cells in the Rapamune Trial

(A–G) Cell surface markers across defined T cell subsets in participants in rapamycin (n=4) and placebo (n=5) groups across four time points, assessed by spectral flow cytometry: (A) CD57, (B) KLRG1, (C) PD-1, (D) LAG3, (E) NKG2A, (F) NKG2D, and (G) CD28. Each value is expressed as fold change from baseline. *P*-values are derived from an unpaired t-test between placebo and rapamycin at each time point. Statistically significant *p*-values are indicated.

5.2.4 *In vitro* validation of the Rapamune Trial

To validate the effects of rapamycin from this small clinical trial, I used the *in vitro* immune cell DNA damage assay developed in Chapter 4 (Figure 4.5). Healthy NHS-BT donor PBMCs were activated for 3 days (anti-CD3/28) and treated with 10 nM rapamycin either continuously (RRR), during (DRD), and/or after (DRR) DNA-damaging zeocin treatment, and the mean fluorescence intensities of p21, p53, and p16 were measured in T cells at both 4 and 24 hours of recovery using spectral flow cytometry (Figure 5.18A). Data are presented as heatmaps in Figure 5.18 and as individual graphs in Figure 5.19.

Zeocin treatment induced high levels of both p21 and p53 at 4 hours of recovery in CD4⁺ and CD8⁺ T cells which declined after 24 hours of recovery (Figure 5.18B–C, Figure 5.19). By contrast, zeocin treatment decreased p16 levels in both T cell subsets (Figure 5.18B–C, Figure 5.19). Consistent with the clinical trial, in which rapamycin decreased p21 levels across most cell types (Figure 5.16A), continuous rapamycin treatment (RRR) strongly suppressed levels of p21 at both 4 and 24 hours after recovery from zeocin treatment in both CD4⁺ and CD8⁺ T cells (Figure 5.18B–C, Figure 5.19). In the Rapamune trial, p53 levels increased across most cell types after 16-week rapamycin treatment (Figure 5.16B). However, *in vitro*, rapamycin restricted p53 levels in both CD4⁺ and CD8⁺ T cells, when administered continuously (RRR), during (DRD), and both during and after zeocin treatment (DRR), at both recovery time points (Figure 5.18B–C, Figure 5.19). This suggests that the induction of p53 in the clinical trial may not reflect effects on DNA damage. Finally, whereas p16 increased in CD8⁺ T cells in rapamycin compared to placebo groups in the trial (Figure

5.16C), rapamycin treated continuously (RRR) or only during zeocin treatment (DRD) decreased p16 levels at 4 hours and 24 hours recovery respectively during *in vitro* DNA damage (Figure 5.18B—C, Figure 5.19). Overall, this *in vitro* assay suggests that continuous rapamycin treatment limits both the acute and sustained increase of p21 in response to DNA damage in primary human T cells (Figure 5.18), consistent with the results from the Rapamune Trial (Figure 5.16A). The observation that *in vitro* rapamycin treatment limited DNA damage-induced p53 levels (Figure 5.18), but p53 was increased in immune cells from the rapamycin group in the trial (Figure 5.16B), may therefore reflect DNA damage-independent mechanisms that remain to be explored.

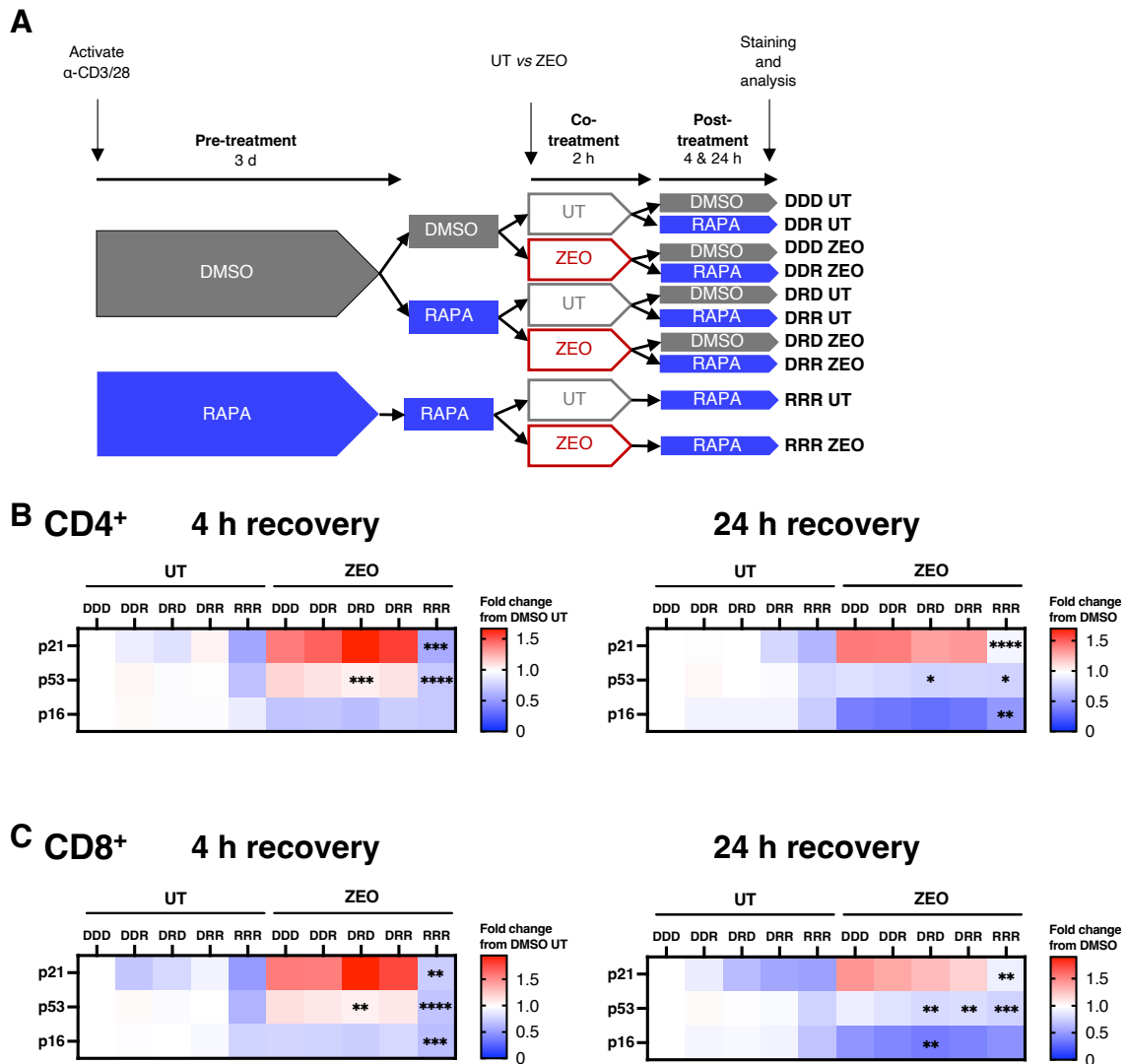


Figure 5.18 Heatmaps for *in vitro* validation of Rapamune trial results using healthy NHS-BT donor PBMCs

(A) NHS-BT PBMCs were activated for 3 days with 1 μ g/ml α -CD3/28 and afterwards treated with zeocin (ZEO, 200 μ g/ml) or untreated (UT), and recovered for 4 or 24 hours before analysis by spectral flow cytometry. Cells were treated either with 10 nM rapamycin (RAPA) or DMSO control either continuously (RRR), co- (DRD), or co- and post-treatment (DRR) with zeocin. (B–C) Geometric mean fluorescence intensity of p21, p53, and p16 in CD4⁺ (B) and CD8⁺ (C) T cells at 4 hours (left) and 24 hours (right) recovery from zeocin across different conditions. All values are expressed as fold change from the DMSO→DMSO→DMSO untreated condition for each recovery time point. All *p*-values represent comparisons with the DMSO→DMSO→DMSO zeocin (ZEO) condition and are derived from a two-way ANOVA with Dunnett's multiple comparisons test, *n*=4 healthy donors.

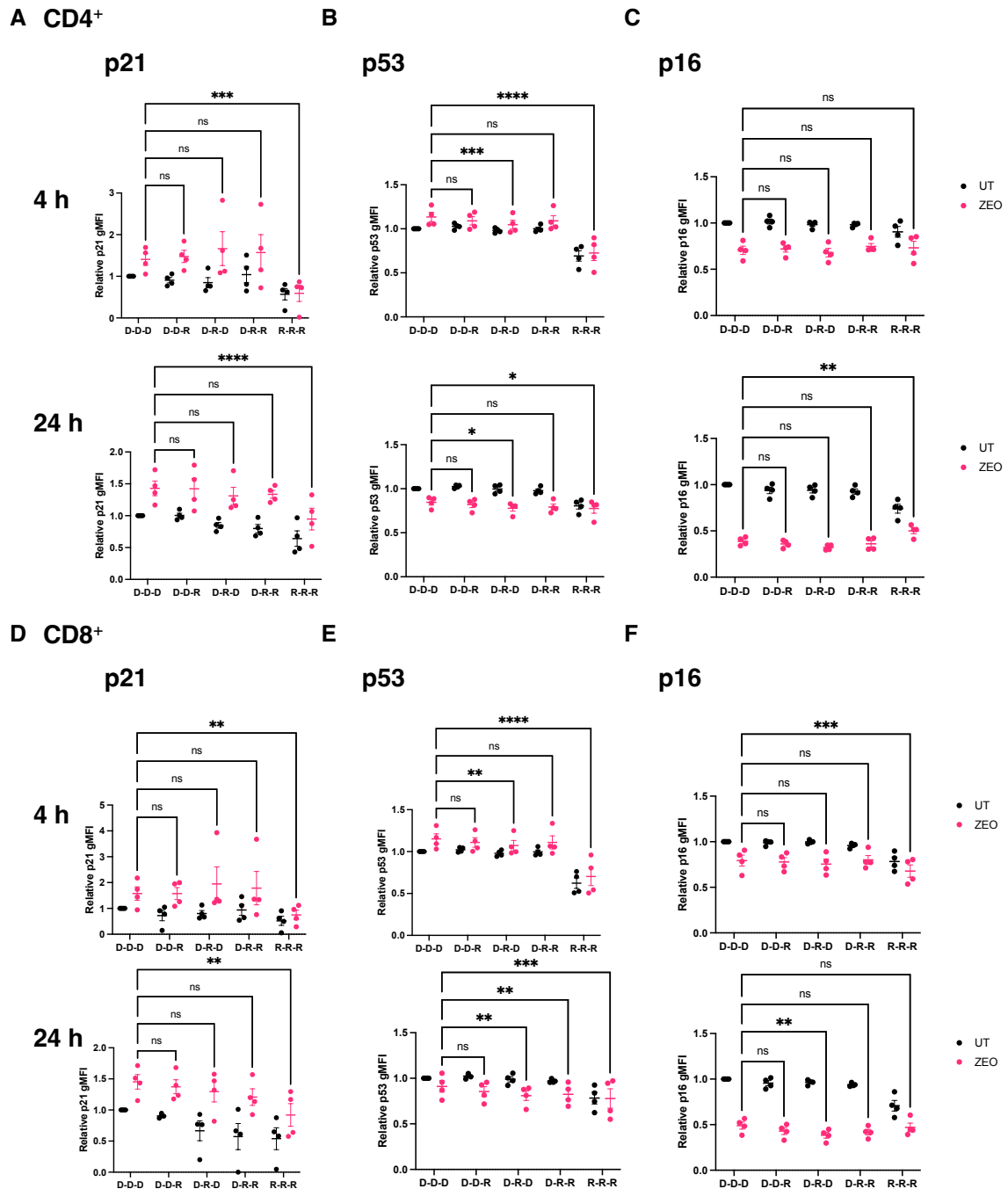


Figure 5.19 Individual graphs for *in vitro* validation of Rapamune trial results using healthy NHS-BT donor PBMCs

Data the same as in Figure 5.18. (A–F) Geometric mean fluorescence intensity of p21 (A, D), p53 (B, E), and p16 (C, F) in CD4⁺ (A–C) and CD8⁺ (D–F) T cells at 4 hours (upper) and 24 hours (lower) recovery from zeocin (pink points), across different rapamycin treatment conditions, as assessed by spectral flow cytometry. All values are expressed as fold change from the DMSO→DMSO→DMSO untreated condition for each recovery time point. *P*-values are derived from a two-way ANOVA with Dunnett's multiple comparisons test, *n*=4 healthy donors.

