Université de Montréal

Study of the Role of the p16^{INK4a} Gene in Tumor Progression and Tissue Regeneration/Function Following Exposure to Ionizing Radiation.

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Cette thèse intitulée:

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Résumé

La sénescence est un important mécanisme cellulaire qui prévient la tumorigenèse et se caractérise par un arrêt permanent du cycle cellulaire orchestré principalement par les inhibiteurs des cycline-kinases dépendantes (i.e p16^{INK4a}). La sénescence est une caractéristique importante du vieillissement, mais un déséquilibre dans son induction peut être délétère pour la régénération tissulaire et paradoxalement pour la progression tumorale. L'irradiation (IR) est couramment utilisée comme approche thérapeutique dans le cancer. Chez les enfants survivants du cancer, l'exposition à l'irradiation et à la chimiothérapie entrainent le développement d'importants effets secondaires, lesquels sont associés à une forme de vieillissement prématuré. La formation de cellules sénescentes, en inhibant la prolifération tissulaire et en sécrétant des cytokines proinflammatoires, pourrait être en être responsable. Notre groupe a précédemment démontré que le gène p16^{INK4a} est augmenté de manière tardive (environ 8 semaines) suite à une exposition à l'irradiation. Il n'a pas encore été étudié si cette expression retardée survient en réponse aux dommages causés par l'irradiation sur l'homéostasie tissulaire ou à titre de mécanismes de suppression tumorale. Un objectif de cette thèse visait donc à déterminer s'il était possible de moduler/inhiber l'expression de p16^{INK4a} dans le but d'accroitre la régénération tissulaire sans nécessairement accroitre les risques d'incidence du cancer. En effet, ceci pourrait être possible dans la mesure ou la sénescence induite par p16^{INK4a} est également irréversible

III

in vivo. Nos résultats ont démontré que l'inhibition de l'expression de p16^INKa (suite à l'administration de tamoxifen chez les souris p16L/LCre), induit à la fois une augmentation de la régénération tissulaire mais malheureusement également une augmentation de l'incidence du cancer. Nous voulions également connaitre l'impact de l'accumulation de ces cellules sénescentes sur les tissus, plus spécifiquement sur la fonction des cellules immunitaires de la rate. Nous avons démontré que des altérations (dépendantes de p16^INK4a) au sein du microenvironnement splénique pouvaient altérer les fonctions intrinsèques des macrophages, des cellules dendritiques et des lymphocytes T. En outre, l'élimination systémique des cellules p16^INK4a positives (modèle de sourie p16-3MR) a conduit à une restauration partielle de la fonction de ces cellules immunitaires. La combinaison de ces données nous permet de mieux comprendre le rôle et la fonction du gène p16^INK4a dans le processus de sénescence induite par l'irradiation. Nos résultats suggèrent qu'il est envisageable d'utiliser des agents pharmacologiques tels que des composés sénolytiques, capables d'induire l'apoptose chez les cellules sénescentes spécifiquement, afin de potentiellement diminuer les effets du vieillissement prématuré induit par la sénescence cellulaire chez les survivants du cancer.

Mots-clés: Rayonnement ionisant, sénescence, microenvironnement splénique, cellules stromales, INK4a / ARF, fonction hématopoïétique, phagocytose, prolifération cellulaire, activité b-gal, prolifération des cellules T.

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Abstract

Senescence is an important cellular mechanism that prevents tumorigenesis and is characterized by a permanent cell cycle arrest orchestrated by cyclin-dependent kinases inhibitors (i.e p16^{INK4a}). Senescence is an important hallmark of aging and unbalanced levels of senescence is considered deleterious for tissue regeneration, and paradoxically for tumor progression. Irradiation (IR) is commonly used therapeutic approach in cancer treatment. Together with surgery and chemotherapy, it has helped to increase the life expectancy of patients and, in some cases, leads to complete remission. However, long-after therapy, children who survive cancer encounter alterations in the integrity of tissues/organs associated with premature aging. The accumulation of senescent cells may be responsible for this accelerated aging by limiting tissue proliferation and secreting pro-inflammatory cytokines. Our group has previously demonstrated that the p16^{INK4a} gene is increased in a delayed manner (approximately 8 weeks) following exposure to IR. It has not yet been investigated whether this delayed expression occurs in response to IR-induce damage of tissue homeostasis or as tumor suppression mechanisms. One objective of this thesis was to determine whether it was possible to modulate / inhibit the expression of p16^{INK4a} in order to increase tissue regeneration without necessarily increasing the risk of cancer incidence.

Indeed, this may be possible since p16^{INK4a}-induced senescence is also irreversible *in vivo*. Our results demonstrated that the inhibition of p16^{INK4a} expression in conditional-p16^{INK4a} null mice, induces both an increase in tissue regeneration but unfortunately also an increase in the incidence of cancer. We also

wanted to know the impact of the accumulation of these senescent cells on the tissues, more specifically on the function of the immune cells in the spleen. We have demonstrated that alterations (p16^{INK4a}-dependent) within the splenic microenvironment can alter the intrinsic functions of macrophages, dendritic cells and T cells. In addition, the systemic elimination of p16^{INK4a} positive cells (mouse model p16-3MR) has led to a partial restoration of the function of these immune cells. The combination of these data allows us to better understand the role and function of the p16^{INK4a} gene in the irradiation-induced senescence process. Our results suggest that it is conceivable to use pharmacological agents such as senolytic compounds, capable of inducing apoptosis in senescent cells specifically, in order to potentially reduce the effects of premature aging induced by cellular senescence in cancer survivors.

Keywords: ionizing radiation, senescence, splenic microenvironment, stromal cells, INK4a/ARF, p16^{INK4a}, hematopoietic function, phagocytosis, cell proliferation, T cell proliferation, regeneration.

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ANOVA:	Analysis of variance
APC:	Antigen-presenting cells
ARF:	Alternative Reading Frame
ASF1a:	Antisilencing function 1a
ATM:	Ataxia-telangectasia mutated
ATR:	ATM and rad3 related
BCL-2:	B-cell lymphoma 2
BER:	Base excision repair
BM:	Bone marrow
BMDM:	Bone marrow-derived macrophages
BrdU:	5-bromo-2'-deoxyuridine
CDK:	Cyclin-dependent kinase
CDKIs:	Cyclin-dependent kinase inhibitors
CDKN2a:	Cyclin-dependent kinase inhibitor 2a
C/EBPβ:	CCAAT/enhancer-binding protein-β
CFSE:	6-carboxy-succinimidyl-fluorescein-ester dye
CHK1:	Checkpoint kinase-1
CHK2:	Checkpoint kinase-2
COX:	Cyclooxygenase
CTLA-4:	Cytotoxic T lymphocyte-associated molecule-4
CTZ:	Coelenterazine
DAPI:	4,6-diamidino-2-phenylindole
DCs:	Dendritic cells
DDR:	DNA-damage response
DMP1:	D binding myb-like protein 1
DNA-SCARSDNA:	Segments with chromatin alterations reinforcing senescence
DSB:	Double strand breaks
DTR:	Diphtheria toxin receptor
eGFP:	Enhanced green fluorescence protein

Ets:	E-26 transformation-specific
EV:	Extracellular vesicles
FBS:	Fetal bovine serum
FDCs:	Follicular dendritic cells
FOXO:	The forkhead box O family
FRCs:	Fibroblastic reticular cells
GATA4:	GATA binding protein 4
GCV:	Ganciclovir
Gy:	Gray
H3K9Me:	Lysine 9 methylated histone H3
TERT:	Telomerase reverse transcriptase
HIRA:	Histone repressor A
HIV-1:	Human immunodeficiency virus type 1
HMGA:	High mobility group A
HP1:	Heterochromatin protein 1
HR:	Homologous recombination
HRP:	Horseradish peroxidase
HSC:	Hematopoietic stem cell
Id:	DNA Binding 1
IF:	Immunofluorescence
IGFBP-7:	Insulin-like growth factor binding protein 7
INF-γ:	Interferon gamma
IL:	Interleukin
ILT2:	Ig-like transcription 2
IP:	Intraperitoneal injection
IR:	Ionizing radiation
ITSE:	INK4a transcription silence element
KC:	keratinocyte chemoattractant
KIR:	Killer immunoglobulin-like receptors
KLRG:	Killer cell lectin-like receptor subfamily G

LAG-3:	Lymphocyte activation gene 3
LPS:	Lipopolysaccharides
LCMV-Arm:	Lymphocytic choriomeningitis virus Amstrong strain
MAdCAM-1:	Addressin cell adhesion molecule 1
MCP-1:	Chemoattractant protein-1
Mdm2:	Mouse double minute 2 homolog
MEFs:	Mouse embryo fibroblasts
mH2A:	Histone variant macro H2A
MiRNA:	Micro RNA
MLR:	Allogenic mixed lymphocyte reactions
MMM:	Marginal metallophilic-macrophages
MMPs:	Matrix Metalloproteinases
MMR:	Mismatch repair
MRCs:	Marginal reticular cells
mTOR:	Mechanistic target of rapamycin
MVEs:	Intracellular multi-vesicular endosomes
MZ:	Marginal Zone
MZM:	Marginal zone macrophages
NER:	Nucleotide excision repair
NF1:	Neurofibromatosis type I
NF-κB:	Nuclear factor of the kappa light
NHEJ:	Nonhomologous end joining
NK:	Natural killers
NLRs:	Nod-like receptors
8-oxo-dG:	8-oxo-2-deoxyguanosine
OIS:	Oncogenic-induced senescence
MAPK:	Mitogen-activated protein kinase
PAMPs:	Pathogen associated molecular patterns
PALS:	Periarteriolar lymphoid sheath
PcG:	Polycomb group protein

PD:	Population doublings
PD1:	Programmed death 1
PD-L1:	Programmed death-ligand 1
PDNP:	Podoplonin
PE:	Phycoerythrin
PGE$_2$:	Prostaglandin E$_2$
PI:	Protease inhibitors
PI3K:	Phosphatidylinositol kinase
PML:	Promyelocytic leukemia
PPARα:	Peroxisome proliferator-activated receptor alpha
PRR:	Pathogen recognition receptors
PPRE:	Peroxisome proliferator response element
PRC:	Polycomb transcription factor
Rb:	Retinoblastoma
RE:	p53-responsive element
RIPA:	Radioimmunoprecipitation assay buffer
RLRs:	RIG-I like receptors
ROS:	Reactive oxygen species
RPMI:	Roswell Park Memorial Institute medium
RT:	Room Temperature
SA-β-gal:	Senescence-associated β-galactosidase
SASP:	Senescence associated secretory phenotype
Sp1:	Specificity protein 1
SSB:	Single strand Breaks
SSBR:	Single-strand break repair
TAM:	Tamoxifen
TGFβ:	Transforming growth factor beta
Tim-3:	T-cell immunoglobulin mucin domain-3
TIS:	Senescence induced by genotoxic stressors
TK:	Thymidine kinase

TLR:	Toll like receptor
TNF-α:	Tumor necrosis factor alpha
TOR:	Target of rapamycin
TRANCE:	Tumour necrosis factor family member
UTR:	The 3`-untranslated region
UV:	Ultraviolet
VEGF:	Vascular endothelial growth factor
WHO:	World health Organization
X-gal:	5-bromo-4-chloro-3-indolyl--D-galactopyranoside
YB-1:	Y box-binding protein 1

To all my Family

-A mi familia

Greg and Emma Flora-

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Chapter 1: Introduction

1.1 Senescence as a hallmark of aging

In the recent decades, the proportion of the population over the age of 60 is higher than in any time in history and continues to grow and may outpace the younger population for many years to come. The societal effects of this demographic transformation are unknown but as the aged population worldwide increases, the incidence of age-related diseases will also rise. The World health Organization (**WHO**) highlights the importance to develop an effective health response to new disease patterns in aging populations to reduce the economic and social challenges [1].

Aging is defined as the time-dependent functional deterioration of physiological integrity leading to diminished function and augmented vulnerability to death. This phenomenon gathers important characteristic hallmarks that contribute to the aging process; these are genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (Figure 1.1) [2]. Among these, senescence is one of the most relevant factors affecting the aging population for many reasons. First, senescent cells accumulate *in vivo* during aging contributing to autonomous and non-autonomous effects that modulate the tissue microenvironment. Secondly, this accumulation limits the tissue regeneration capability, which influences the functional deterioration of organs. Despite a young chronological age of individuals, senescent cells can increase at a faster accumulation rate after exposure to stressors like

radiation, chemotherapy, high-fat nutrition; environmental toxicants such as arsenic or cigarette smoke provoking an accelerate-like age phenotype [3-9]. Lastly, clearance of senescent cells from tissues can lead to an extension of life span by rejuvenating organs and potentially improving functional as discussed in more details in future sections [10-13]. Although, senescence is considered a deleterious phenomenon in aging, in early stages of life plays an important role in embryogenesis, tissue regeneration, wound healing and cancer suppression.
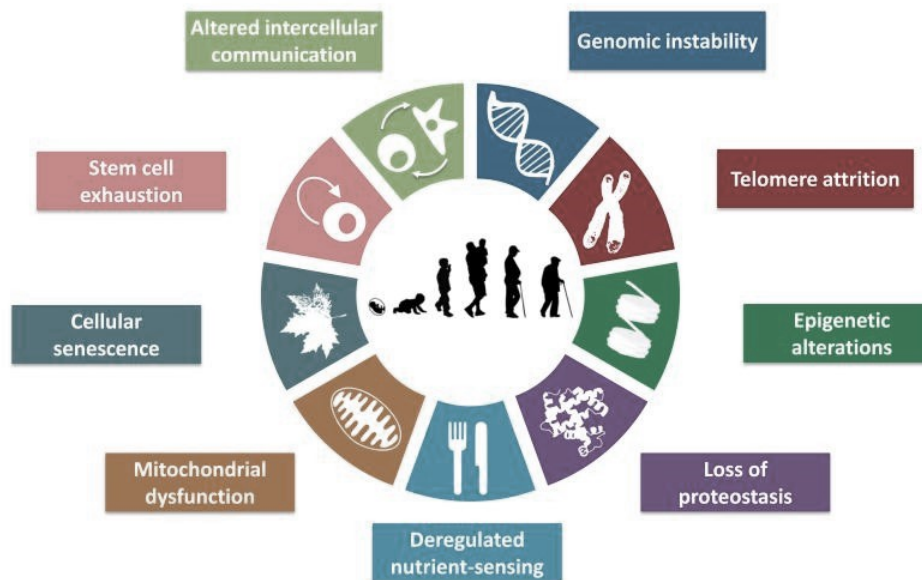
Figure 1.1 The hallmarks of aging

During normal aging individuals harbor typical characteristics that cooperate to impair the function of tissues/organs. The word senescence can be traced to the Latin word *senex*, meaning old man. In cellular biology, senescence refers to a state of permanent cell growth arrest with characteristic features [8,9,14-17]. Adapted from Lopez-Otin *et al*., 2013 [2].

1.2 Senescence phenotype

Experiments in cultured cells have identified a series of characteristics or markers in senescent cells that are summarized below. Moreover, not all senescent cells express all the markers, but when analyzed together they can define this state.

1.2.1 Cell cycle arrest

The cell cycle consists essentially of four basic phases: checkpoint Gap 1 (G1), DNA synthesis (S phase), checkpoint G2 and mitosis (M phase). In S phase, cells replicate the genomic DNA, which is distributed to two daughter cells in M phase. Most of the time the irreversible arrest of senescence occurs predominantly in G1/S checkpoint, triggered by telomeric-DNA damaged by eroded telomeres, or telomeric-independent DNA insult such as oncogene activation, loss of tumor suppressor genes and/or DNA-damage inductors [8,9,18-20]. The molecular basis of this G1/S arrest results from the accumulation of cyclin-dependent kinases (CDK)-inhibitors p16^{INK4a}, p19^{ARF} and p21^{Cip1} proteins. p16^{INK4a} protein arrests the cell cycle by limiting cyclin-dependent kinases 4 and 6 (**CDK4/6**) activity, impeding the retinoblastoma protein (**Rb**) phosphorylation. As consequence, Rb remains associated with the E2 factor (**E2F**) transcriptional factor localizing it to the cytoplasm, thus repressing transcriptional activity required to overpass G0/G1/S phase transition (Figure 1.2) [21]. In addition, Rb activation in some cells organize the formation of cytologically detectable regions of heterochromatin associated with senescence foci (**SAHF**). This facultative heterochromatin accumulates in many irreversible proliferation-arrested cells. In fact, SAFH is though to contribute to this permanent arrest of

proliferation by repressing-expression of proliferation-promoting genes necessary for the progression of the cell cycle [22-24].
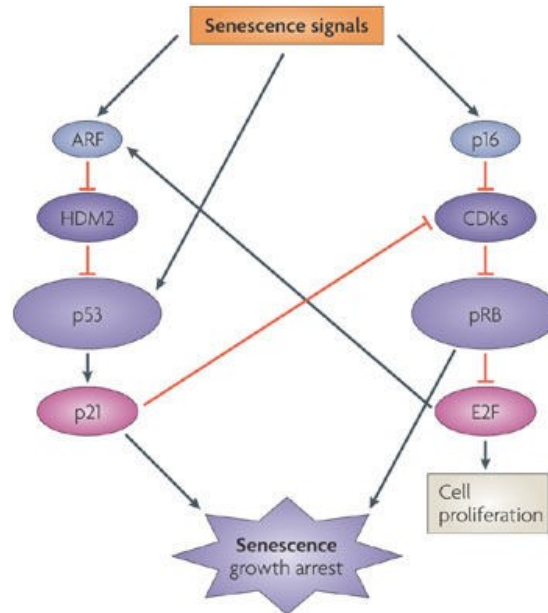
Figure 1.2 Molecular pathways of senescence

p16^{INK4a} regulates cell cycle progression through hypophosphorylation of Rb. p16^{INK4a} binds to CDK4 and 6 to disable interaction of D-type cyclins. This maintains Rb in a hypophosphorylated state with E2F repressionally bound and thus inhibiting cell cycle progression and arrest in G0/G1 phase. Adapted from Campisi & Di Fagagna, 2007[8].

In contrast, p19^{ARF} inhibits the E3 ubiquitin ligase activity of mouse double minute 2 homolog (**MDM2**), leading to stabilization of p53 and inducing p53-dependent growth arrest or apoptosis depending on the biological context. In wild-type mouse embryo fibroblasts (MEFs), p19^{ARF} and p16^{INK4a} both accumulate significantly after passaging, but spontaneous escape from senescence occurs through loss of the ARF–p53 axis rather

than INK4a–Rb [25]. *P16^INK4a* expression is highly dynamic with temporal and spatial compartmentalization with low level expression during embryogenesis, is undetectable in young healthy and proliferating cells, but its expression dramatically increases in chronological aging, wound healing and under cellular stress conditions [6,26-29]. In addition, cell arrest can occur in S and G2/M phases of the cell cycle depending on the cell type and the origin of stimulus [8,18-20]. Notably, Irradiation (IR)-induced DNA damage can also induce senescence during G2/M transition triggering p53/p21[Cip1] response [30,31]. However, long-term G2 senescent cells can undergo G2 slippage, bypass the M phase and directly entering G1 phase, consequently becoming senescent as tetraploid cells that fail to subsequently divide (4N DNA content) [31,32].

1.2.2 Altered morphology

The senescent cells are accompanied by changes in their morphology. Such changes include enlarged, irregular and flattened cytoplasm (*in vitro*), with increased vacuoles and lysosomal content associated to high expression of the lysosomal hydrolase β-galactosidase (encoded by *GLB1* gene) [18,23,33].

1.2.3 Senescence-associated β-galactosidase activity

The β-galactosidase is a lysosomal hydrolase enzyme whose function is to cleave the β-glycosidic bond of a large variety of substrates such as gangliosides, glycoproteins and glycosaminoglycans. In 1995, Dimri and colleagues (1995) proposed using SA-β-gal to differentiate senescent fibroblasts and keratinocytes from quiescent and pre-senescent

cells. SA-β-gal remains one of the most extensively used biomarkers of cellular senescence *in vitro* and *in vivo* [34]. In normal young cells, its activity can be determined using chromogenic substrate the 5-bromo-4-chloro-3-indolyl β-D-galacto-pyranoside (**X-Gal**) at pH 4. In contrast, in senescent cells its activity is evaluated at pH of 5-6 (Figure 1.3). SA-β-gal presents limitations as parameter to define senescence both *in vitro* and *in vivo*, regardless of being considered a specific marker for senescence. For example, this enzymatic staining can be performed only on fresh frozen tissues; and *in vitro* SA-β-gal activity can be detected in cells in various nonsenescent states. Therefore, it is important to consider other senescent markers such as *p16^{INK4a}* expression, DNA-damage foci and the senescence-associated secretory phenotype (**SASP**), see below [6,10,23,34-41].

1.3 Epigenetic stress of senescent cells

1.3.1 SAHF a hallmark of senescence

Chromatin constitutes one of the most complex and dynamic macromolecular structures in the nucleus of a cell. The genomic DNA can be associated to a wide range of proteins and RNA molecules working in concert to mediate gene transcription or repression. The packaging of chromatin can be loosely packaged euchromatin and thus favoring access to the transcriptional machinery, or tightly packaged heterochromatin thereby limiting gene expression. Narita *et al*., (2013) observed that the nuclei of senescent cells contain 30–50 bright, punctate DNA-stained dense foci (Figure 1.3) that can be readily

distinguished from chromatin in normal cells [23,42-47]. Cytologically, individual SAHF appears as a compacted chromosome observed as punctuated DAPI- dense foci (Figure 1.3). In senescence, these heterochromatin structures are known as SAHF which correspond to highly compacted chromatin and they are not associated with cells undergoing quiescence. Importantly, these structures have also been shown to be different from constitutive heterochromatin because centromeres, telomeres, and other constitutive heterochromatin regions are not included in SAHF [23,43,48]. Experimental evidences in senescent cells have suggested that SAHF are responsible for sequestrating and silencing genes associated with the progression of the cell cycle; for example, E2F genes such as cyclin A related with proliferation [23,42-47]. Thus, the formation of heterochromatin in senescence is thought to contribute to permanent growth arrest [23,49] [23,43,48].
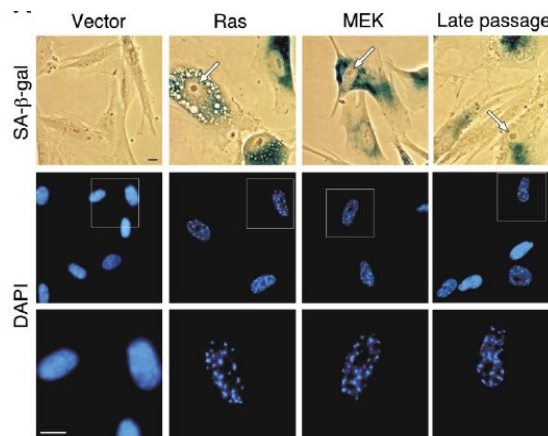
Figure 1.3 Senescent cells express specific characteristics

Cellular senescence induced by oncogene overexpression *H-Ras^V12* (*Ras*) and *Mek1^Q56P (Mek)* or telomere attrition (late passage) possesses specific features, altered morphology with a larger and flattened shape, a multivacuoled cytoplasm

(arrow) SA-β-gal expression and DAPI-dense foci termed senescence-associated heterochromatic foci (**SAHF**). Adapted from Narita *et al.*, 2003 [23].

SAHF also contribute to the tumor suppressive function of senescence by limiting the extent of DNA damaging signaling which may prevent senescent cells from undergoing apoptosis induced by high DNA damage signaling. Some senescent cells can also be identified by the formation of foci associated with a persistent activation of the general response to "DNA Damage Response (DDR)" [50]. These DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS) contain proteins associated with DNA damage such as ATM, ATR, DNA-PK, phosphorylated histone H2AX (γ-H2AX) and p53 binding protein 1 (53BP1) [50,51]. Another distinct type of senescence-specific nuclear structures associated often at the periphery of PML nuclear bodies [51-53]. PML bodies contribute to senescence by recruiting pRB/E2F complexes and suppressing E2F target gene expression [53-55].

1.3.2 The nuclear Lamina and senescence

The integrity and shape of the nucleus is supported by an intricated mesh-like structure named the nuclear lamina. The nuclear lamina besides providing mechanical support, is involved directly or indirectly in functions such as chromatin structure, DNA replication, cell division and nuclear compartmentalization [56]. Topologically, genes generally expressed at low levels are organized into large areas known as *lamina associated domains*. These domains are depleted for active histone marks but include large domains of H3K9me3 and H3K27me3 enrichments and DNA hypomethylation [57].