5.3 Discussion

The major findings of this chapter are presented in Figure 5.20. A 27-colour spectral flow cytometry panel was used to identify age-associated immune subsets and their senescence phenotypes in healthy donors. Out of all senescence markers studied, the age-associated immune cell subsets all upregulated p21 levels compared to their naïve, early-differentiated counterparts (Table 5.4). This suggests that the form of senescence which immune cells undergo with ageing may be p53- and p21- driven, hinting – importantly – towards a more DNA damage-induced type of senescence (DDIS), akin to the DDIS fibroblasts studied in Chapter 3 (Figure 3.3). This provides further evidence that pursuing drugs which ensure genome stability, so-called genoprotectors, is a relevant strategy for targeting immunosenescence in human ageing (Kell et al., 2023). The limitation of examining age-associated subsets from healthy NHS-BT donor PBMCs in this study is that they are from nominally healthy donors and do not come with meta-data of donor age or sex, masking the effects of these characteristics. Secondly, the age-associated subsets identified from NHS-BT PBMCs may not have represented the same age-associated subsets from individuals with advanced age or those who with age-related diseases.

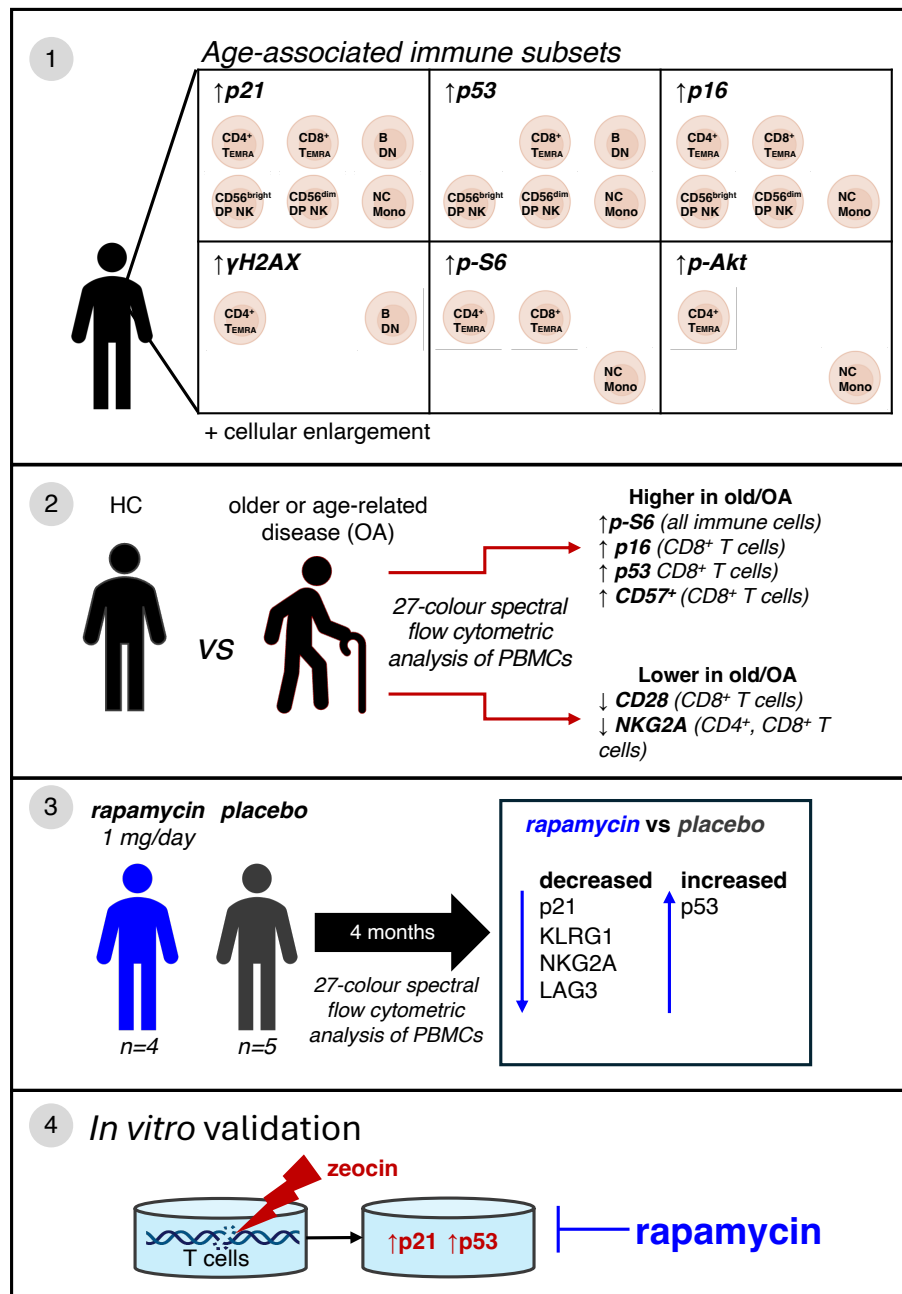


Figure 5.20 Summary of findings in Chapter 5

1) Age-associated immune subsets from healthy individuals display cellular enlargement and p52/p21 upregulation, indicating senescence. 2) PBMCs from older individuals or those with osteoarthritis (OA), an age-related disease, display an exacerbation of immune cell senescence and mTOR hyperactivation compared to healthy controls (HCs). 3) Low-dose rapamycin treatment in a small trial modulated markers of cell senescence (p21, p53) and immune exhaustion (KLRG1, NKG2A, and LAG3) in immune cells. 4) Rapamycin limits p21 and p53 levels after *in vitro* DNA damage.

Abbreviations (see also List of abbreviations): TEMRA (T effector memory cell re-expressing CD45RA); B DN (double negative IgD⁻CD27⁻ B cell); DP NK (CD57⁺CD16⁺ Natural Killer cell); NC mono (non-classical monocyte).

To test whether features of immune cell senescence were exacerbated during ageing and an age-related disease, I investigated these senescence biomarkers in a cohort comprised of individuals of advanced age and/or with osteoarthritis (Figure 5.10), and the other, a cohort of older men at baseline for the Rapamune trial (Figure 5.13). Compared to healthy controls, these cohorts had particularly high levels of p-S6, indicating high mTORC1 activity, across all immune subsets, and displayed exacerbated features of cellular senescence, such as high p16 and p53, loss of CD28 and gain of CD57 and KLRG1, particularly in the CD8⁺ T cells (Figure 5.9 and Figure 5.12). The observation that features of cell senescence were apparent predominantly in the CD8⁺ rather than CD4⁺ T cells is consistent with the literature describing their differing susceptibility to undergo senescence. Namely, CD8⁺ TEMRA cells accumulate peripherally at a faster rate than do CD4⁺ TEMRA cells in human ageing (Callender et al., 2020, Czesnikiewicz-Guzik et al., 2008). As discussed in section 1.3.2.1, the enhanced proclivity of CD8⁺ T cells towards senescence is partly due to their inability to respond to DNA damage by promoting mitochondrial biogenesis (Callender et al., 2021).

To investigate how *in vivo* treatment of low-dose rapamycin affects steady-state features of immunosenescence, I analysed PBMCs from a single-blind, placebo-controlled small clinical trial in older men of low-dose (1 mg/day) rapamycin for 4 months. This is the first UK trial evaluating the effects of *in vivo* rapamycin treatment on peripheral features of immunosenescence in humans. A previous study in the USA observed that 6-week treatment with the rapalogue RAD001 (everolimus) caused a reduction in the proportion of circulating PD-1⁺ CD4⁺ and CD8⁺ T cells (Mannick et al., 2014). In the current study, no changes in PD-1 were observed in the rapamycin-treated group compared to placebo (Figure 5.17C). However, there was a significant reduction in both KLRG1⁺ and NKG2A⁺ CD4⁺ T cells, and near-significantly LAG3⁺ CD4⁺ T cells (Figure 5.17B,D,E). Like PD-1, these three cell-surface proteins are all immune checkpoint inhibitors, each with roles in

limiting T cell activation (Table 5.2). Therefore, though this study did not recapitulate the same effect on PD-1 specifically, there were similar functional effects of rapamycin in downregulating other immune inhibitory receptors. Thus, low-dose rapamycin may perhaps potentiate a less-exhausted T cell phenotype.

Rapamycin treatment caused a consistent reduction in p21^{Cip1/Waf1} levels across multiple immune cell types (Figure 5.16A). Paradoxically, p53 expression (Figure 5.16B), and in some cases p16 in CD8⁺ T cells (Figure 5.16C), was higher with rapamycin treatment at 4 months across immune subsets, compared to individuals in the placebo group. To investigate whether these changes could be attributed to effects on DNA damage, I evaluated the impact of rapamycin on p21, p53, and p16, after DNA-damaging zeocin treatment of immune cells *in vitro* using healthy NHS-BT donor PBMCs. Consistent with the trial, rapamycin strongly inhibited p21 expression after DNA damage (Figure 5.18 and Figure 5.19), suggesting that the trial result could be attributed to an attenuation of DNA damage-dependent p21 upregulation. However, γ H2AX-marked DNA damage was not affected by *in vivo* rapamycin treatment, potentially because γ H2AX was already low in this immune-unchallenged context; other markers of DNA damage, such as 53BP1 and 8-oxo-dG, or use of the comet assay, may be informative in understanding whether rapamycin affected other types of DNA damage.