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Therefore, the nuclear lamina is considered to be a repressive chromatin environment associated to heterochromatin regions, including low gene density and repressed gene expression regions, with enrichment for repressed histone marks [58] [57].

1.3.3 The Lamina A and B

The critical components of the nuclear lamina are Lamins A/C and B, alterations in their expression or functionality lead to disturbances in the integrity of the nuclear lamina and therefore are the caused of abnormal nuclear morphology, DNA damage, and chromosomal aberrations. In the context of accelerated aging or acute-induced senescence using multiple models of progerias the nuclear lamina instability is a key player in SAHF formation. For example, a single genetic alteration of Lamin A has a profound effect of on the mechanisms of aging and chromatin structure [59-62]. Specifically, progeria cells harbor mutations in Lamin A resulting in a reduced expression of Lamin B1. Similarly, replicative senescent fibroblasts, oncogene-induced senescence, and cells with high levels of chronic DNA damage decreased Lamin B1 expression [62-64]. The loss of Lamin B1 expression leads to the disassociation between the nuclear lamina and *lamina associated domains* during accelerated-aging (progeria) and chronic-induced senescence [61,65]. In fact, it was recently shown that SAHF formation is aided by this loss in anchoring of chromatin to the nuclear lamina [56-65]. SAFH formation is suggested to be the result of a structural rearrangement of the three dimensionally architecture change rather than a redistribution of epigenetic marks [61]. Loss of Lamin B1 also appears to cause a redistribution of histone marks, including an enrichment in H3K27me3 and H3K4me marks within Lamin B1-associated domains and depletion in H3K27me3

marks in enhancers and genes [61,65]. Experimental observations linked to the exclusive acute-stress SAHF formation, with upregulation of heterochromatin markers such as HP1 and histone H2AX and absence of heterochromatin relaxation.

1.3.4 The senescence-associated secretory phenotype

The senescent cells bearing DNA-SCARS remain metabolically active, nonetheless upregulate a wide range of genes as part of the senescence-associated secretory phenotype (**SASP**) [15,66-68]. The most remarkably increased genes encode a wide range of secreted inflammatory proteins such as cytokines (e.g. interleukin (IL) IL-1α, IL-6, and IL-1β), chemokines (e.g. IL-8, MCP-1, KC), growth factors (e.g. bFGF, HGF/SF, IGFBPs), proteases (e.g. matrix metalloproteinase -MMP-) and other non-soluble extracellular matrix proteins (e.g. collagens, fibronectin, laminin); in addition to upregulation of genes, senescent cells secrete exosomes [9,67,69-71]. The augmented secretion of SASP confers multiple cell-autonomous and non-cell-autonomous activities. These biologically active factors provide both beneficial and/or deleterious responses in neighboring cells [9,15]. Understanding this apparent paradox may be particularly important depending on the senescence inducing stimuli and cellular context [69].

1.3.5 Regulation of SASP expression

The general understanding of how the expression of SASP is regulated remains poorly understood, and varies depending on the cell type and trigger of cellular senescence [72]. SASP secretion is regulated through DDR-dependent and DDR-

independent mechanisms. In persistent DDR, signals emanating from DNA-SCARS activate nuclear factor of the kappa light (NF-κB) and CCAAT/enhancer-binding protein-β (C/EBPβ) transcription factors of SASP genes including of IL-1α, IL-6 and IL-8 (Figure 1.4) [67,73]. However, the DDR response is not considered to be the only regulator of SASP, since the DDR is activated immediately after damage, whereas SASP, like other aspects of the senescent phenotype such as the activity of β-galactosidase, takes several days [72,67,73]. So, SASP phenotype requires autocrine positive (continuing reading below).
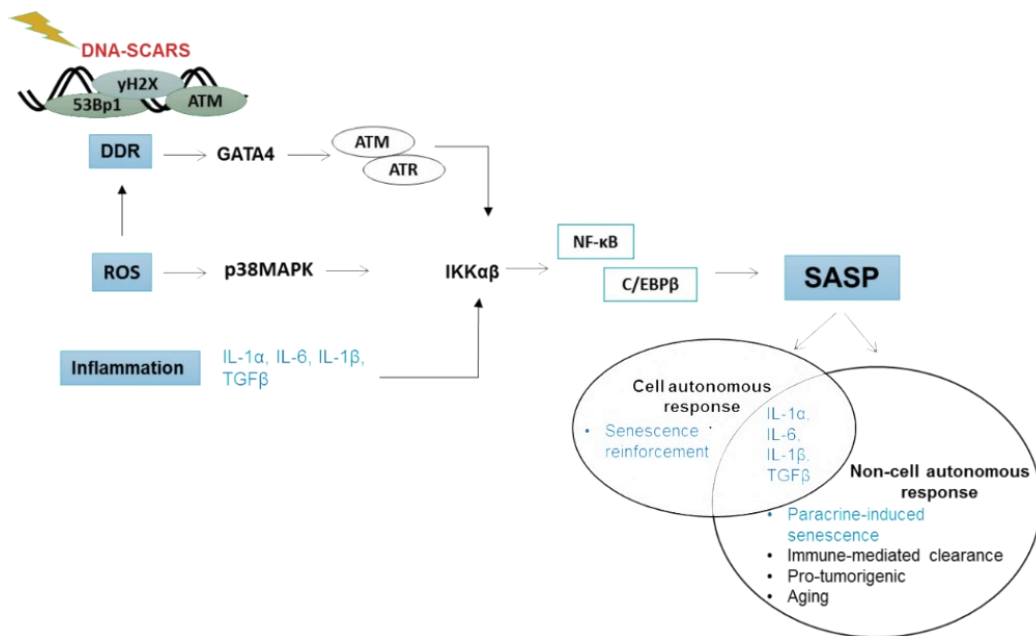
Figure 1.4 DDR-dependent and DDR-independent mechanisms that control the senescence-associated secretory phenotype (SASP).
Different networks and their components interact to activate NF-κB and/or C/EBPβ transcriptional activators of SASP factors. Adapted from Lujambio (2016) [74].

1.3.6 Cell-autonomous reinforcement of senescence

SASP can reinforce the senescent phenotype in an autocrine manner through an amplification system. IL-1α is an essential positive regulator of IL-6/IL-8 expression. The IL-1α signalling network acts through p38MAPK and/or mammalian target of rapamycin (**mTOR**) pathways and therefore stimulates the expression and activity of NF-κB and C/EBPβ [75]. In addition, recent evidence suggests that other SASP factors such as IL-6, IL-8, CXCL1 (also named GROα, KC) and insulin-like growth factor binding protein 7 (**IGFBP-7**) act in an autocrine manner reinforcing senescence in a positive feedback loop [66,68].

1.3.7 Non-cell-autonomous activity of SASP

The SASP possesses the capacity to transmit signals to the normal neighboring cells. The paracrine-induced senescence is orchestrated by cytokines IL-1α, IL-1β, IL-6, VEGF and the transforming growth factor beta (**TGFβ**) signaling (Figure 1.4) [66,68,76]. Importantly, Ohanna *et al*., 2011 has shown that SASP factor MCP-1, (CCL2) expressed by senescent melanoma cells, induces DNA lesions in naive melanoma cells [77].

Paracrine effects of SASP in early stages of life are beneficial and play an important role in processes such as tissue regeneration, embryogenesis, tissue remodeling, limb regeneration (in salamanders) and wound healing and tumour suppression [78]. However, under the conditions of aging, SASP signaling is considered deleterious. An increased chronic inflammation favors the accumulation of senescent cells, which affects the repair,

turnover and regeneration of tissues leading to progression and age-related pathologies [79]. In addition, the SASP factors secreted by senescent cells affect vital processes such as growth, migration, tissue architecture, blood vessel formation and differentiation, processes that are highly controlled and whose deregulation can promote tumor growth [80].

Table 1.1 Senescence mediator pathways

Pathways	Mediators	Major functions
DDR pathway	ATM	• Double strand break (DSB) repair • Serine/threonine protein kinase • phosphorylates the C-terminal tail of the histone variant H2AX (γ-H2AX), p53 • phosphorylates CHK2
	ATR	• Single strand break (SSB) repair • phosphorylates CHK1
	CHK1	• Cell cycle arrest activation of p53 • Replication fork stability
	CHK2	• Cell cycle inhibition, p53 activation • DSB repair
P16^{INK4a}/Rb pathway	p16^{INK4a}	• Inhibits cyclin-dependent kinases 4 and 6
	Rb	• Inhibits cell cycle by repression of E2F transcription factor
P19^{ARF}/p53/ p21^{Cip1} pathway	p19^{ARF}	• Activation of p53 by limiting Mouse double minute 2 homolog (**Mdm2**) activity
	p21^{Cip1}	• Inhibits the activity of cyclin-CDK2, -CDK1, and -CDK4/6 complexes • Its expression is promoted by activated p53.
	p53	• Activates DNA repair • inhibits cell cycle progression.
	Mdm2	• Negative regulator of p53.

1.4 Molecular pathways leading to senescence

The cellular growth arrest in senescent cells is orchestrated and maintained by multiple tumour-suppressor genes from three important pathways: DDR, p19^{ARF}/p53/p21^{Cip1} and p16^{INK4a}/Rb (Figure 1.5) [9,14,81]. These molecular cascades can interact with each other, but they can also limit the progression of the cell cycle independently, responding to different stimuli and depending on the cell type.

1.4.1 DNA damage response: sending a SOS to repair, die or senesce

In specific situations, DNA-damaged or stressed cells are locked permanently in G1/S phase transition to limit the risks of becoming cancerous tumours [81,82]. A common factor in different senescence types is the presence of persistent DNA damage foci or DNA-SCARS; these lesions can impair genome replication and transcription and a wider-scale compromised genomic integrity and/or organismal viability [8].
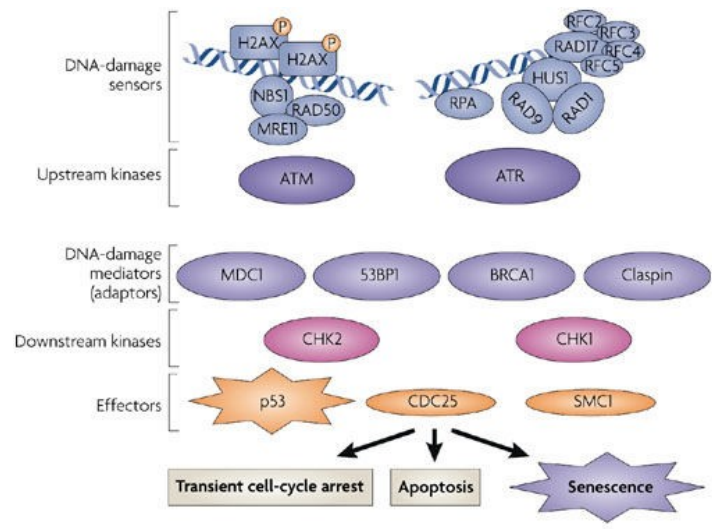
Figure 1.5 DNA damage response (DDR)

DNA damage response requires diverse sensors, mediators and effectors to define the fate of cells with persistent DNA damage. Adapted from Campisi and d'Adda di Fagagna, 2007 [8].

The wide diversity of DNA-lesion types requires specific repair mechanisms; mispaired DNA bases are replaced with correct bases by mismatch repair (MMR), and small chemical alterations of DNA bases are repaired by base excision repair (BER). More complex lesions, such as pyrimidine dimers and intra-strand crosslinks are corrected by nucleotide excision repair (NER). SSBs are repaired by single-strand break repair (SSBR), whereas DSBs are processed either by nonhomologous end joining (NHEJ) or homologous recombination (HR). The main DNA-damage sensor histone γ-H2AX is essential for the recruitment of 53BP1, BRCA1, MDC1, and the MRE11-RAD50-NBS1 complex (Figure 1.5) [82,83]. Subsequently, those complex are targets for the kinases ATM or ATR. In turn, both ATM and/or ATR transmit the DNA damage signal to their targets CHK2 and CHK1 respectively, which phosphorylate and activate several cell cycle inhibitor proteins, including the tumor suppressor gene p53 [83]. In turn, phosphorylated p53 tetramers bind to specific DNA sequences, named the p53-responsive element (**RE**) and trigger the transcriptional activation of a multitude of target genes [84]. Importantly, activated p53 can play a pivotal transcription-independent role in apoptosis [85].

1.4.2 p19^*ARF*/p53 pathway

1.4.3 p53 activation

During cellular stress response, levels of p53 rise dramatically, which leads to different cell fates: apoptosis, autophagy, cell cycle arrest, DNA repair or senescence. The p53 protein is tightly regulated by an array of posttranslational modifications such as phosphorylation, ubiquitination, methylation, sumoylation, neddylation and acetylation; all targeting and modifying the C-terminal region of p53 [85]. In normal cells, p53 is a short-lived protein that is constantly monoubiquitinated by an E3-ubiquitin ligase MDM2, promoting the export of nuclear p53 to cytoplasm for proteosomal degradation. Upon genotoxic stressors, rapid phosphorylation of p53 at Serine 15 (Ser-15) by ATM, and at Ser-20 by Chk2 results in dissociation of p53 from MDM2, leading to p53 stabilization, tetramerization and activation of its downstream processes. Likewise, phosphorylation of MDM2 at Ser-395 by ATM attenuates the capability of MDM2 in for subsequent p53 degradation, thereby enabling p53 accumulation [86,87].
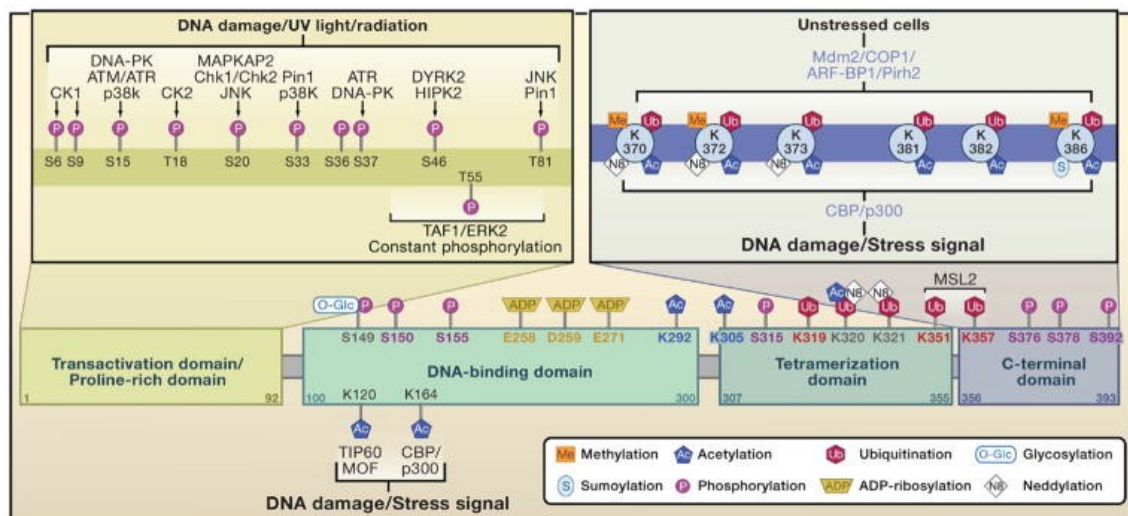
Figure 1.6 p53 posttranslational regulation

Postransductional modifications of p53, methylation (Me), sumoylation (S), neddylation (N8), glycosylation (O-Glc), and ribosylation (ADP) Modified from Kruse and Gu, (2009) [86].

1.4.4 p53 and apoptosis

Acetylation of p53 on lysine 164 (K164) by CBP/p300 is required for the cell cycle arrest and apoptosis [88,89]. Upon mono-ubiquitylation, p53 translocates from the nucleus to the cytoplasm and then localizes in the mitochondria and induces apoptosis [90]. At the outer mitochondrial membrane p53 interacts with antiapoptotic members of the Bcl family such as Bcl-XL and Bcl-2 to promote the oligomerization of the proapoptotic factors Bak and Bax [91]. In turn, Bak/Bax drives mitochondrial membrane permeabilization and release of cytochrome c [92].

1.4.5 p53/p19^{ARF} and senescence

The transcriptional activation of specific genes for cell cycle arrest and/or senescence programs mediated by p53 activation depends on the post-translations modifications and the combinations with other transcription factors. As a result, p53 increases the expression of cell cycle inhibitors and senescence mediators, p21^{Cip1} and of *p19^{ARF}*. p19^{ARF} binds and retains MDM2 in the nucleolus; thus, when p19^{ARF} is present p53 accumulates. In contrast, p53 activation leads to an autoregulatory negative feedback loop by promoting transcription of its own inhibitor *Mdm2* [93,94].

1.4.6 $p16^{INK4a}$/Rb pathway regulation

1.4.7 The INK4a/ARF/INK4b locus

The *INK4a/ARF/INK4b* locus is located on chromosome 9 (p21) in humans, while the homolog in mice resides in chromosome 4, and encodes three important tumor suppressors genes: *the* cyclin-dependent kinase inhibitors (CDKIs) *p15^{INK4b} (INK4b)*, *p16^{INK4a} (INK4a)* genes and p14^{ARF}(*ARF*) [95]. It is presumed that *p15^{INK4b}* and *p16^{INK4a}* arose from an evolutionary duplication event as both genes share high sequence homology and both proteins are almost indistinguishable in structure and biochemical properties; by acting as antagonist of the cyclin-dependent kinases (CDKs) that regulate progression through the G1 phase of the cell cycle. The *p15^{INK4b}* gene has its own promoter and reading frame that is physically different from *p16^{INK4a}* and *p14^{ARF}*. Interestingly, *p16^{INK4a}* and *p19^{ARF}* have different first exons 1α and 1β which bear no homology in their sequences and are transcribed from their own promoter [96]. E1α and 1β are spliced to a common second and third exon; although *p16^{INK4a}* and *p14^{ARF}* share two exons, *p14^{ARF}* mRNA is translated in an alternative reading frame (ARF) (Figure 1.7) [96,97].
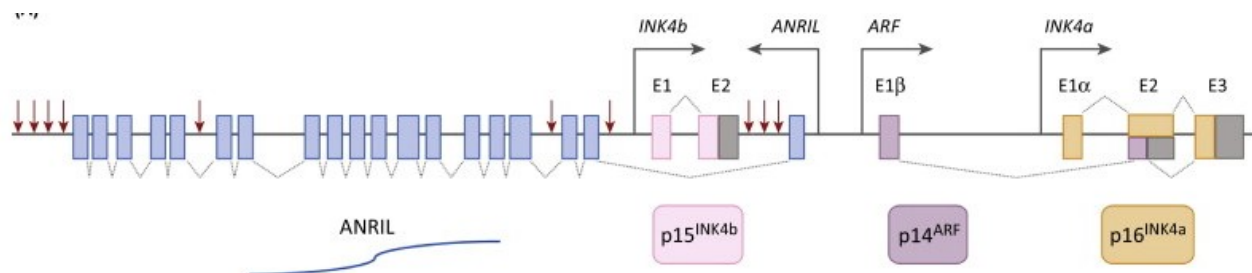
Figure 1.7 The *INK4B-ARF-INK4A* locus

The genomic structure of the *INK4b-ARF-INK4a* locus encodes three tumor suppressor genes *p15^INK4b^*, *p19^ARF^* (p14^ARF^ in humans) and *p16^INK4a^* which are color coded to represent the gene product that they are spliced into. Arrows represent the promotor of each gene. Adapted from Martin *et al*., 2014 [98].

1.4.8 Transcriptional regulation of INK4a/ARF/INK4b locus

p16^INK4a^ and p19^ARF^ inhibit cell cycle and play critical and tightly controlled roles to maintain tissue homeostasis and prevent cancer. In normal cells p16^INK4a^ levels are relatively low or undetectable, but its elevated expression in mice is enhanced during aging or in response to cellular stressors [6]. Intriguingly, normal young mice exposed to IR treatment experienced a premature senescence program, with a gradual increase of *p16^INK4a^* and *p19^ARF^* starting at 4 weeks post-IT treatment and reaching higher levels at 8-12 weeks although the reason for this delay is currently unknown [99,100]. *p16^INK4a^* transcriptional regulation is controlled at multiple levels of , and most of this regulation occurs through diverse regulatory elements and their combinatorial action [101-103].

1.4.8.1 TRANSCRIPTIONAL REGULATION OF P19^ARF^ PROMOTER

*p19^ARF^* promoter is located in the *INK4a/ARF/INK4b locus* and contains a variety of putative binding sites for transcription factors such as Sp1, cyclin D binding myb-like protein 1 (DMP1), Pokemon and the E2F family but its activation has remained poorly understood [104]. The *p19^ARF^* protein exists at low or undetectable levels in most normal cells and tissue types in embryogenesis [26,105]. Like *p16^INK4a^*, the expression of *p19^ARF^* has been shown to increase mice aging in [6]. Importantly, it has been proposed that the *p19^ARF^*

gene responds to low and chronic stress. *p19^ARF* is induced in response to oncogenic stress mainly by a sustained mitogenic signals from *Ras^V12*, *H-ras, c-Myc*, *E2F-1*, *E1A*, *v-Abl*, *T_{121}* oncogenes [106,107]. Overexpression of oncogenic *Ras^V12* acts through the Raf-MEK-ERK pathway and AP-1 signaling to activate *Dmp1*. In turn, DMP1 activates the *p19^ARF* promoter only Ras^V12 activated the *Dmp1* promoter [108]. It is important to note that many of these conclusions stem from highly tractable cell culture models, but the *in vivo* relevance is less clear in most cases.

1.4.8.2 REGULATORY DNA-MOTIFS FOR *P16^INK4A* EXPRESSION

INK4a promoter contains its own activators sites to promote the subsequent recruitment of RNA polymerase and transcription of *p16^INK4a*. Although the exact mechanism of activation of *INK4A* is somewhat unclear, few models have been proposed (Figure 1.8) .
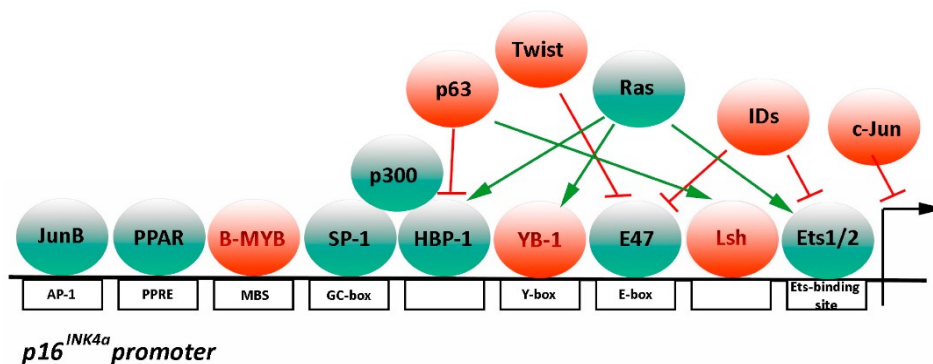
Figure 1.8 Transcriptional activators and repressors of *p16^INK4a* promoter
p16^INK4a expression requires the recruitment of transcriptional factors (green) to binding sites within *INK4a* promoter indicated by white rectangles. Transcriptional repressors (red) have an opposite function. Adapted from D'Arcangelo *et al*., 2017 [109].

Serving as an example, Ets-binding site binds E-26 transformation-specific 1 (Ets1) and E-26 transformation-specific 2 (Ets2) transcriptional factors which in turn can be activated by a variety of stress-associated signals, in particular the Ras/MAPk signaling pathway [22]. In addition, Ets1, Ets2, E47 and/or c-Myc transcriptional factors can induce $p16^{INK4a}$ expression by binding the E-box site located on the promoter. In replicative senescence, $p16^{INK4a}$ transcription is activated by increased expression of Ets1/2 and decreased expression of DNA Binding 1 (**Id**) proteins[22]. Id1 is a helix loop helix transcription factor that can negatively regulate $p16^{INK4a}$ expression by binding to phosphorylated Ets1/2 transcription factors. However, how Ets1/2 and Id1 protein regulation and expression change during aging is still elusive [110]. Alternatively, AP1 binding site promotes $p16^{INK4a}$ expression by binding JunB. Conversely, c-Jun acts as a JunB antagonist, downregulating $p16^{INK4a}$ expression. Another important regulatory element within the $p16^{INK4a}$ promoter is the Sp1-binding site, a GC-rich region containing at least five putative GC boxes that represent the putative binding target sites for Sp transcription factors. These are enhanced during cellular senescence mainly due to an increase in Sp1 binding affinity. Sp1 positively regulates $p16^{INK4a}$ transcription by directly binding to DNA and recruiting P300 to the $p16^{INK4a}$ promoter [111,112].