Whereas *in vivo* rapamycin treatment in the trial caused an increase in p53 expression in immune cells (Figure 5.16B), *in vitro* treatment of rapamycin robustly inhibited the induction of p53 after DNA damage (Figure 5.18), suggesting that the induction of p53 in the trial may be independent of effects on DNA damage. Notably, this study assessed total p53 rather than any phosphorylated forms, such as at Ser¹⁵ and Ser²⁰. Indeed, analyses of cells deficient in PTEN, which rapidly undergo cell senescence, point towards a relationship between mTOR, phosphorylated p53, and p21 upregulation (Jung et al., 2019). In PTEN-deficient human MCF7 cells, mTORC1 and mTORC2 physically associate with p53 and

directly phosphorylate it at Ser¹⁵, thus driving p21 upregulation and senescence (Jung et al., 2019). Accordingly, rapamycin treatment of cells decreased total and phospho-p53 and p21 expression and prevented senescence (Jung et al., 2019). Given this relationship between mTOR and p53 phosphorylation, investigations into the *in vivo* effect of rapamycin on phospho-p53 may be helpful.

p53 plays a myriad of roles outside of its position as ‘guardian of the genome’; one meta-analysis found that p53 binds a striking 3661 genes as a transcription factor (Fischer, 2017). Many of these genes are involved in activating the autophagy pathway. One screen in mouse embryonic fibroblasts found that p53 bound to a wide range of autophagy genes including, but not limited to, *Atg2b*, *Atg4a*, *Atg4b*, *Atg7*, *Atg10*, *Ulk1*, *Ulk2*, and *Tsc2* (Kenzelmann Broz et al., 2013). Others have identified that p53 increases expression of DRAM1, a lysosomal membrane protein that induces macroautophagy (Crighton et al., 2006). My observation that the immune cells from participants in the rapamycin group showed elevated p53 (Figure 5.16B) in a way that is independent of DNA damage (Figure 5.18), and the evidence that p53 induces autophagy, is provocative. Importantly, autophagy was increased in rapamycin-treated γ H2AX⁺ cells in the DNA damage assay in Chapter 4 (Figure 4.25), and boosting autophagy is a known strategy for improving immune function in an aged context (Puleston et al., 2014, Alsaleh et al., 2020). It is therefore plausible that the increase in p53 expression with rapamycin in the trial may be indicative of the induction of autophagy and could be confirmed using the flow cytometry-based LC3 autophagy assay utilised in Chapter 4.

Unlike in the trial, where rapamycin increased p16 expression in CD8⁺ T cells (Figure 5.16C), rapamycin decreased p16 levels in the *in vitro* assay (Figure 5.18). One explanation for this is that induction of the p16-Rb pathway is potentially indicative of deeper senescence, whereas p53-p21 upregulation represents an earlier stage of senescence induction which, unlike the p16-Rb pathway, is sensitive to reversal by rapamycin treatment

(Beauséjour et al., 2003). This is consistent with data from the Cox lab, where chronic pan-mTOR treatment using AZD8055 was sufficient to rejuvenate peri-senescent HF043 fibroblasts, but not those in deep replicative senescence (Cox lab unpublished data).

This study using the Rapamune trial samples is limited. Firstly, the study tested a small number of participants per treatment arm, meaning that statistical power was reduced. As this is an ongoing study, the analysis of PBMCs from more participants in the future will be invaluable to confirm the results from this thesis. Furthermore, due to the small numbers and inconsistency in blood sampling, similar time points were pooled together (i.e., weeks 2-3 and 4-5), thus masking any changes occurring between these time points. Finally, all analysis was performed using manual biaxial gating, and therefore involved a bias in identifying differences in pre-defined populations. This study would strongly benefit from the use of dimension reduction analyses, such as t-SNE or UMAP, or analysis by scRNA-seq techniques.

In summary, *in vivo* rapamycin treatment attenuated several features of immunosenescence, including those related to cellular senescence and immune inhibitory checkpoints. The effect of rapamycin on p53 upregulation is potentially independent of DNA damage and may indicate induction of non-canonical pathways, such as autophagy. Taken together, these data may provide a mechanistic explanation for how rapalogues modulate immunosenescence and improve immunity in older people.

6

Discussion

Genoprotectors as a strategy for alleviating immunosenescence, and a new model for the origin of mTOR hyperactivation during ageing

6.1 Thesis summary and impact

6.1.1 Summary: mTOR inhibition as a strategy to protect from DNA damage and promote immune resilience in ageing

In this thesis, I observed that WRN, a geroprotective protein whose transcript expression increases in replicative and DNA damage-induced senescence of fibroblasts, may interact with the mTOR and protein synthesis pathways, suggesting that WRN's geroprotective effects may be mediated through its interactions with mTOR (Chapter 3). Consistent with a role of mTOR in senescence, both senescent fibroblasts (Chapter 3) and age-associated peripheral immune subsets (Chapter 5) displayed high levels of phosphorylated mTOR targets, DNA damage, cellular enlargement, and upregulation of the cyclin kinase inhibitor, p21. Using *in vitro* assays of DNA damage with both immune cells (Chapter 4) and fibroblasts (Chapter 3), revealed that mTOR inhibition by rapamycin and AZD8055 limited DNA damage, cell death, and senescence, potentially through induction of autophagy. Moreover, in the first study of its kind in the UK, I assessed the effects of *in vivo* rapamycin treatment in humans on peripheral features of immunosenescence (Chapter 5). Here, low-dose rapamycin treatment attenuated immune cell expression of a marker of DNA damage-induced senescence, p21, and immune co-inhibitory molecules, such as KLRG1, NKG2A, and LAG3. Taken together, these data suggest both gero- and genoprotective roles of mTOR inhibition and provide some mechanistic bases for the immunoprotective benefits of this anti-ageing intervention. The major results of this thesis are summarised in Figure 6.1.

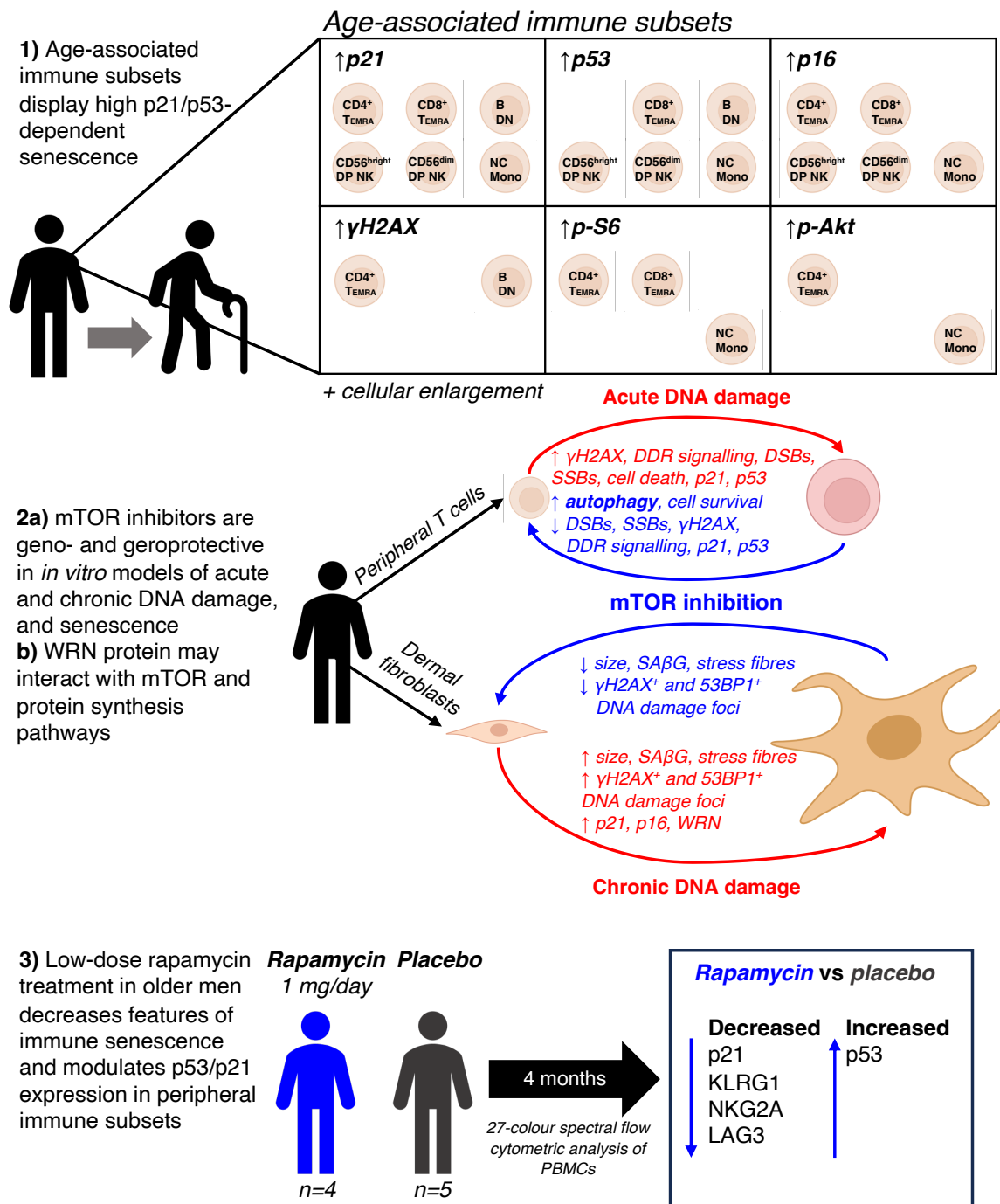


Figure 6.1 Summary of major thesis findings

Abbreviations (see also List of abbreviations): TEMRA (T effector memory cell re-expressing CD45RA); B DN (double negative IgD⁻CD27⁻ B cell); DP NK (CD57⁺CD16⁺ Natural Killer cell); NC mono (non-classical monocyte); DDR (DNA damage response); DSB (double-strand break); SSB (single-strand break); SA β G (senescence-associated β -galactosidase).

6.1.2 Thesis impact

The genoprotective effects of mTOR inhibitors make them amenable for use in a wide range of clinical scenarios where the induction of DNA damage leads to pathology. For example, cancer treatments such as radio- or chemotherapy lead to widespread DNA damage of healthy tissue; thus, treatment with a genoprotector, such as low-dose rapamycin, after remission from the original tumour may attenuate the accelerated ageing associated with such cancer therapies (Wang et al., 2024). Furthermore, infection with SARS-CoV-2, the virus behind the COVID-19 pandemic, induces DNA damage and senescence by degrading DDR enzymes (Gioia et al., 2023); heightened virus-induced senescence in this way strongly contributes to disease mortality (Camell et al., 2021, Lee et al., 2021). Perhaps prophylactic treatment of older care home residents with genoprotectors such as low-dose mTOR inhibitors may provide a much-needed boost to immune resilience in this vulnerable population during future pandemics (Cox et al., 2020). Likewise, since mTOR inhibitors improve vaccine responses in older people (Mannick et al., 2018, Mannick et al., 2021, Mannick et al., 2014), future vaccine drives could consider administering short-term mTORi treatments prior to immunisations against pathogens that particularly affect the older population, such as influenza, coronaviruses, and VZV. Infections by other pathogens, such as *Salmonella* Typhi, *Leishmania*, and some Gram-negative bacteria (which release cytolethal distending toxin), all drive pathology through the induction of DNA damage and senescence (Ibler et al., 2019, Mathiasen et al., 2021, Covre et al., 2018); low-dose mTOR inhibition in these contexts of infection could possibly limit disease progression. Though largely untested in humans, research in mice at least suggests that rapamycin improves immune control of Leishmaniasis (Khadir et al., 2018). Finally, in addition to progeroid diseases resulting from a DNA repair deficiency (Werner syndrome, Rothmund-Thomson syndrome, Bloom syndrome, Cockayne's syndrome, Fanconi's anaemia, and Ataxia-Telangiectasia), chronic viral infection and rheumatic diseases display decreased DNA

repair factor expression in immune cells (Zhao et al., 2018, Shao et al., 2009, Li et al., 2016). It is possible, though unexplored, that rapamycin could limit DNA damage and attenuate pathology in these diseases, in addition to progeria.

Given the known physiological roles of senescent cells and DNA damage, caution must be taken in administering genoprotective low-dose mTOR inhibitors (de Magalhaes, 2024). For example, inhibiting seno-conversion of virus-infected cells may disrupt their removal by the immune system (section 1.3.1.3). Additionally, genoprotectors may disrupt the intentional induction of DNA damage by adaptive immune cells during VDJ recombination, potentially leading to immunodeficiency (section 1.3.1.1); however, as the thymus (where T cell VDJ recombination occurs) atrophies with age, it is likely that late-life administration of low-dose rapamycin would not impact this. Finally, senescent cells play critical roles in tissue regeneration in response to damage (Chen et al., 2023); thus, genoprotectors may have unforeseen consequences such as by hindering wound healing, a process which is already impaired in older people (Demaria et al., 2014, Wicke et al., 2009).

6.1.3 Future directions: testing immunosenescence interventions using human immune challenge studies

In each results chapter I have discussed experimental limitations of the data presented; these will not be repeated here. This section will identify broader limitations and future directions of the work presented in this thesis, and outstanding questions in the field.

Several immunosenescence interventions have shown efficacy in human settings (section 5.1.2). The limitation of these studies, including the Rapamune trial, are the wide range of immune challenges, outcomes, and markers utilised by the different research groups to measure their impact on immunosenescence. Future studies in the field could benefit from the development of ‘gold standard’ approaches to measure the effectiveness of a given

intervention on attenuating immunosenescence in humans *in vivo*. One solution could be to develop human immune challenge (HIC) models. For example, some HIC models measure pre-existing cutaneous immune memory to an antigen (e.g. VZV (Vukmanovic-Stejic et al., 2018)). Other, newer HIC models challenge participants with novel antigens to which participants are immunologically naïve, such as keyhole limpet haemocyanin (KLH), to measure cutaneous primary and subsequent memory immune responses in a controlled setting (Drennan et al., 2023). These HIC models could be developed to test interventions on specific features of immunosenescence, such as immune memory responses to pre-existing or novel antigens, or resolution of inflammation, and outcomes compared to real clinical data on participant health. Panels of immunosenescence interventions could then be tested and compared with each other. HIC models are particularly useful as they allow for tissue-specific immune responses to be interrogated, unlike this and many other studies, where only circulating immune cells are sampled. A drawback of HIC models is that they are restricted to studying immune responses only in accessible organs such as the skin; however, as non-invasive tissue sampling technology advances, other organs may be investigated in the future. The use of humans as the model animal also reduces the need for non-human animal models, thus improving the translatability of the intervention. In all immunosenescence studies and HIC models, immune responses and efficacy of interventions should be stratified by participant sex, as anti-ageing interventions have dramatically sexually dimorphic effects (Bartke et al., 2024). Of note, the role of sex and ethnicity has not been explored in this thesis. However, the Rapamune Trial is ongoing (NCT05414292) and aims to recruit women over the next few years.

Many of the mechanistic studies in this thesis have been performed using *in vitro* models and assays. However, future work could employ a HIC model to evaluate whether mTOR inhibitors modulate DNA damage and senescence and improve immune responses in people *in vivo*. For example, immunogenic KLH could be injected into cutaneous regions

that had either been pre-treated with bleomycin to induce local DDIS, or into a control cutaneous region, to measure whether antigen-specific memory responses are affected by local DDIS. Furthermore, the effects of whether topical rapamycin cream could rescue the KLH responses in the bleomycin-induced DDIS cutaneous region could be measured (Chung et al., 2019). Finally, immunosenescence interventions such as rapamycin would benefit from measuring their effects on composite measures of immunosenescence, such as the IMM-AGE score, a clinically relevant immune metric predicting all-cause mortality (Alpert et al., 2019). This could be determined using samples from the Rapamune trial in the future.

6.1.4 A call for genoprotectors

Taken together, the evidence from this thesis supports a drive for drug discovery campaigns with the aim of identifying compounds that promote genome stability (so-called, genoprotectors), and the assessment of their efficacy in improving immune responses in older people using replicable human immune challenge models.

6.2 Evidence of genoprotection by known immunosenescence interventions

The major outcome of this thesis is the evidence that low-dose mTOR inhibition may be immunoprotective due to its ability to promote genome stability (genoprotection). In addition to mTOR inhibition, a variety of treatment interventions have been reported to restore immunity in older people. Like mTOR inhibition, the efficacy of many of these can be partly explained by their ability to promote genome stability. This serves as further indirect evidence that inhibiting DNA damage and promoting DNA repair improves immune resilience in old people. This section will consider some of these immunoprotective treatments (spermidine, metformin, p38 MAPK inhibitors, and vitamin D₃) and offer a

genoprotective mechanism of action that may contribute to their success. Given this evidence and the major outcomes of my thesis, I further discuss the potential merit in exploring novel interventions, such as nicotinamide riboside (NR), DREAM complex inhibition, enoxacin supplementation, and increased physical activity, as strategies to target immunosenescence, given the evidence for their effects in limiting DNA damage. These interventions, along with their relevant effective *in vitro* and *in vivo* doses, are summarised in Table 6.1.

6.2.1 Like mTORi, known interventions against immunosenescence are genoprotective

6.2.1.1 Spermidine

Spermidine is a naturally derived polyamine that improves antigen-specific B and T cell responses in cells from old donors, through increasing autophagy (Zhang et al., 2019, Alsaleh et al., 2020). Spermidine is used as a substrate for the post-translational hypusination of eIF5A, a factor that enhances the translation of otherwise hard-to-translate poly-proline sequences. One such poly-proline-containing protein is TFEB, a transcription factor that drives the expression of autophagy genes. Thus, spermidine treatment increases autophagy in both old B and T cells through increased production of TFEB (Zhang et al., 2019, Alsaleh et al., 2020). As discussed, dysfunctional mitochondria accumulate in T cells from old people, and these produce ROS that damage the DNA and promote telomere attrition (Sanderson and Simon, 2017). Promoting autophagy by a different drug, metformin, has been shown to restore mitochondrial function in CD4⁺ T cells in old people (Bharath et al., 2020); by extension the autophagy-activating effect of spermidine may also restore mitophagy and mitochondrial health, thus protecting the DNA from ROS-mediated damage. Rejuvenating autophagy by spermidine may therefore be considered as an intervention that protects the genome, i.e. that is genoprotective.

In addition to their action through autophagy, polyamines such as spermidine may directly limit DNA damage and promote DNA repair. For example, spermine (spermidine's derivative) reduces the presence of DNA oxidation products after damage by H₂O₂ (Muscari et al., 1995). Spermidine was also found to promote homologous recombination repair (HRR) mediated by RAD51 after DNA damage (Lee et al., 2019). Consistent with a core role for polyamines in DNA repair, inhibition of the polyamine biosynthesis pathway using DFMO (which inhibits ornithine decarboxylase) prolonged the presence of DNA damage after ionising radiation or PARP inhibition by olaparib (Lee et al., 2019). Though several polyamines improved HRR after DNA damage, spermine was found to be most efficient (Lee et al., 2019). Spermidine and other related polyamines may therefore elicit genoprotective effects and alleviate immunosenescence by acting as antioxidants, promoting repair, and reducing ROS burden through increased autophagy. Spermidine supplementation in humans is tolerated at doses tested up to 15 mg/day, though it is noted that supplementation does not detectably change blood spermidine levels, despite eliciting its beneficial anti-ageing effects (Schwarz et al., 2018, Senekowitsch et al., 2023, Wirth et al., 2018). Current clinical trials investigating whether spermidine supplementation improves vaccine responses in old people (e.g. NCT05421546, spermidine at 6 mg/day) will be greatly informative in translating *in vitro* studies to population interventions.

6.2.1.2 Metformin

The anti-diabetic drug metformin is a potential geroprotector affecting a number of the hallmarks of ageing (Kulkarni et al., 2020); it may also improve immune resilience in older people (Justice et al., 2021). Metformin has polypharmacological effects, one of which I propose is genoprotective. Metformin is an AMPK activator and thus induces autophagy via activating phosphorylation of ULK1 (Kulkarni et al., 2020). In this way it can induce the removal of dysfunctional mitochondria (sources of genotoxic ROS) to prevent DNA

damage. Consistent with this, treating old donor T cells with metformin restored their autophagy and mitochondrial bioenergetics (Bharath et al., 2020).

Metformin can affect the DNA damage response by driving a pseudo-DDR that protects genome stability. Epidermoid carcinoma cells treated for 2 days with metformin accumulated γ H2AX foci and high levels of the phosphorylated DDR orchestrator, p-ATM (Ser¹⁹⁸¹), in the absence of any detectable DNA lesions (Vazquez-Martin et al., 2011). Furthermore, metformin treatment of A549 lung cancer cells modulated the expression of p-Chk2, p53 and p53R2 (a p53 target gene) after UVC-induced DNA damage (Lee et al., 2016). Importantly, supplementation of metformin in *Drosophila* diminished the proportion of γ H2AX⁺ and 8-oxo-dG⁺ intestinal stem cells undergoing acute paraquat-induced oxidative stress or chronological ageing, indicating the genoprotective effects of metformin (Na et al., 2013). Though some *in vitro* studies have used high metformin concentrations (5 mM) that would not be attainable *in vivo*, it is encouraging that a well-tolerated treatment regimen in older people was associated with upregulated expression of several DNA repair pathways in muscle biopsies (base excision repair, mismatch repair, and BRCA-mediated repair) (Kulkarni et al., 2018). Metformin may therefore ensure DNA stability by limiting damage by oxidative stress and further kickstarting a DDR and DNA repair.