1.5 Types of senescence

The senescent phenotype depends on the stimulus that can differentiate two types of senescence. Currently, senescence is grouped in two categories, one of them is named replicative senescence and it is closely related to the finite number of cell divisions. This

is mainly regulated by telomere shortening which diminishes with each division leading an increase of the reactive oxygen species (ROS) levels and DDR [113,114]. The other type is premature senescence which is caused by a stressful exogenous stimulus such as DNA damage, oncogene activation, paracrine induced senescence [18,19].

1.5.1 Replicative senescence

1.5.2 Hayflick limit

The concept of cellular senescence was described for the first time in 1961 by Leonard Hayflick and Paul Moohead who observed a decline in the proliferative capability of normal embryonic human fibroblasts cultured *in vitro* after multiple passages. This finite number of cell cycle divisions or populations doubling (PD) corresponded approximately 50–60 rounds, and would be known as the Hayflick´s limit [115].

1.5.3 Telomere length defining the limit

Years later after Hayflick´s observations, Dr. Olovnikov hypothesized the molecular mechanism of Hayflick´s limit was the result of telomere attrition [116-118]. In normal eukaryotic cells, the telomeres protect the ends of chromosomes. These complex structures are composed of multiple proteins and range from 4000 to 15000 nucleotides in a tandem repeat sequence 5'-TTAGGG-3' [119]. To avoid telomere attrition, telomeres require a specialized mechanism and enzyme for its maintenance: the ribonucleoprotein

complex RNA-dependent DNA polymerase known as telomerase reverse transcriptase (TERT) [120]. In humans, hTERT is inactivated in most post-mitotic cells and its expression is limited to embryonic stem cells, while in adults is confined to stem cells compartments [121]. Consequently, in its absence differentiated cells experience a steady decay of telomere length of 100-200pb per cell division, ticking as a cellular "molecular clock" (Figure 1.9) [114,121,122]. The cellular micro-environment also plays an important role in regulating the telomeric length and telomerase activity. Excessive oxidative stress causes a faster telomere attrition and antioxidants decelerate it [123,124]. When telomeres reach critical lengths, exposing the functional DNA ends, the DDR is engaged, followed by the activation of ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR) kinases. These responses incur a growth arrest effected mainly by p53 ultimately leading to a cell-cycle arrest [125]. Cells can bypass the cell cycle arrest by oncogene activation and/or telomerase activation and can then undergo malignization [49].
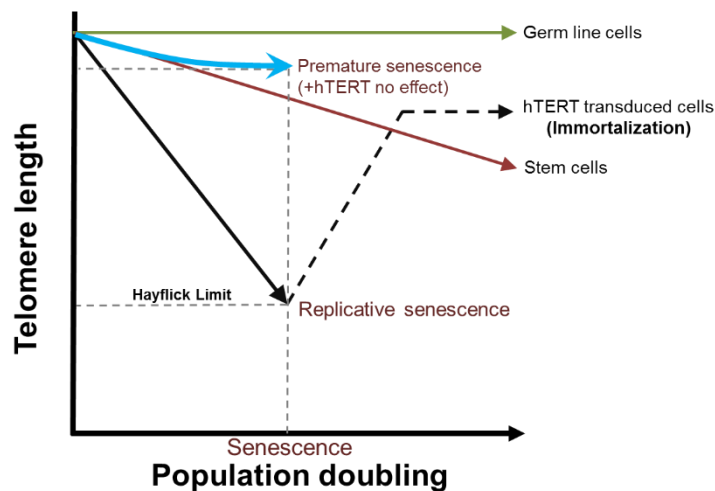
Figure 1.9 Replicative senescence *in vitro*.

Normal cells undergoing replicative senescence *in vitro* have low levels of telomerase or are telomerase negative and consequently experience telomere shortening with each cell division (black line) reaching a critical length of telomere triggering replicative senescence at the Hayflick´s limit. This proliferative checkpoint can be overcome by inactivation of Rb/p16^{INK4a} or p53 pathways and ectopic expression of hTERT leading to cell immortalization (dashed line). Pluripotent stem cells are telomerase positive (red line), so their telomeres length shorten in stem cells at rates slower than telomerase-negative somatic cells. Adapted from Cong *et al.*, 2002 [125].

1.5.4 Premature senescence

Regardless of the number of cell divisions, senescence can be induced prematurely by a plethora of non-telomeric stimuli including direct DNA-damaging stressors such as IR, chemotherapy drugs, reactive ROS, ultraviolet (UV), oncogene activation and high glucose concentrations [20,67,69,126-132]. Moreover, senescent cells bearing persistent DNA damage foci secrete pro-inflammatory cytokines that provoke non-cell autonomous induced premature senescence to neighboring normal cells [76,133,134].

Table 1.2 Types of senescence

Types of senescence		Inductor
Replicative senescence	Replicative senescence	Telomere erosion-dependent DNA damage
Premature senescence	ROS-induced senescence	8-oxo-2-deoxyguanosine, SSB and DSB.
	DNA damage-induced senescence	Single- and double-strand DNA breakage
	Oncogene induced senescence (OIS)	DNA replication stress
	Paracrine-induced senescence	IL-1β, IL-1α, TGF- β ligands, VEGF, CCL2, CCL20

1.5.5 Oxidative stress induces senescence

During aging, cells undergo an intracellular accumulation of ROS [135,136]. This occurs due to a decrease in the activity of the enzymes responsible for eliminating or neutralizing these species. The increase in ROS is known to cause as much DNA damage as faster telomere shortening [137]. Intracellular oxidative stress occurs when an increase in the number of reactive molecules, causes nucleic acid, protein, and lipid damage [138]. ROS are highly reactive atoms or molecules with one or more unpaired electron(s) in their outermost shell and are formed when oxygen interacts with other specific molecules. Free radicals are unstable particles such as superoxide ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), hydroperoxyl radical ($HO_2^{\bullet}$), nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), peroxyl ($ROO^{\bullet}$), hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), and lipid peroxide (LOOH) [139].

The pioneering work of Packer & Fuehr (1977) provided the first experimental evidence of the role of oxygen levels on *in vitro* senescence [140]. They used the WI-38 human fibroblast strain established by Hayflick and correlated the number of PDs with the amount of oxygen tension in the environment. Cells grown at 10% oxygen had a better capacity to proliferate as indicated by increased number of PDs while cells at 50% O_2 possessed a diminished life span and therefore, diminished PDs [140]. Particularly in mice, fibroblasts cultured *in vitro* will senesce after only 15-20 PDs despite having exceptionally long telomeres (>20 kb). This premature senescence makes it unlikely that telomere length plays a prominent role in normal mouse aging [141]. This response is suggested to be due to stressful culture conditions as a response of exposure to atmospheric oxygen concentrations of 20%, which is above biological levels of 3%. Mouse cells possess differences in oxygen sensitivity compared to human cells; murine fibroblasts have weaker protective mechanisms against ROS and growing at high levels of oxygen exhibit more damage than human cells [142]. In recent studies, high oxygen levels *in vitro* culture of MEFs have been demonstrated to accelerate the accumulation of DNA mutation, mostly by genomic rearrangements [143]. On the other hand, telomere shortening in murine cells is accelerated in response to ROS exposure and can be blocked by adding antioxidant in primary cell cultures lines.

According to the free radical theory of aging, oxidative DNA damage caused by ROS play a pivotal role in the aging process, these un-stable molecules make a significant contribution to genomic instability by causing single-strand base oxidative modification, the 8-oxo-2-deoxyguanosine (8-oxo-dG), which leads to single-strand nick formation.

These nicks or gaps are an important source of a subset of single-strand breaks (SSB) which in turn are at risk in becoming in double-strand DNA breaks (DSBs) if they persist until the next round of DNA replication, and thereby participates in downstream premature senescence signaling [144].

1.5.6 Ionizing Radiation

Direct damage to DNA, either by irradiation or by the use of damaging agents, can induce senescence. Normally the cellular response to damage includes the arrest of the cell cycle to carry out the repair. If the magnitude of the damage is very large, the response may include irreversible apoptosis or arrest of the cycle. Thus, the activated mechanism depends on the type of damage, the dose administered and the cell type treated [145,146].

Radiation is defined as the transmission of energy through waves or particles. IR corresponds X-rays and fast-moving subatomic particles, like alpha particles (α), beta particles (β) and neutrons (n). α particles are doubly charged and are relatively heavy with two protons and two neutrons and can be stopped by a sheet of paper. They lack energy to penetrate the outer dead layer of skin but are considered hazardous if ingested. β particles are much smaller with one negatively charged electron and can be stopped by a layer of clothing or millimetres of aluminum. Without any protection in the body, β particles can penetrate for a few centimetres and cause greater damage than α particles. The γ and X-rays are very short electromagnetic waves with high energy; both possess high penetrance ability in biological tissues and enough energy to ionize atoms and

disrupt molecular bonds in cells. In fact, exposure to radiation induces massive DNA damage, by depositing high energy on the DNA backbone resulting in SSB and/or DSB, and indirectly after electrons are removed from neutral water molecules to produce OH$^{\bullet}$. These waves can be effectively blocked by several metres of concrete or few inches of lead. The intensity of X-rays is measured as the absorbed amount of energy per unit mass of tissue, defined as the dose of radiation and measured in gray (Gy), where 1Gy = 1 joule/kg of tissue.

1.5.7 Oncogene-Induced senescence

Sustained chronic proliferative capacity is the most fundamental hallmark for cancer development. Accordingly, oncogene-induced senescence (OIS) evolved as a powerful tumour suppressor mechanism to restrain aberrant cell proliferation of damaged or stressed cells bearing genetic alterations in proto-oncogenes and/or tumor suppressor genes [147-149]. The first association between, constitutive activation of oncogenic *Ras* (*H-Ras*^{V12}) and OIS was described by Serrano *et al*., (1997) [18]. They observed that overexpression of *H-Ras*^{V12} on normal human and murine fibroblasts induced a transient arrest in growth accompanied with DNA damage and followed by activation of DDR and a strong growth arrest response by accumulation of *p15*^{INKb}, *p19*^{ARF}, *p53* and *p16*^{INK4a} tumor suppressor genes [18,150]. The induction of OIS is associated with senescent hallmarks such as heterochromatin rearrangements, oncogene-induced DNA-damage foci, SASP and an increase in senescence-associated β-galactosidase (SA-β-Gal) activity [18,23,151,152]. Normally, upon mitogenic signals the members of the *Ras* proto-

oncogene family (K-*Ras*, N-*Ras* and H-*Ras*) activate downstream mitogen-activated protein kinase (MAPK) and phosphatidylinositol kinase (PI3K) pathways which participate in the control of proliferation, differentiation, and survival of eukaryotic cells (Figure 1.10) . Genetic alterations within these growth-promoting genes transmit aberrant mitogenic signals; single mutations in *Ras* typically at codon 12, 13, or 61 can lead to hyperproliferative signaling. Overexpression of *Ras* effectors Raf, Mek1/2, Braf, PI3K, Akt provokes a burst in proliferation [18,147,153-156]. Consequently, an aberrant DNA hyper-replication promotes telomere-independent DNA damage through the accumulation of ROS and the collapse of DNA replication forks at genomic instability sites [147,152,157,158].
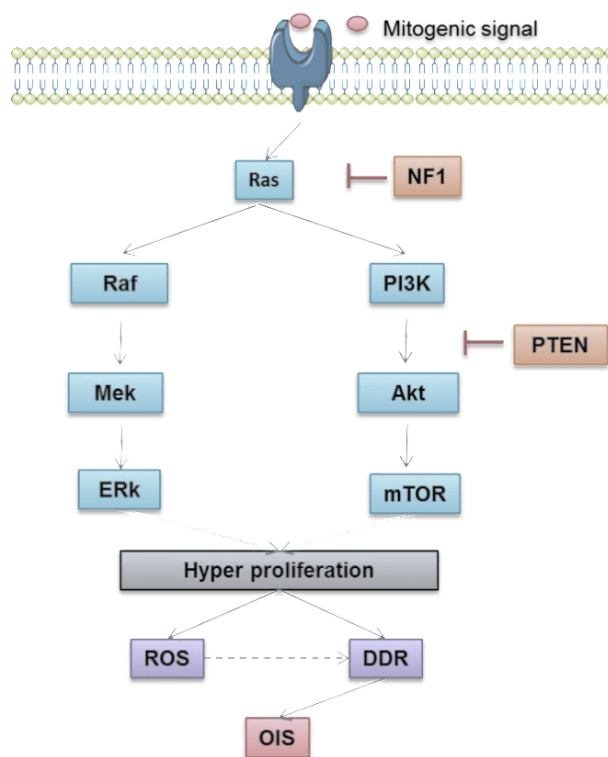
Figure 1.10 Multiple pathways are involved in oncogene-induced senescence.

Ras activation promotes Raf/MEK/ERK and PI3K/Akt/mTOR pathways leading to cell growth. Ras activity can be repressed by NF1, and downstream PTEN constrain PI3K activity.

Phosphatase tensin homolog (PTEN) and neurofibromatosis type I (NF1) are tumor-suppressor genes that negatively control cell proliferation signaling (Figure 1.3). Their inactivation triggers sustained activation of PI3K cascade leading cells to senesce [159-161]. Promyelocytic leukemia (PML) protein inactivation also leads to senescence [132,162-165]. pVHL protein promotes the senescence program through stabilization and accumulation p27^{KIP}, a cyclin-dependent kinase inhibitor which in turn leads to the Rb pathway [166,167].

1.6 The meaning of senesence *in vivo*

1.6.1 *The senescence is considered a double-edged sword*

The paradigm of senescence as a "double-edged sword" is based on the observation that senescent cells have beneficial effects for tumour suppression, tissue homeostasis, limb and tissue regeneration, embryogenesis, and wound healing [6,26,28]. However, senescent cells can prevail for longer periods and accumulate during aging with deleterious role. Primarily, because of the decline in tissue regenerative ability from stress; and secondly because the production of chronic SASP factors by senescent cells are thought to drive undesired senescence in neighboring cells and favors age-related pathologies degenerative such as atherosclerosis, idiopathic pulmonary fibrosis, osteoarthritis, esteatosis, diabetes osteoporosis, neurodegeneration and atherosclerosis and cancer [10,13,168-170].

At the bright side, developmental senescence does not seem to be triggered by DNA damage response or *p19^ARF/p53* or *p16^INKa* activation. Instead, embryogenic senescence is mediated mainly by p21^{Cip1} and TGFβ/SMAD- and PI3K/FOXO-signaling cascade[170,171]. The SASP program secreted from this kind of senescent cells leads their own subsequent disposal by the immune system, especially macrophages, NK and granule exocytosis [170-172]. Although the molecular mechanism to detect and selectively eliminate transitory senescent cells remains poorly understood, SASP-dependent recruitment of immune cells promotes infiltration of various immune cells such monocytes, macrophages, neutrophils, natural killers (NK), and T cells into places of chronic tissue inflammation [173-176].

The cellular senescence program is considered a potent tumour suppressor mechanism; consistent with this role, senescence is controlled by multiple tumour-suppressor genes from three important pathways DDR, p19^{ARF}/p53/p21^{Cip1} and p16^{INK4a}/Rb. Nevertheless, senescent cells are not merely non-dividing cells but, due to their active metabolism are known to act as non-cell autonomous or secretory tumor suppressor mechanism by active disposal [177-179]. The first evidence for the clearance of senescent cells by the immune system was provided by implantation of pre-neoplastic hepatoblasts co-expressing oncogenic *H-Ras^V12* and conditional turning off shRNA restoring system of *p53* expression in mice. Rapidly after transplantation mice developed liver carcinomas and prompt involution after *p53* re-expression of endogenous p53-induced senescence. In addition, this was accompanied by the induction of inflammatory cytokines and

subsequent elimination of senescent hepatocarcinoma cells by Induction of innate immune responses against tumor senescent cells, including macrophages, neutrophils and NK [180]. Importantly, p53 also seems to contribute to limiting the excessive activation of SASP. Indeed, Coppe *et al*., (2008) found that p53 is not required for SASP but, rather, suppresses its amplification, indicating a cell non-autonomous tumour suppressive activity of p53 [67]. Wang *et al*., 2011 have shown that pre-malignant senescent hepatocytes bearing *N-Ras*^{GV12} are cleared by induction of antigen-specific immune responses against premalignant senescent in neoplastic lesions engaging CD4^{+} T cell-dependent program execute by monocytes/macrophages [176].

In the context of liver acute or chronic injury, hepatocellular carcinoma depends of sequential progression from disease/injury to fibrosis and to cirrhosis culminates in neoplasia. In response to liver injury by carbon tetrachloride (CCl_4) treatment, stellate cells are induced to proliferate and produce extracellular matrices to repair the damage, but then undergo p53-dependent senescence and p53-driven SASP program to be selectively eliminated by immune cells. In the absence of p53-dependent senescence cirrhotic livers secrete factors that polarize macrophages towards pro-tumorigenic M2-like macrophages, while factors secreted by p53-expressing senescent stellate cells induce an M1 phenotype, which is associated with antitumor activity [177-179].

Cancer is a life-threatening disease that requires intensive care and treatment such as chemotherapy and IR. Long after therapy individuals develop side effects, which are significantly pronounced in the immune system organs like the bone marrow (BM). IR is one of the most widespread approaches in clinic for cancer treatment, transplantation, or

diagnosis. However, a range of long-term side-effects of radiation therapy have been described, notably in multiple organs, including tissues from the immune system. Importantly, long term IR exposed individuals encounter adverse physiological responses, such as cancer, gastrointestinal failure, bone marrow failure and auto-immune diseases [181]. The hematopoietic cells are among the most radiosensitive cells; consequently, the spleen and bone marrow (BM) are very radiosensitive organs [182,183]. Within few hours after IR, irradiated hematopoietic progenitor cells are eliminated mainly by apoptosis thereby causing a hematological crisis [184,185]. Long after IR, side effects manifest as an accelerated-age like phenotype of the hematopoietic/immune system. IR-induced residual BM injury manifested by the accumulation of senescent hematopoietic stem cell (HSC) and stromal cells [99,100,186]. Not surprisingly, both HSC and stromal cells from the hematopoietic niche display typical markers for senescence and aging, such DNA damage foci, increased SA-β-gal activity, pro-inflammatory factors and upregulated levels of p16^{INK4a} and p19^{ARF} [12,100,187]. As mentioned before, the accumulation of senescence is an important hallmark of aging and may account for the decline in proliferative potential and function of progenitors/stem cells observed by a reduced colony forming units (CFU) capacity of the bone marrow environment. Interestingly, atomic bomb survivors were at risk to develop age-related diseases and possess immunological alterations that support an accelerated aging of the immune system phenotype [188]. Long after radiation exposure, atomic bomb survivors experienced a decline of naïve T cell, impairments in the proportion of CD4$^+$ T cells and an increased proportion of the memory CD8$^+$ T cell population [188,189]. Furthermore, among atomic bomb survivors, some experienced long-lasting persistent expression of proinflammatory factors such as tumour

necrosis factor alpha (TNF-α), IL-6, Interferon gamma (INF-γ) and ROS. They also experienced augmented anti-inflammatory IL-10 cytokine levels, and increased levels immunoglobulins (IgA and IgM) [188,190,191].

Table 1.3 Genetically modified mice to target senescent cells *in vivo*

Mouse model	Genetic construct
P16-ATTAC	**P16^{INK4a} promoter-eGFP-FKBP:CASP8** [10] - *eGFP*: functional enhanced green fluorescence protein - FKBP–Casp8: FK506-binding-protein–caspase 8 fusion protein - AP20187 induces cell death through synthetic drug that induces dimerization FKBP–Casp8.
P16-3MR	**P16^{INK4a} promoter-RLUC-RFP-TK** [169] - RLUC: *Renilla luciferase* - RFP - *TK is* thymidine kinase a suicide gene that induces selectively death after Ganciclovir treatment. - 3MR mice are wild-type as the cassette was inserted using a bacterial artificial chromosome.
ARF-DTR	**P19^{ARF} promoter-ARF-DTR-LUC** [192] - DTR: diphtheria toxin receptor - LUC is Luciferase - Diphtheria toxin treatment mediates conditional cell knockout

1.6.2 *The consequences of senescence on age-related diaseases*

Senescence is a beneficial anti-tumour mechanism for early life stages but might have deleterious and pleiotropic effects on aging. Such a dualistic relationship between senescence and cancer/aging is complex and depends on its temporal organization. Thus, to define the benefits of selective elimination of senescence on aging and tissue/organs age-related degeneration, scientists have engineered transgenic mice in

which they can selectively induce elimination of $p16^{INK4a}$-positive and $p19^{ARF}$-expressing cells (Figure 1.5) [10,169].

In support of this, selectively ablation of $p16^{INK4a}$-positive alleviates the senescent cell burden, delayed aging features, extends health span and ameliorates multiple age-related diseases such as atherosclerosis, idiopathic pulmonary fibrosis, osteoarthritis, steatosis, diabetes [11,13,193-198]. Similar observation was obtained by selectively elimination of $p19^{ARF}$-positive cells from lung tissue which ameliorate aging associated phenotypes including reduced tissue elasticity, morphological changes, and gene expression [192]. Furthermore, these age-related pathologies have a strong association to the immune system function. In one hand, immune cells experienced immuno-senescence by intrinsic changes during aging increasing susceptibility to infections and a decline of senescence removal. Another hand, age-related tissue changes can produce non-cell autonomous effects on immune cells through pro-inflammatory factors promoting auto-reactive responses [10,13,169,199-201].

Senescent macrophages and DCs are also capable of secretion of a myriad range of SASP factors including cytokines and chemokines known to influence the immune system both positively and negatively regarding T cell activation. Upregulation of NF-kB activity leads to accumulation of cyclooxygenase (COX)-2 and its product, prostaglandin E_2 (PGE_2); PGE_2 is a soluble component of SASP [69,202,203]. In aging, both mice and humans activated macrophages are associated with increased factors secretion which potentially impact responses of T cells in their proximity [204-209].

The presence of senescent cells at sites of tissue injury possess the remarkable ability to regulate positively tissue regeneration/repair [134,210]. These senescent cells secrete several SASP factors to the microenvironment to activate their own clearance by attracting macrophages and leading to tissue regeneration. This reparation mediated by the presence of senescent cells and their secretome requires a tightly regulated presence. Therefore, senescent cells need to be cleared to promote appropriated tissue regeneration [211]. Interestingly, macrophages and senescent cells have been implicated in the pathogenesis of age-related diseases such as cancer, fibrosis, osteoarthritis, atherosclerosis; and their selective elimination by clodronate liposome treatments reduces drastically the age-related inflammatory [212].

1.7 The spleen

In higher vertebrates, the spleen is the largest secondary lymphoid organ in the body. Its microarchitecture possesses a complex compartmentalized parenchyma, which corresponds to functionally and morphologically different structures: the white pulp, marginal zone **(MZ),** red pulp and its intricate vasculature system [213-215]. These are specialized, and dynamic environments supported by an elevated perfusion and different lymphoid and non-lymphoid cell-to-cell interactions, cell types, extracellular molecules, reticular fibers and splenic conduits. The spleen serves as an efficient filter to clear undesired blood-borne antigens and old or damaged cells from the blood and has the

capacity to generate and innate and/or adaptive immune response. In addition, the spleen is a storage site for iron and cells like erythrocytes and platelets [216].

1.7.1 The splenic architecture

1.7.2 Marginal Zone

The elevated perfusion of the spleen permits this organ, through the MZ, to provide efficient immune surveillance of the circulatory system [217]. Strategically interposed between the lymphoid tissue of the white pulp and the circulation, the splenic MZ contains B cells enmeshed with macrophages, DCs and granulocytes in a stromal reticular cell network [218]. All these cells readily interact with circulating antigens as a result of the low flow rate of the blood passing through the MZ. The MZ is a specialized filter-like structure at the interface among the lymphoid white pulp and the more innate scavenging red pulp [219]. This efficient constraint of undesired particles is allowed by a complex mesh-like microarchitecture and the blood flow that constantly traffics through the MZ and marginal sinus. In mice, the MZ is divided into an inner and an outer compartment which are densely packed with actively phagocytic cells like macrophages and DCs four or five layers of B lymphocytes and structural network of marginal reticular cells (MRCs) (Figure 1.12) [213].