6.2.1.3 *p38 MAPK inhibitors*

MAPK signalling occurs in response to growth factors, leading to cell proliferation; alternatively, members of the MAPK family are implicated in stress responses, including following DNA damage (Yacoub et al., 2001). Consistent with this, and the high levels of DNA damage in senescence, p38 MAPK is more active in both senescent T cells and non-immune cells compared to non-senescent counterparts (Lanna et al., 2014, Callender et al., 2018, Davis et al., 2016). p38 MAPK inhibitors also successfully dampen the

inflammatory SASP of senescent cells, partly through inhibiting p38-DDR signalling (Alimbetov et al., 2016). p38 MAPK inhibitors, such as losmapimod and BIRB 796, improve both senescent phenotypes and responses to immune challenge in older people (Chambers et al., 2021a, Vukmanovic-Stejic et al., 2018). p38 MAPK inhibition increases autophagy and telomerase expression, and reduces levels of intracellular ROS in senescent T cells (Henson et al., 2014). Thus, by restoring T cell telomere length and removing genotoxic ROS, p38 MAPK inhibition is a genoprotective strategy. Further evidence for the genoprotective effects of p38 MAPK inhibition is evidenced by the effects of SB203580 treatment, which rejuvenates both the senescent morphology and proliferative potential of DNA repair-deficient Werner Syndrome fibroblasts (Davis et al., 2005), although this treatment may have off-target effects (Lali et al., 2000). Furthermore, long-term (>3 month) treatment with current p38 MAPK inhibitors (such as losmapimod) is unfavourable due to hepatotoxicity, though short-term treatment prior to immune challenge may be beneficial. Although current p38 MAPK inhibitors are limited by their low therapeutic index and specificity, drugs with higher specificity and selectivity for MAPK isoforms are in development (Davis et al., 2016).

6.2.1.4 Vitamin D₃

Vitamin D₃ supplementation has been shown to improve cutaneous immune responses in old people, in part due to the effects of reducing inflammatory monocyte recruitment to the site of antigen challenge (Chambers et al., 2021b). Interestingly, vitamin D₃ modulates DNA repair factor activity and cellular senescence (Graziano et al., 2016). For example, fibroblasts that have undergone oncogene-induced senescence (OIS) fail to repair DNA damage due to insufficient expression of BRCA1 and the vitamin D receptor (VDR); VDR knockdown recapitulated both BRCA1 insufficiency and the senescence phenotypes observed in OIS (Graziano et al., 2016). Intriguingly, treatment of OIS fibroblasts with active

vitamin D₃ (1,25(OH)₂-D₃) enhanced the recruitment of DNA repair factors to DNA lesions after ionising radiation (Graziano et al., 2016). This reveals an axis connecting vitamin D₃, DNA repair factor activity, and senescence. Furthermore, vitamin D₃ treatment reduced DNA damage foci in myometrial stem cells isolated from rats developmentally exposed to diethylstilbestrol (DES) (Elkafas et al., 2020). In the cited study, *in vitro* treatment with vitamin D₃ enhanced expression of both DNA damage sensors (in the MRN complex) and DDR effector proteins (RAD51 and BRCA2) after DES treatment (Elkafas et al., 2020). Additionally, vitamin D₃ antagonised the DNA-damaging effects of DNA-damaging treatment by upregulating MRE11 and other DDR genes (Elkafas et al., 2020). These data suggest that vitamin D₃ is genoprotective and its supplementation in humans could improve immune responses in old people by limiting DNA damage. This vitamin D₃-DNA repair axis deficiency may be particularly pertinent in older adults, where vitamin D deficiency is common. Importantly, supplementation in humans elevates vitamin D₃ blood concentrations similar to those used in *in vitro* studies that demonstrate improved DNA repair (Table 6.1); however, the effect of supplementation on DNA damage readouts in either immune or non-immune cells in people remains to be explored.

6.2.2 Novel genoprotective interventions for immunosenescence

6.2.2.1 Nicotinamide riboside supplementation

Energy homeostasis is critical for correct immune function, yet immune energetics have remained largely ignored within the extensive body of research on NAD⁺. NAD⁺ levels decline during normal ageing and this contributes both to increased DNA damage and mitochondrial dysfunction, both processes discussed in this review to be drivers of immunosenescence (Yoshino et al., 2018). The age-related decline in NAD⁺ is due, in part, to the increase in its consumption by NADases, such as CD38, which are upregulated in tissue-resident macrophages in response to the SASP (Covarrubias et al., 2020, Chini et

al., 2020). Additionally, NADases, such as DNA damage-induced poly-ADP ribosyl polymerases (PARPs), become hyperactivated in senescence due to the increase in DNA lesion burden, leading to NAD⁺ decline (Yoshino et al., 2018, Fang et al., 2014). The overactivation of these NAD-consuming enzymes restricts the activity of other pro-longevity NAD-dependent enzymes, the sirtuins: e.g. SIRT1, an NAD-dependent deacetylase that regulates mitochondrial biogenesis by deacetylating PGC-1 α (Fang et al., 2016). Restoring SIRT1 activity by NR supplementation increases mitophagy, removal of ROS-producing dysfunctional mitochondria, and lifespan in progeroid worms mutant for the DNA repair factor, *wrn* (*wrn-1(gk99)*), and in progeroid mice that are deficient in the DDR master regulator, *Atm* (Fang et al., 2016, Fang et al., 2019). Furthermore, in progeroid *Tfam^{fl/fl}* *Cd4^{Cre}* mice that accumulate senescent T cells with dysfunctional mitochondria, NR supplementation reversed tissue inflammation and senescence, although the explicit effect of this treatment on immune responses was not reported (Desdin-Mico et al., 2020). NR supplementation also attenuated inflammaging in *Atm^{-/-}* progeroid and wildtype old mice, and restored their lymphoid vs myeloid haematopoietic output (Zong et al., 2021). Restoring sirtuin activity in this way may be beneficial in immunosenescence, since antigen-experienced senescent CD8⁺CD28⁻ T cells, for example, have decreased SIRT1 expression compared to naïve T cells, which partly drives their glycolytic and cytotoxic aged phenotype (Jeng et al., 2018). It would be interesting to investigate whether NR supplementation may alleviate immunosenescence in other DNA repair-defective models, e.g. the *Vav-iCre^{+/-}*; *Ercc1^{-fl/fl}* mouse model (Yousefzadeh et al., 2021b).

The impact of NR supplementation on mitophagy is also accompanied by better DNA repair. In ATM-knockdown primary neurons treated with DNA-damaging etoposide, NR reduced 53BP1 staining (Fang et al., 2016). Mechanistically this was due to an increase in SIRT1-mediated deacetylation of Ku70 in the DNA-PK complex during DSB repair by NHEJ (Fang et al., 2016). Similarly, in *wrn-1(gk99)* mutant worms, NR treatment decreased the RAD51

signal 24 hours after ionising radiation, this time suggesting an improvement in HR-mediated repair of DSBs (Fang et al., 2019). Accordingly, NR supplementation starting at 2 years-old in wildtype mice reduced the accumulation of γ H2AX⁺ DNA-damaged muscle stem cells (Zhang et al., 2016). Taken together, these data suggest that NR may be genoprotective by improving both mitochondrial quality and DNA repair. Notably, *in vitro* studies use relatively high concentrations of NR (0.5 – 1 mM) for improvement of DNA repair (Fang et al., 2016, Fang et al., 2019). Though current supplementation regimens in older adults robustly increase the NAD⁺ levels of peripheral immune cells, investigations into the effective doses of NR for *in vivo* genoprotection are needed, as questions remain as to the general translatability of the findings from cell and animal models (Martens et al., 2018).

6.2.2.2 Enhancing DNA repair

I have discussed the evidence that persistent DNA damage is a primary driver of immunosenescence during ageing, and how existing interventions that ameliorate immunosenescence may be genoprotective by acting to promote DNA repair. Pharmacological interventions that target DNA repair pathways are common in the field of cancer chemotherapy, and often work to elicit cancer cell death by inhibiting DNA repair. However, drugs that act to promote DNA repair to elicit genoprotection have received less attention.

6.2.2.2.1 DREAM complex inhibition

There are a limited number of regulatory mechanisms for suppressing overall DNA repair capacities in somatic cells. One of these is the DREAM complex, formed by the Dp/Retinoblastoma(Rb)-like/E2F and the MuvB subcomplexes (Bujarrabal-Dueso et al., 2023). The DREAM complex directly binds and represses up to 65% of genes involved in

the DDR, as observed in *C. elegans*, including *atm-1*, *chk-1*, *parp-1*, *rad-50*, *rad-51*, and *rpa-1* (Bujarrabal-Dueso et al., 2023). Furthermore, in *C. elegans*, loss-of-function mutations in genes that encode components of the DREAM complex (namely, *lin-52*, *dpl-1*, *efl-1*, and *lin-35*) conferred resistance to UV-induced DNA damage and death and displayed enhanced repair of UV-induced DNA lesions. Pre-treatment of human U2OS cells with small molecule inhibitors of DYRK1A, harmine hydrochloride (10 μ M) or INDY (25 μ M), caused inhibition of the DREAM complex and protection from DNA damage-induced cell death following either UV or MMS treatment (Bujarrabal-Dueso et al., 2023). Further *in vivo* evidence of genoprotection by DREAM complex inhibition was observed in DNA-repair-deficient *Ercc1*^{-/-} mice, which experienced reduced retinal apoptosis and γ H2AX-marked DNA damage when treated with harmine hydrochloride (Bujarrabal-Dueso et al., 2023).

DREAM complex inhibition may therefore be a potent genoprotective strategy. However, quantitative data of the effects of harmine in humans *in vivo* is lacking. Furthermore, there are no studies exploring its effects on cellular senescence or immunosenescence. A recent study of oral administration of harmine hydrochloride in 10 healthy male subjects achieved peak blood concentrations of 0.532 ± 0.448 μ M, indicating much lower and highly variable levels than those used in the early study which demonstrated genoprotection (Dornbierer et al., 2023, Bujarrabal-Dueso et al., 2023). However, the effects of harmine on promoting human pancreatic beta proliferation have spurred a recent phase I trial in diabetes patients, which will hopefully provide more data on its pharmacodynamic and *in vivo* effects (Wang et al., 2022a, Rosselot et al., 2024).

6.2.2.2.2 Enoxacin

Another drug that improves DNA repair is enoxacin, a fluoroquinolone antibiotic that boosts DNA repair by enhancing the RNAi machinery component, DICER, thereby increasing the generation of DNA damage response non-coding RNAs (DDRNs) (Gioia et al., 2019). Deficiency of the RNAi machinery impairs the DDR and increases genome instability

(Swahari et al., 2016). However, treatment of cells with 50 μ M enoxacin prior to ionising radiation caused a rapid accumulation of 53BP1 at DSBs which corresponded with a subsequent decrease in DNA lesions (as measured by comet assay and γ H2AX levels) after the acute DDR, indicating a boost in overall DNA repair (Gioia et al., 2019). Thus, enoxacin appears to be genoprotective by enhancing DSB repair. In the context of ageing, enoxacin has been reported to increase *C. elegans* lifespan (Pinto et al., 2018), but little is known on its immune effects. It is of note that the SARS-CoV-2 N-protein binds to DDRNAs, and by doing so outcompetes the 53BP1-DDRNA binding that is necessary for 53BP1 recruitment to DSBs (Gioia et al., 2023). In this way, SARS-CoV-2 infection was recently demonstrated to increase DNA damage and induce cellular senescence, driving disease pathology (Gioia et al., 2023). It may be that enoxacin treatment, by increasing DDRNA expression, may be able to promote 53BP1-mediated repair of DNA lesions, thus preventing senescence and potentially limiting morbidity and even mortality in COVID-19 patients. Most importantly, enoxacin is already well tolerated in older adults, and current treatment regimens increase enoxacin blood concentrations to a level relatively comparable to those used in the *in vitro* studies for eliciting genoprotection (Table 6.1) (Hamel et al., 2000). Given the urgent call for novel drugs attenuating immunosenescence, future studies investigating enoxacin's effect on DNA damage/repair in immune and non-immune cells, and on immune system function in older people, may be beneficial. More generally, I propose that genoprotective drug discovery campaigns may be a valuable approach towards attenuating immunosenescence.