The MZ possesses multiple professional antigen presenting cells (APC), macrophages and DCs filter the blood content flowing in the MZ from antigens, apoptotic debris and pathogens. Then, these cells can migrate into the white pulp, where they deliver or

present antigen(s) to B and T cells [213]. Two special macrophage populations are associated with the MZ, CD169+ MOMA-1+ metallophilic-macrophages (MMM) and SIGN-R1+MARCO+ marginal zone macrophages (MZM). MMM occur as a tight network at the inner layer of MZ and are directly adjacent to the marginal sinus and white pulp [220]. As APC, their main function is to uptake antigens and presentation to T lymphocytes, they are also optimally equipped to activate B-cell response and act as promoters of germinal centre [221]. MZM are found in the outer MZ toward the red pulp tightly bound to the reticular meshwork, and appear to exhibit high phagocytic activity but they are hitherto considered not to be determinant for the generation of T cell responses.
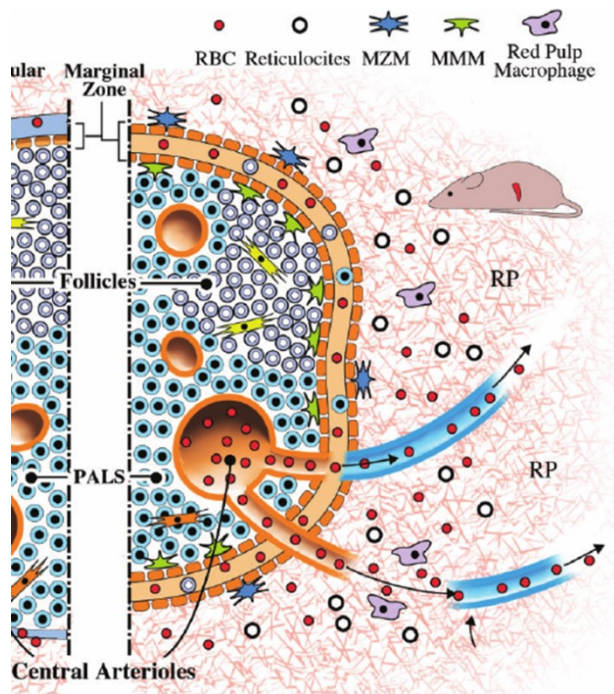
Figure 1.11 Murine splenic microarchitecture.

The mouse spleen organization is characterized by a well-defined-MZ that clearly delimits the red pulp (RP) and the PALPs. FDC, follicular dendritic cell; MMM,

marginal metallophilic macrophages; MZM, marginal zone macrophages. Figure originally published in Del Portillo *et al*., 2012 [222].

DCs are determinant for antigen presentation and priming T cells responses. The $CD8\alpha^+CD11b^-$ DCs population reside in the outer part of the MZ and in the T-zone within the white pulp and they have a role in the activation and tolerization of cytotoxic T cells [223]. $CD8\alpha^-CD11b^+$ DCs are mainly localized in the marginal zone and red pulp; this population of DCs is specialized in the activation of $CD4^+$ T cells. Further, both populations of DCs are also known to interact and engulf apoptotic material entering the spleen from circulation.

The MZ is also enriched by presence of region-specific MRCs unidentified stromal cell population [224]. These cells are at the edges of the B cells follicles forming a functional conduit networks that can deliver antigens to the follicles; they also support follicle structures by the secretion of important chemokines. MRCs express several markers in common with other subsets of stromal cells located in the white and red pulp such as ER-TR7, desmin, laminin, VCAM-1, MAdCAM1. Importantly, MRCs appears to be unique in the expression of the tumour necrosis factor family member TRANCE (RANKL) [225].

1.7.3 White pulp

In mice, the splenic white pulp consists of lymphoid accumulations segregated into T cell zone or periarterial lymphatic sheath (PALS) and B-cell zone (follicles). PALS are

surrounding the central arterioles and divided into inner and outer PALS. The lymphocytes of the inner part are largely CD4$^+$ T cells, though smaller amounts of CD8$^+$ T cells and disperse DCs and B cells. The outlier layers of PALS are small lymphocytes B and T cells [213]. Lymphoid follicles are B-cell-rich compartments.

Table 1.4 Splenic cells

Cells	Markers	Location in the spleen
Macrophage	F4/80^{high}CD68$^+$CD11b$^{low/-}$ macrophages	Red pulp
	SIGN-R1$^+$MARCO$^+$ MZM	MZ
	CD169$^+$ MOMA-1$^+$ MMM	MZ
	CD68$^+$ macrophages (CD11b$^-$F4/80$^-$CD68$^+$)	White pulp
	IgM^{hi} IgD^{low} CD21^{low} CD23$^-$ CD1d^{low} B cells	MZ
DC	CD8α$^+$CD11b$^-$ DCs	T cell zone
	CD8α$^-$CD11b$^+$ DCs	Red pulp and MZ
	CD11c$^+$ DCs	Red pulp
Stromal cells	ER-TR7$^+$ reticular cells	Red pulp and MZ
	FDC or CD35$^+$	B zone (follicle) – white pulp
	FRCs or gp38$^+$	T zone (PALS) – white pulp
	MRC	MZ

The gp38$^+$ fibroblastic reticular cells (FRCs) and CD35$^+$ follicular dendritic cells (FDCs) are region-specific stromal cells that support the microarchitectural in the white pulp. These resident stromal elements have far more sophisticated role in the immunological function of the spleen than merely providing a static tissue scaffold. For example, they form an important network to support the segregation, migration and function of T and B cell. The T cell zone is supported by a network of gp38$^+$ stromal cells known as FRCs. Functionally, they form the so-called conduit system, a reticular collagenous network that

allows the transport and distribution of small molecules or particles of approximately 70 KDa, lymphocytes and DCs. This cellular scaffold also produces various extracellular matrix components, that are determinant in the chemotaxis and homeostasis of T cells into PALS by secretion of CCL19, CCL21, and IL-7 [226,227].

In the follicles, B cells are supported by a network of CD35$^+$ stromal cells, which play a key role in promoting recruitment, survival, segregation and activation of B-cell [228]. CD35$^+$ stromal cells facilitate B cells recruitment to the follicles by secretion of CXCL13 chemokine, the ligand for CXCR5 expressed by B cells [229]. FDC support the maintenance of the germinal centers and the production of high-affinity antibodies by B cells [227]. This stromal cell type can trap and present small antigens and deliver to B cells [230].

1.7.4 Red pulp

The red pulp is composed by a dimensional cellular meshwork of venous sinuses and splenic cords. Approximately, 75% of the splenic volume is composed of red pulp, where sinuses are the primary vascular structures and account 30% of its volume. The splenic cords are composed mainly of fibroblasts, reticular fibres, ER-TR7$^+$ reticular cells and associated F4/80^{hi}CD68$^+$CD11b$^{lo/-}$ macrophages. Other phagocytic splenic red pulp cells are CD11c$^+$ and CD8α-CD11b$^+$ DCs cells [216]. Like the MZ, the phagocytosis in the red pulp is determinant for the clearance of apoptotic particles, foreign material, and/or damaged and aged erythrocytes [165,231]. Splenic red pulp F4/80^{hi}CD68$^+$CD11b$^{lo/-}$ macrophages are specialized in the phagocytosis of damaged

or aged red blood cells after containing surface alterations. Based on the constant blood flow and the splenic circulation, every erythrocyte transits the spleen every 20 min. This place the red pulp in a pivotal position to subject the red blood cells to several types of quality control [161,232].

1.7.5 Effects of aging on the spleen

The immune system activity and efficiency decline with aging; a phenomenon termed immunosenescence [233]. Intrinsic and extrinsic changes in the immune system are manifested with an increased susceptibility to infection diseases and cancer; and paradoxical accompanied by an increase in the incidence of autoimmune disorders. The microenvironment plays a pivotal role in supporting and facilitating the development and activation of immune response. However, the splenic architecture undergoes significant age -related changes[234,235]. Distribution and organization of several immune and stromal cells change in the tissue architecture which may also contribute towards immunosenescence [227,236-239]. In the MZ, macrophages and FDC experience an altered organization, with disturbances in their density, distribution and ultimately function [236,237,239,240]. One of the most significant splenic age-related alterations is the MZ disorganization. MZM loss the continuous boundary between the follicle and MZ and became thicker in density[235,236,238-241]. In addition, marginal zone B cells are also disturbed with age, but contradictories observations within murine strains [238,239].

The white pulp also experiences age-related changes with an increase in the overall size and a considerable attrition in their organization and a merging of the T-cell and B-cell areas[236]. The aged splenic environment also contributes to the defects in CD4$^+$ T cell functions attributable to a decline in the migration capacity of young CD4$^+$ T subpopulation in aged environment [236,242]. In conjunction, the increased status of inflammation contributes to impairments in tissue organization, maintenance and importantly, immune response. In addition, FRC possess a decreased secretion of CCL19 and CCL12 after an immunogenic challenge. FDC from aged mice that undergo antigenic challenge with West Nile Virus present a reduced production of CXCL13 [234]. Aged splenic-FDC also have defect in their ability to trap and present complexes to B cells[243]. All these age-related changes within the spleen could potentially contribute to the age-dependent deficiencies in functional immunity.

1.8 T cell activation

T lymphocytes are the key mediators of the adaptive immune response and its activation is highly regulated and dependent of several interactions among T cells, APC, stromal cells and soluble factors present in the microenvironment. Appropriate T cell activation by DC antigen presentation requires the signal I and II. In addition, cytokine secretion by DCs dictates the priming and polarization of T cells. Via co-stimulation, the first signal (signal I) involves histocompatibility complex (MHC), which binds antigen fragments and carry them to the cell surface and present them to the T cell receptor (TCR)

[244]. The second signal (Signal II) requires costimulatory proteins CD80 (B7-1) and CD86 (B7-2) from the DCs which bind to CD28 receptor expressed on naive T cells (Figure 1.12). Once T cells are activated additional co-inhibitory molecules consisting of the programmed death-1 (PD-1; CD279) receptor and its ligands, programmed death-ligand 1 and 2 (PD-L1, also termed B7-H1; CD274) and PD-L2 (B7-DC; CD273) are recruited to the immune synapse disrupting the positive signals to prime T cells. This negative feedback mediates T cell tolerance and prevents autoimmunity. The sub-optimal co-stimulation of DCs leads to T cell anergy or generation of T regulatory cells.
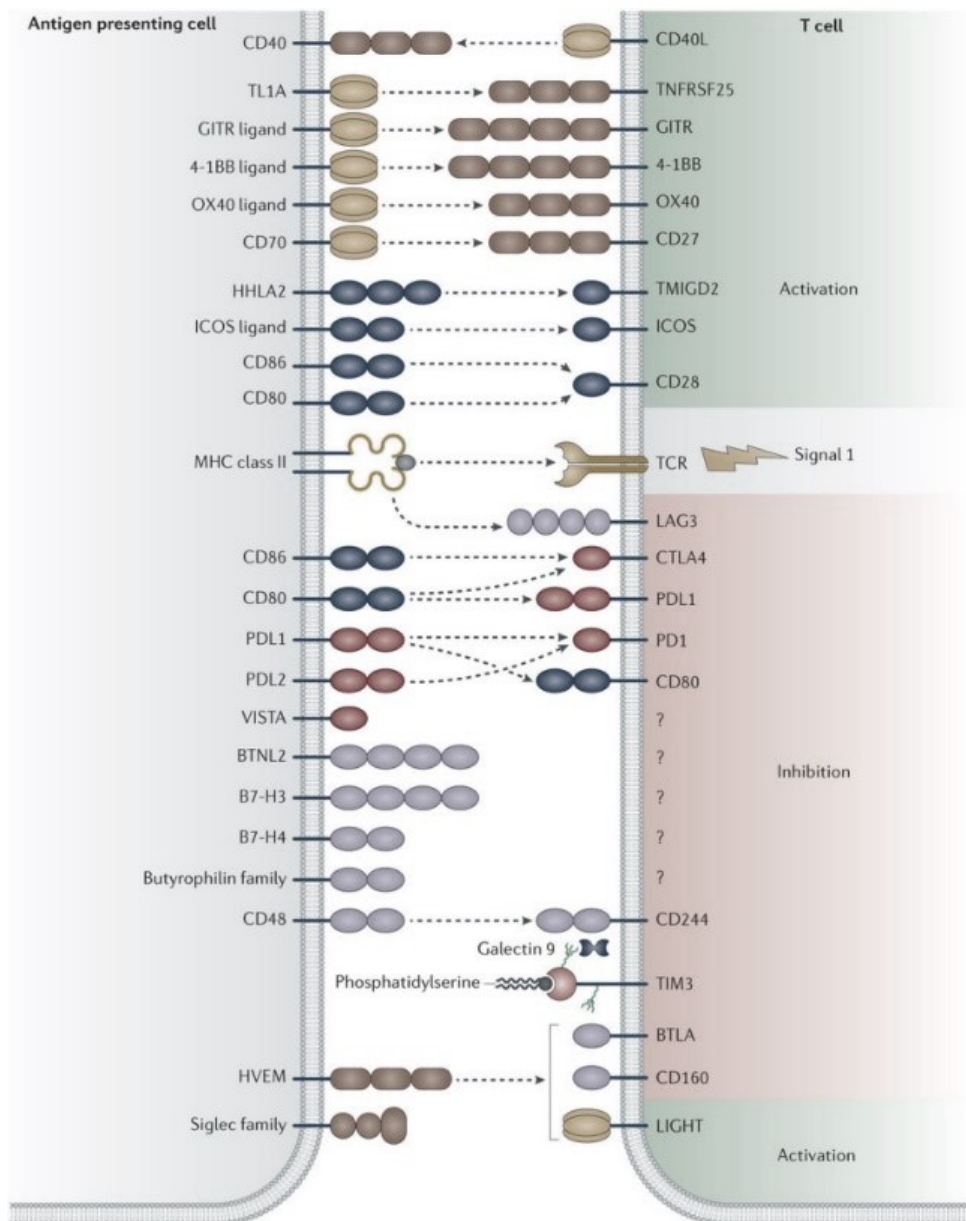
Figure 1.12 Priming of T cells activation