6.2.2.3 Lifestyle interventions

In addition to pharmacological targeting of immunosenescence, other interventions promoting lifestyle changes can target mTOR and possibly be genoprotective. These include increased physical activity (Duggal et al., 2019) and caloric restriction (Vermeij et al., 2016). Currently, trials have not assessed whether these interventions improve

functional immune responses in older people. However, there is evidence that a 16-week combined physical exercise training programme reduces basal levels of DNA strand breaks and oxidative DNA damage, as measured by the comet assay, in circulating lymphocytes (Soares et al., 2015). Accordingly, regular physical activity with old age appears to protect from a range of features of immunosenescence. In one observational study, older cyclists displayed a significantly lower proportion of both CD4⁺ and CD8⁺ TEMRA cells than did age-matched healthy older adults who did not partake in regular physical activity; furthermore, this active cohort possessed lower serum IL-6 levels, suggesting lower levels of inflammaging (Duggal et al., 2018).

Lifestyle interventions such as caloric restriction are unlikely to have high uptake on a population level. However, other dietary interventions, such as high adherence to the Mediterranean diet (MedDiet), are more feasible; indeed, the MedDiet is positively associated with protection from age-related immune changes (Conway et al., 2024). Intriguingly, components of the MedDiet, such as olive oil-derived oleocanthal, downregulate mTOR activity, which suggests one potential mechanism of action for the diet's anti-ageing effects (Khanfar et al., 2015). More generally, interventions targeting immunosenescence must balance the intervention's cost, feasibility, and likelihood of population-level adherence.

Table 6.1 Summary of immunosenescence interventions that may be genoprotective

Intervention	Evidence for beneficial effect on immunosenescence	Proposed mechanism(s) of action	Evidence for drug genoprotection	Effective dose	
				<i>In vitro</i>	<i>In vivo</i> (human)
Spermidine	Improved vaccine-induced HCV- and RSV- specific responses in CD8 ⁺ T cells from old people (Alsaleh et al., 2020); improved flu-specific immune memory in old mice (Puleston et al., 2014)	Enhances hypusination of eIF5A and translation of TFEB, leading to induction of autophagy-related gene expression (Zhang et al., 2019); increases autophagy in B cells and T cells from old individuals (Zhang et al., 2019, Alsaleh et al., 2020).	Promoted DSB repair by homologous recombination in U-2 OS cells (Lee et al., 2019)	0.01–0.5 mM (Lee et al., 2019, Alsaleh et al., 2020, Puleston et al., 2014)	1.2 mg/day (Schwarz et al., 2018), though higher doses (15 mg/day) can be tolerated (Senekowitsch et al., 2023)
Metformin	Improved survival (in women) from COVID-19 (Bramante et al., 2021) and other studies, reviewed in (Justice et al., 2021)	Multiple targets, many of which act through AMPK activation (Kulkarni et al., 2020)	Elicited pseudo-DDR in cancer cells (Vazquez-Martin et al., 2011); decreased % γ H2AX ⁺ and %8-oxo-dG ⁺ intestinal stem cells in aged or paraquat-treated <i>Drosophila</i> (Na et al., 2013)	5 mM (Vazquez-Martin et al., 2011, Lee et al., 2016)	500 mg twice daily, then increased to 2000 mg daily at the end of 2 weeks led to average plasma concentration 18 μ M metformin (Kulkarni et al., 2018)
p38 MAPK inhibitors	Improved response to VZV challenge in skin in old people (Chambers et al., 2021a, Vukmanovic-Stejic et al., 2018)	Improves efferocytosis of macrophages promoting immune resolution after blister challenge (De Maeyer et al., 2020); decreases CCL2 expression from skin to decrease inflammatory monocyte recruitment to allow adaptive T cell responses (Chambers et al., 2021a)	Restored telomerase expression and reduced reactive oxygen species in senescent T cells (Henson et al., 2014); restored young morphology and proliferation in senescent DNA repair-deficient Werner syndrome fibroblasts (Davis et al., 2005)	3 μ M losmapimod (De Maeyer et al., 2020, Chambers et al., 2021a); 500 nM BIRB 796 (Henson et al., 2014); 10 μ M SB203580 (Davis et al., 2005)	15 mg twice daily losmapimod (De Maeyer et al., 2020, Chambers et al., 2021a)
Vitamin D ₃	14-week vitamin D ₃ supplementation improved cutaneous response to VZV challenge in old people (Chambers et al., 2021b)	Decreases inflammatory monocyte recruitment to site of challenge and allowed resident memory T cell adaptive response (Chambers et al., 2021b)	1,25(OH) ₂ treatment increased recruitment of DNA repair factors to DNA lesions after ionising radiation in human fibroblasts (Graziano et al., 2016); vitamin D ₃ upregulated DNA sensors and effectors and diminished DNA damage in myometrial stem cells isolated from rats	100 nM (Graziano et al., 2016, Elkafas et al., 2020)	6400 IU of vitamin D3 per day for 14 weeks; average serum concentration of 25(OH)D after supplementation was ~

			developmentally treated with diethylstilbestrol (Elkafas et al., 2020)		125 nM (Chambers et al., 2021b)
Nicotinamide riboside	Not yet assessed	Restores NAD ⁺ levels that naturally decline with age (Fang et al., 2019) Activates SIRT1 to promote mitophagy (Fang et al., 2019)	NR enhanced SIRT1-mediated deacetylation of Ku70 in the DNA-PK complex to promote NHEJ-mediated repair of DSBs in ATM-insufficient human neuroblastoma cells (Fang et al., 2016); NR promoted RAD51-mediated HR repair of DSBs (Fang et al., 2019); NR supplementation in aged mice decreased %γH2AX ⁺ muscle stem cells (Zhang et al., 2016)	0.5–1 mM NR (Fang et al., 2016, Fang et al., 2019)	500 mg of NR twice daily for 6 weeks increased PBMC concentration of NAD ⁺ by 60% compared to placebo; median NAD ⁺ levels from 7.7 to 12.2 pmol/mg protein (Martens et al., 2018); 1 g/day NR for 21 days increased blood NAD ⁺ by >2-fold (47.75 μM supplementation versus placebo 20.90 μM) (Elhassan et al., 2019)
Harmine hydrochloride	Not yet assessed	Inhibits DYRK1A and DREAM complex assembly (Bujarrabal- Dueso et al., 2023)	Improved survival from UV- and MMS- induced DNA damage (Bujarrabal-Dueso et al., 2023)	10 μM (Bujarrabal- Dueso et al., 2023)	0.532 ± 0.448 μM (Bujarrabal-Dueso et al., 2023)
Enoxacin	Not yet assessed	Increases activity of RNAi machinery, DICER; promotes the generation of non-coding RNAs recruited to sites of DNA damage (DDRNs) (Gioia et al., 2019)	Treatment of HeLa cells prior to ionising radiation increased accumulation of 53BP1 foci at DNA lesions and decreased tail moment in neutral comet assay (readout for DSBs) (Gioia et al., 2019)	50 μM (Gioia et al., 2019); <i>C. elegans</i> lifespan extension when grown on plates supplemented with 100 μg/ml (312.19 μM) enoxacin (Pinto et al., 2018)	400 mg twice daily enoxacin increased average plasma drug concentrations of 15.86 μM (SD 3.53, range 10.26–24.06 μM) (Hamel et al., 2000)
Physical activity	Not yet assessed	Decreases basal DNA lesions and oxidative DNA damage in circulating leukocytes (Soares et al., 2015)		16-week combined physical exercise training programme (Soares et al., 2015)	

6.3 A new model for the origin of mTOR hyperactivation during ageing

6.3.1 Sexually dimorphic impacts of ageing and mTOR inhibition

mTOR inhibition is one of the most potent life extension strategies documented, and the findings from this thesis offer a potential mechanism for some of its anti-ageing effects. However, it is currently unclear why age-related mTOR hyperactivation, which is targeted by mTOR inhibitors to extend life, originally evolved.

The anti-ageing effects of mTOR inhibition are sex biased. While late-life rapamycin treatment increases lifespan in both male and female mice, the percentage increase in lifespan is consistently higher in females than males (Harrison et al., 2009, Miller et al., 2014). Similarly, genetic deletion of mTOR signalling in mice extends the lifespans of females, but not males, in *Rps6kb1^{-/-}* (Selman et al., 2009), *Igf1r^{+/-}*, (Bokov et al., 2011), and *Mtor^{+/-} Mlst8^{+/-}* models (Lamming et al., 2012). Thus, there are sex-specific effects of mTOR inhibition, which could possibly be explained by sex differences in ageing.

While the experimental research in this thesis does not address the role of sex in ageing and immunosenescence, there is a great disparity in longevity between men and women. In general, biological women (here defined as people bearing two X chromosomes) live longer than men (XY) (Rochelle et al., 2015) though, paradoxically, frailty is higher in women than men of the same age (Park and Ko, 2021). The sexual dimorphism in lifespan is consistent across other mammalian species, albeit to varying extents (Lemaitre et al., 2020), and could be related to sexual dimorphisms in immunosenescence. For example, older men have higher levels of chronic inflammation (inflammageing) than women; furthermore, T cells from older women reveal enhanced proliferation after activation compared to those from men; additionally, women have higher levels of B cells and immunoglobulins, compared to men (Klein and Flanagan, 2016). Broad profiling of chromatin accessibility in immune cells found that age-related changes occurred in two

distinct stages: while there were no sex differences during the first stage (during the late-thirties), the second stage occurred 5-6 years earlier, and more strongly, in men than women (Marquez et al., 2020). Since immunosenescence can drive systemic ageing and premature mortality in mice (Yousefzadeh et al., 2021b, Desdin-Mico et al., 2020), this exacerbation of immunosenescence in males may contribute to the disparity in longevity between the sexes. With relevance to the findings of my thesis, one explanation for the sex differences in immunity and ageing could be related to DNA damage, as lymphocytes from men have a greater DNA lesion burden compared to age-matched women, as measured by the alkaline comet assay (Bajpayee et al., 2002). Another example and potential explanation of the sexual dimorphism in ageing is the existence of the menopause, a phenomenon in which some female mammals live for a significant post-reproductive period. However, the evolutionary basis of the menopause, and how it may relate to extended longevity in women, is unclear.

In this section, I explore how mTOR hyperactivation during ageing may be related to sexual dimorphisms in senescent cell accumulation due to deregulated X chromosome inactivation; furthermore, I argue that an age-related mTOR hyperactivation programme may have evolved as a female-specific evolutionary adaptive strategy for the evolution of menopause.

6.3.2 Sex-specific dynamics of senescent cell accumulation and X chromosome inactivation

The accumulation of senescent cells during ageing is sexually dimorphic. Old male mice accumulate senescent cells in their tissues earlier than do old female mice; however, this difference is lost after a further 5 months of ageing (equivalent to ~10 human years) (Yousefzadeh et al., 2020). Given the known role of senescent cells in driving ageing

(section 1.2.5), this delay in senescent cell accumulation in females, if extended to humans, could plausibly contribute to their superior longevity. The biological basis of this delay is not understood, though could be explained by deregulation of X chromosome inactivation, which controls expression of a component of mTOR activity, *RPS6KA6* (Table 6.2).

In mammals, sex is determined by the Y sex chromosome: while biological males possess XY sex chromosomes, females possess two X chromosomes (XX). For female cells therefore to avoid overdosage of genes on the X chromosome (termed X-linked genes), one X chromosome in each cell is inactivated and associated with a repressive compartment of the nuclear membrane (Grigoryan et al., 2021). This process of X chromosome inactivation (XCI), discovered in 1961 (Lyon, 1961), is orchestrated by the long non-coding RNA, *XIST*, which is expressed by the inactivated X chromosome (Xi). This process does not occur in male cells, and so is sex specific.

XCI becomes deregulated during ageing. For example, the expression of *XIST* decreases during the senescence of female donor cells, suggesting that XCI may become deregulated with cell ageing (Abdelmohsen et al., 2013). Furthermore, haematopoietic stem cells (HSCs) from old female mice display decreased *XIST* expression and deregulated XCI (Grigoryan et al., 2021). Loss of *XIST* expression may be functionally relevant to ageing, as genetic deletion of *XIST* in HSCs in mice led to an ageing-like increase in blood malignancies (Yildirim et al., 2013). These data suggest that *XIST* expression and hence XCI may be deregulated during ageing, leading to an overdosage of X-related genes which could affect senescence.

The X chromosome contains several genes which, when overexpressed, are either geroprotective or pro-senescence; importantly, geroprotective X-linked genes significantly outnumber pro-senescence X-linked genes (Table 6.2). Perhaps the decrease in *XIST* expression during cell ageing in females reactivates the Xi and causes overdosage of X-linked geroprotective genes, thereby protecting females against seno-conversion, delaying

deleterious senescent cell accumulation, and thus affording a female survival advantage (Figure 6.2). However, further XCI deregulation with age could then cause overexpression of pro-senescence X-linked genes such as *RPS6KA6*, a gene involved in mTOR kinase activity (Table 6.2) (Lopez-Vicente et al., 2009). The overdosage of pro-senescence *RPS6KA6* may then lead to mTOR hyperactivation and the rapid accumulation of senescent cells in females that is comparable to males (Figure 6.2). This links female ageing with an mTOR hyperactivation programme and could possibly explain why females benefit more from mTOR inhibition as an anti-ageing intervention (Miller et al., 2014).

Table 6.2 Geroprotective and pro-senescence genes on the X chromosome

Cellular senescence effect	Gene	Name	Cell type investigated
Geroprotective	<i>G6PD</i>	Glucose-6-Phosphate Dehydrogenase	Human fibroblasts (Ho et al., 2000)
	<i>MAGEA2</i>	Melanoma Antigen Gene (MAGE) Family Member A2	Human fibroblasts (Peché et al., 2012)
	<i>MECP2</i>	Methyl-CpG Binding Protein 2	Human mesenchymal stem cells (Squillaro et al., 2010)
	<i>TMSB4X</i>	Thymosin Beta 4 X-Linked	Human nucleus pulposus cells (Wang et al., 2015)
	<i>DKC1</i>	Dyskerin Pseudouridine Synthase 1	Human lung adenocarcinoma cells (Kan et al., 2021)
Pro-senescence	<i>AR</i>	Androgen Receptor	Human dermal papilla cells (Yang et al., 2013b)
	<i>RPS6KA6</i>	Ribosomal Protein S6 Kinase A6	Human colon carcinoma, fibroblasts (Lopez-Vicente et al., 2009)

X chromosome genes and their effects on senescence identified with CellAge. **Bold gene** denotes involvement in mTOR activity. (Source: genomics.senescence.info/cells/).

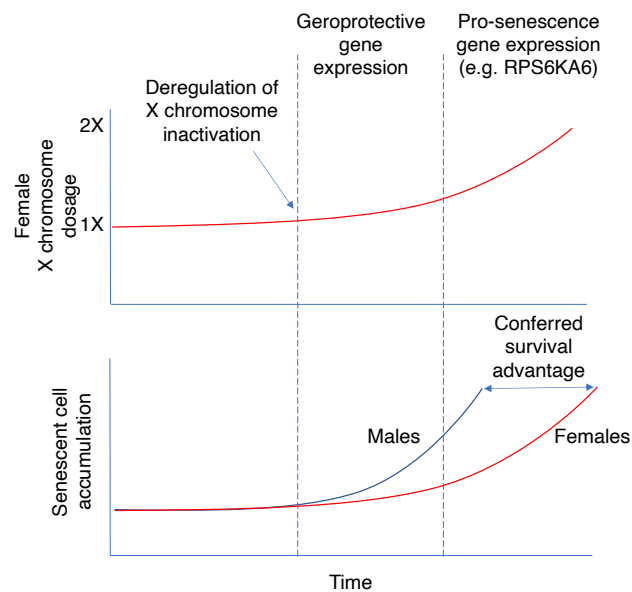


Figure 6.2 Proposed model for the sexual dimorphism in age-related senescent cell accumulation

Initially, XCI deregulation in females increases the dosage of geroprotective genes (Table 6.2), which prevents senescent cell accumulation. As XCI deregulation continues during ageing, however, pro-senescence gene expression (e.g. *RPS6KA6*, which drives mTOR hyperactivity) increases, therefore accelerating senescent cell accumulation to a level comparable to males. The delay in senescent cell accumulation also delays the senescent cell-mediated induction of age-related pathologies, which confers a survival advantage in females.

6.3.3 An mTOR hyperactivation programme may explain the pro-ageing effects of menopause

Female longevity may therefore be controlled by sex-specific dynamics in an mTOR hyperactivation programme following XCI deregulation (Figure 6.2). mTOR hyperactivation may also drive female ovarian ageing and therefore the menopause: *Fmr1*^{-/-} mice, which display premature ovarian ageing and phenocopy the menopause, exhibit high ovarian mTOR activity (as measured by elevated levels of mTOR, S6K, and p-S6K); however, their fertility could be extended with rapamycin treatment (Mok-Lin et al., 2018). These data suggest a central role of mTOR hyperactivation during female ovarian and organismal ageing and have prompted a phase 2 trial of low-dose rapamycin treatment to extend female fertility (5 mg/week for 3 months, NCT05836025). Therefore extended female

longevity compared to males may be explained by a delayed accumulation of senescent cells resulting from deregulation of XCI, an overdosage of X-linked genes (Figure 6.2), and mTOR hyperactivation, which may then lead to accelerated ovarian ageing and the menopause. However, it is unclear why this pro-ageing mTOR hyperactivation may have evolved.

6.3.4 Prolonged female longevity as an evolutionarily adaptive strategy

The induction of the menopause by mTOR hyperactivation may have evolved as an advantageous evolutionarily adaptive strategy. The benefits of a prolonged post-reproductive period (menopause) in women is explained by the Grandmother Hypothesis, whereby older women (grandmothers) confer a survival advantage to their grandchildren through their roles in food gathering and transmission of knowledge to aid their upbringing (Hawkes et al., 1997), termed 'alloparenting'. Alloparenting of grandchildren increases their survival and the propagation of genes shared between grandparent and grandchild, including the genes that specified for the post-reproductive programmes that promoted the alloparenting behaviours. Compared to grandfathers, grandmothers can be maximally certain that they are related to their children, and their daughter's children, as they both physically bear children (Figure 6.3). Grandfathers, on the other hand, have the least certainty about their relationship to their grandchildren (Figure 6.3). Therefore, alloparenting by grandmothers is more likely to benefit genetically related kin and be more evolutionarily advantageous than alloparenting by grandfathers, on average. Furthermore, while male spermatogenesis is largely unaffected by ageing, ageing in females leads to decreased fertility due to long periods of meiotic arrest and the increase of non-disjunction events in female oogenesis (DiLuigi et al., 2008, Subramanian and Bickel, 2008). As mammalian lifespans increased, there was likely a point at which it became evolutionarily more favourable for females to transition into a post-reproductive period in which alloparenting

was preferred over the production of 'sub-optimal' embryos resulting from non-disjunction events. The biochemical basis of this may have occurred through the evolution of a pro-ageing mTOR hyperactivation programme which promotes ovarian ageing, and therefore encouraged alloparenting. This female-specific mTOR hyperactivation programme may then have pleiotropically manifested in males due to the sharing of genetic material across sexes within the same species. This model is presented in Figure 6.4.

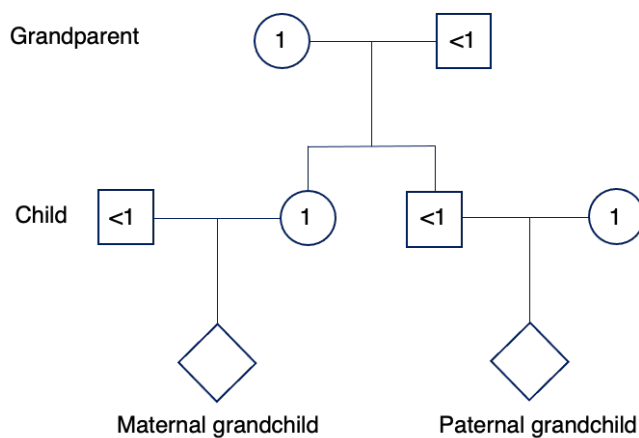


Figure 6.3 Sex differences in kin certainty

Parental certainty of males (squares) and females (circles). Numbers represent parental certainty (maximum 1). Maternal grandmothers have 1×1 parental certainty for their grandchildren. Maternal grandfathers and paternal grandmothers have $(<1) \times 1$ certainty, with paternal grandfathers experiencing the least certainty $(<1) \times (<1)$. Therefore, on average, grandmothers have more kin certainty for their grandchildren than grandfathers, supporting the evolution of genetic programmes that promote alloparenting of related kin in females more than males.

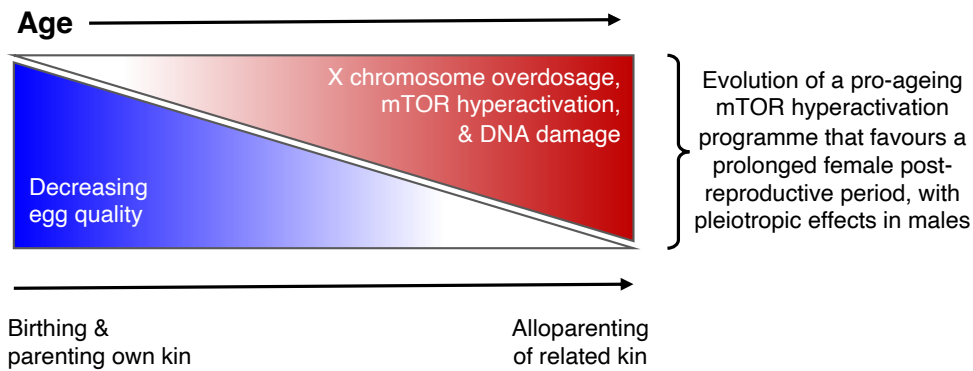


Figure 6.4 Summary of model for the evolution of an mTOR hyperactivation programme

With increasing age in females, egg quality decreases due to extended periods of meiotic arrest leading to increases in nondisjunction events. As a result, it becomes more evolutionarily favourable for females to cease fertility and enter a post-reproductive period where instead they use their resources for alloparenting of their kin, with whom they share maximal certainty that they are related. This transition may be partially coordinated by deregulation of X chromosome inactivation, leading to the overdosage of X-related genes, some of which are geroprotective (Table 6.2) and may partially delay the accumulation of senescent cells in females (Figure 6.2). However, some pro-senescence genes, such as *RPS6KA6*, promote mTOR hyperactivation and senescence induction. This causes an mTOR-dependent, pro-ageing programme in females that favours a post-reproductive period and alloparenting. This mTOR hyperactivation programme for ageing pleiotropically manifests in males as genetic material is shared within the species.

Though testing theories of evolution is complex, there are some elements of this model that could be investigated experimentally. First, the expression of X-linked geroprotective vs pro-senescence genes could be measured in female cells during ageing and entry into senescence using qRT-PCR. Second, to test the hypothesis that XCI deregulation and X chromosome overdosage drives the overexpression of certain geroprotective and pro-senescence genes, and how this affects mTOR activity and senescent cell accumulation *in vitro* and *in vivo*, models with tuneable *XIST* expression could be developed. Third, the data in this thesis suggest that mTOR inhibition is a potent genoprotective strategy: this, taken together with the ideas above, leads to the question as to whether mTOR hyperactivation in female ageing drives a pro-DNA damaged phenotype and senescence induction, which could also be tested experimentally.

More generally, this model predicts that the future success of anti-ageing drugs, such as the mTOR inhibitor rapamycin, would cause an extension of the fertility period in females, and potentially preclude the evolutionary 'need' for a menopause. Though this is difficult to test experimentally, analysis of real-world data already indicates that, with the increase in lifespans over the past 60 years due to improved healthcare, sanitation, and quality of life, both the age of onset of menopause and total reproductive period in women have increased by 1.5 and 2.1 years, respectively (Appiah et al., 2021). Given these sexually dimorphic evolutionary strategies for ageing, it is of the utmost importance to assess anti-ageing interventions using an intersectional approach, to ensure that longevity research benefits all people equally.

7

Supplementary data

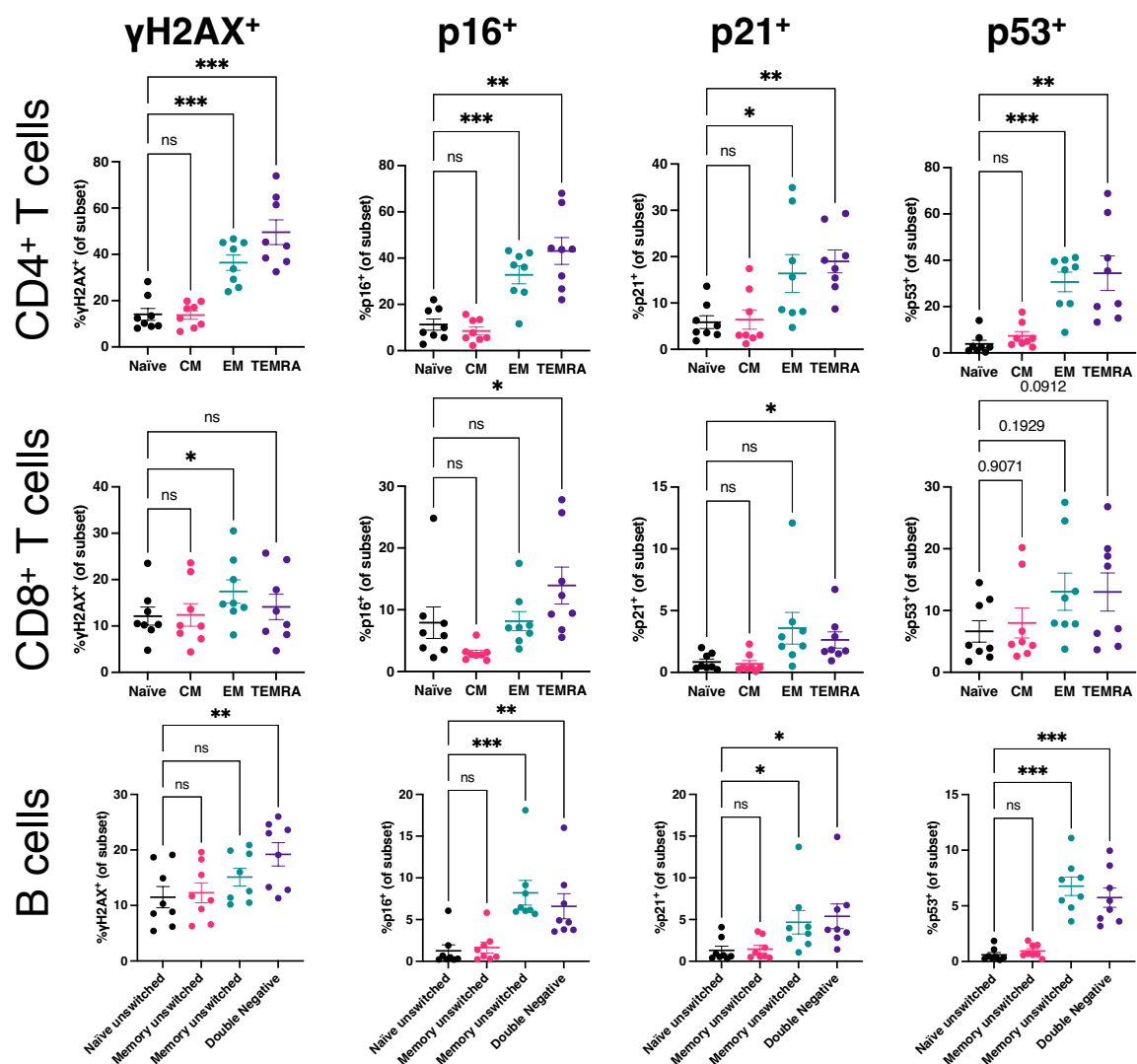


Figure 7.1 Proportion of adaptive immune cells positive for senescence biomarkers in age-associated immune subsets from healthy NHS-BT donors

Adaptive T and B cells in PBMCs from NHS-BT donors were analysed by spectral flow cytometry and gated as in Figure 5.1, and the proportion of cells positive for γH2AX, p16, p21, and p53 within each immune subset across the different cell types was assessed. *P*-values are derived from a one-way ANOVA with comparisons between each subset and its naïve counterpart calculated using Dunnett's multiple comparisons test, *n*=8 healthy NHS-BT donors.

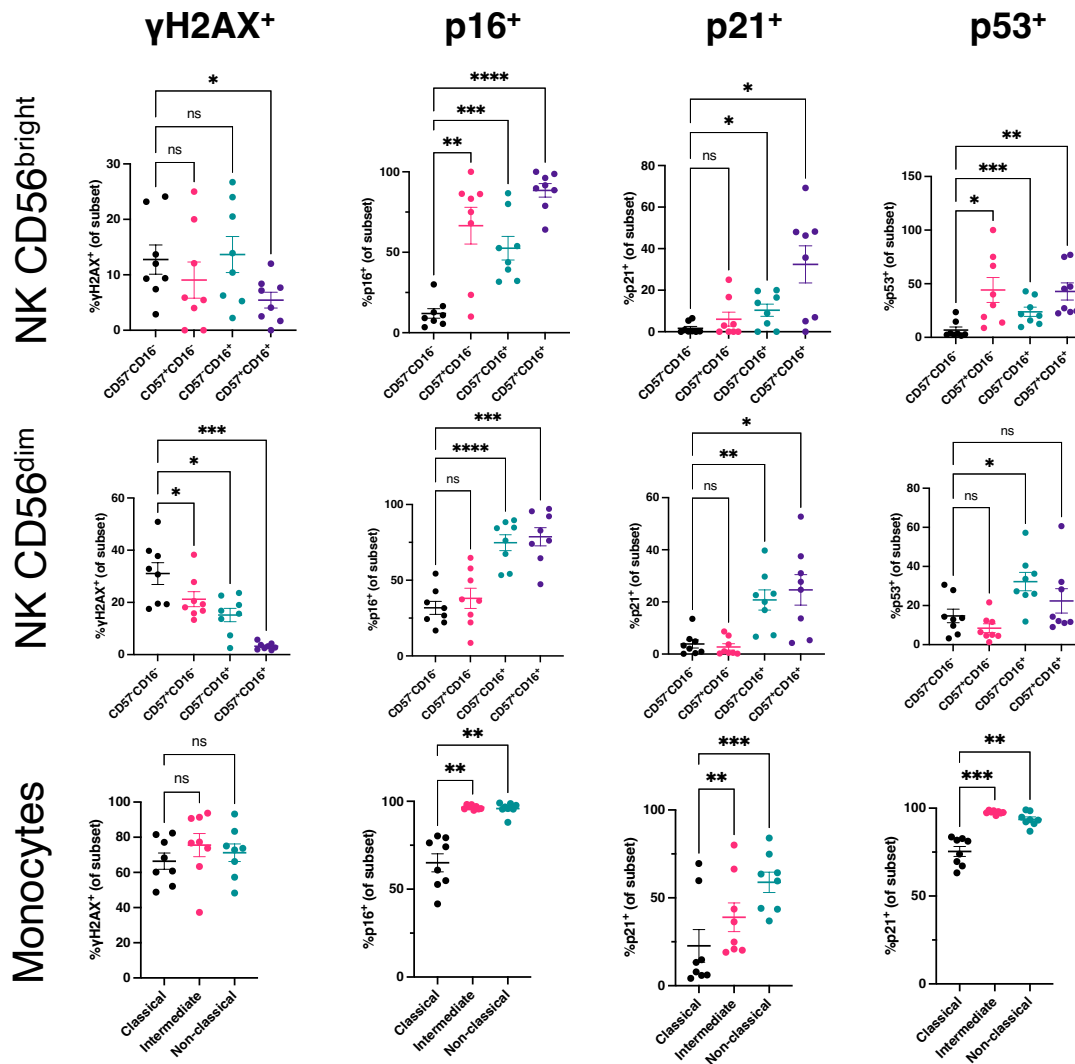


Figure 7.2 Proportion of innate immune cells positive for senescence biomarkers in age-associated immune subsets from healthy NHS-BT donors

Innate monocytes and natural killer (NK) in PBMCs from NHS-BT donors were analysed by spectral flow cytometry and gated as in Figure 5.1, and the proportion of cells positive for γ H2AX, p16, p21, and p53 within each immune subset across the different cell types was assessed. *P*-values are derived from a one-way ANOVA with comparisons between each subset and its naïve counterpart calculated using Dunnett's multiple comparisons test, *n*=8 healthy NHS-BT donors.

8

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