The first step in T cell activation is the engagement of the TCR with a peptide–MHC complex on the surface of an APC. Adapted from Mahoney *et al*., 2015 [245].

1.8.1 Impact of aging in T cell activation

Lymphocytes experience intrinsic age-related alterations that impact their function capability. In normal aging, precursors of T cells migrate from the bone marrow to the thymus where cells finish completing their program to become competent and mature T cells. The effects of aging in T cells are considerable, primarily by thymic involution. Apart from the conspicuous effect of thymic involution on naïve T cells, it has been observed that ageing results in other functional defects: decreased T cell repertoire, decreased IL-2 production [246]. In addition, CD4^{+} and CD8^{+} T subsets of CD3^{+} T lymphocytes experience a gain of negative regulatory receptors, a hallmark for cellular aging [247,248]. This increased expression level of several inhibitory receptor molecules such as programmed death 1 (PD1), killer cell lectin-like receptor subfamily G (KLRG), killer immunoglobulin-like receptors (KIR), Ig-like transcription 2 (ILT2), cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), lymphocyte activation gene 3 (LAG-3) and T cell immunoglobulin mucin domain-3 (Tim-3) [248-252].

1.8.2 Macrophages and dendritic cells

DC and macrophages experienced a decline in their function as a consequence of intrinsic alterations and of aged-related changes in the environment (Table 1.2). The induction of a proinflammatory state in the environment provokes a decline in their capacity to mount specific immune responses [160,165,232,253-256]. In addition, to detect undesired pathogens and/or dangers, macrophages and dendritic cells rely on the expression of numerous germline-encoded pathogen recognition receptors (PRRs). Normal macrophages express several classes of PRRs, including toll-like receptors

(TLRs), Nod-like receptors (NLRs), and RIG-I like receptors (RLRs). These receptors recognise conserved and invariant microbial products referred to as pathogen associated molecular patterns (PAMPs) [257,258]. Once the PRR-PAMPs interact properly, a downstream cascade is activated and elicits macrophages to generate pro-inflammatory cytokines and to initiate the inflammatory response. However, aged macrophages fail to recognize and activate a proper immune response [259]. For example, aged splenic macrophages produce fewer amounts of pro-inflammatory factors in response to lipopolysaccharide (LPS) stimulation [259,260]. This impaired capacity to produce cytokines has been attributed to intrinsic and extrinsic alterations. Intrinsically, aged macrophages present a reduced expression of TLRs, and a decreased activation NF-κB and MAPK. Extrinsically, macrophages are exposed to a persistent pro-inflammatory state in aged mice, downregulating their phagocytic function (table 1.5) [257,261,262].

Table 1.5 Intrinsic age-related changes in the innate immune system

Cell type	Age-related increase	Age-related decrease
Macrophages	- ªPGE$_2$, IL-10, COX-2 [209,263] - Polarisation toward M2-like macrophages [165,232] - Pro-inflamatory cytokine production [264] *p16^{INK4a}* and SA-β-gal activity ref for all this please [265]	- Pathogen detection [259] - Phagocytic capacity [165,232,253-255] - Wound healing [253] - Oxidative burst / microbial killing - Antigen presenting and T cell stimulation [266] - MHC class II - TLR4 expression [260] - Signaling p38MAPK
Dendritic cells		- Decreased antigen presenting - Decreased endocytosis - Impaired capacity to stimulate antigen specific T cells - Lymph node homing/ chemotaxis

Modified from Weiskopf *et al*., 2009 [267]. ª Associated to T cell impaired activation.

Chapter 2. IR-induced senescence in the splenic environment interferes with murine immune cells functions

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2.1 Abstract

Elimination of p16^{INK4a} expressing senescent cells restores tissue homeostasis in mice chronologically aged or treated with genotoxic stressors. Exposure to ionizing radiation (IR) leads to a tissue-wide detrimental increase in p16^{INK4a} expression. Whether IR-induced p16^{INK4a} expression affects immune cell functions is unknown. We here provide evidences that exposure of mice to IR leads to the induction of a senescent associated secretory phenotype (SASP) and the expression of p16^{INK4a} in various cell populations in the spleen. In particular, we observed a reduced capacity of splenic T-cells to proliferate in response to allogenic splenocytes in vitro and to a viral infection in vivo. We found this effect was mostly dependent on the splenic environment as the proliferation of purified T-cells was not abrogated. Furthermore, we found the secretome of splenocytes isolated from irradiated mice to impaired T cell proliferation. Genetic ablation of p16^{INK4a} expressing cells, using the p16-3MR transgenic mouse model, allowed for a reduction of the SASP and the restoration of T-cell proliferation. Moreover, we found F4/80$^+$ macrophages isolated from irradiated spleens to express high levels of p16^{INK4a} and to have a reduced phagocytosis activity, a defect also restored by the elimination of p16^{INK4a} expression. Our data suggests that it may be possible to pharmacologically enhance immune cell functions in irradiated patients.

2.2 Key words:

2.3 Introduction

Cellular senescence is a complex phenotype observed in many tissues at various developmental stages [268,269]. In adults, its main role is likely to irreversibly prevent damaged cells from proliferating [270-272]. Senescent cells are induced during chronological aging and by a wide range of acute genotoxic stressors such as aberrant oncogene expression and DNA damaging agents [131]. Identification of senescent cells in tissues remains very challenging given the absence of an universal marker. Expression of the tumor suppressor p16[INK4a] increases with age in numerous mouse and human tissues and is arguably considered the most reliable marker [6]. Exposure to ionizing radiation (IR) leads to a delayed increase in p16[INK4a] expression in mice tissues and in cancer treated patients [99,176].

Evidence for senescent cell immune clearance *in vivo* has been so far limited to murine hepatocytes expressing an oncogenic form of Ras (Hras[V12]) [176,180]. Concomitant with arrest, and secrete a wide range of cytokines, chemokines and proteases known as the senescence-associated secretory phenotype (SASP) [66,67]. While the induction of senescence is beneficial in instances such as wound healing, the accumulation of senescent cells is predominantly detrimental. For example, the SASP has been shown to contribute to tumor growth and to the development of age-associated diseases [273-275]. Genetic or pharmacological clearance of senescent cells reverses the onset of aging and its associated pathologies in mice [10,13]. It also has the capacity to reduce the side effects of anti-cancer treatments in both mice and humans [276]. Similarly, elimination of IR-induced

senescence was shown to mitigate the premature aging phenotype imposed to murine hematopoietic stem cells [12].

The impact of aging on the immune system is well described and is associated with an increase susceptibility to infection [277-280]. The adaptive response, particularly the proliferation of T-cells and function of dendritic cells is reduced in aged donors [281]. Likewise, deletion of p16^{INK4a} in T-cells specifically was shown to enhanced antigen-specific immune responses, suggesting an intrinsic defect of aged T-cells [277,282].

In comparison, the extent by which premature senescence has an impact on the immune system is less defined. Mice lacking the expression of lamin A, a nuclear scaffolding protein, have an accelerated aging phenotype and display immune deficiencies [283]. Similarly, short-term after exposure to various sub-lethal doses of IR, immune cell numbers and bone marrow niches were shown to be depleted and their proliferative capacity diminished. However, a recent study showed that exposure to IR (up to 4 Gy) at a young age does not impair immune functions at an old age [284]. Yet, in this particular study, irradiated old mice (19 months) were compared to age-matched non-irradiated counterparts which immune functions were likely already diminished.

We previously observed that exposure of mice to IR leads to impaired lymphopoiesis in the bone marrow, an effect we found was cellular nonautonomous and dependent on p16^{INK4a} [7]. Here, we wanted to determine whether IR-induced p16^{INK4a} expression occurs in the spleen if it would interfere with splenic cell functions. Using the described p16-3MR mouse model [169], we show that IR impairs immune cell functions in

the splenic environment, an effect partially mediated by the SASP and reversible upon clearance of p16INK4a senescent cells.

2.4 Results

2.4.1 Exposure to IR induces features of senescence in the spleen

We have previously shown that exposure to IR leads to a deferred (6-8 weeks) p16INK4a expression in several mice tissues including the spleen [158,285]. The reason for such a delay is not clear but is not only a consequence of IR-induced loss of tissue homeostasis. Indeed, we demonstrated that sustained p16INK4 expression is necessary to protect mice against cancer progression [285]. Yet, it is not known if IR-induced p16INK4a expression is associated with a senescence phenotype and whether this can have adverse effects on splenic cell functions. This is particularly relevant knowing the negative impact of IR on the immune system and the importance of the spleen for the latter. We exposed our mice to total body irradiation at the sublethal dose of 6.5 Gy as this is the maximum tolerated dose for all mice to survive without the need for a bone marrow transplant. We also decided to wait a minimum of 8 weeks post irradiation to perform our studies for two reasons. First, to allow p16INK4a expression to rise and second, because it is the minimum time required to regain a steady spleen cellularity although it never completely reaches the level observed prior to irradiation (Supplementary figure 2.1). Hence, we irradiated p16-3MR mice, in which p16INK4a–positive cells can be visualized and eliminated upon

the administration of ganciclovir (GCV), waited for 8-9 weeks, treated mice (or not) with GCV for five consecutive days and sacrificed animals the day after the last dose (Figure 2.1A). Using both luminescence reporter activity and qPCR data, we found that p16^{INK4a} expression is increased 3-4-fold in irradiated spleens and eliminated in mice injected with GCV (Figure 2.1B-D). We then performed a cytokine array using splenic cell lysates. Samples were collected and the secretion of classical SASP markers (IL-1a, IL-6, MCP-1, KC, VEGF) measured. For most of these markers, the concentration was found diminished in GCV treated mice (Figure 2.1E). Although the SASP has been typically described as pro-inflammatory, we did observe an increase in IL-10 secretion, a cytokine with pleiotropic immunosuppressive effects (Figure 2.1E). Despite our best attempt, we failed to detect the expression of the senescence-associated Beta-galactosidase in irradiated spleen sections (data not shown).

2.4.2 Attrition of CD3$^+$ and B220$^+$ cell populations in the irradiated spleen

Days after exposure to IR, massive cell death is observed in splenic T and B-cells and to a lesser extent in the other cell populations (Supplementary figure 2.1). This is not surprising given that lymphocytes are amongst the most highly radiosensitive cells. Here, we wanted to determine if exposure to IR would induced p16^{INK4a} expression in these cells and if it would have an impact on their absolute number upon reconstitution. Spleens were collected from control and irradiated mice treated or not with GCV and dissociated at the single cell level. Cells populations were then isolated using magnetic columns (over 90% purity as determined by flow cytometry) and mRNA was extracted for qPCR analysis.

We found p16^{INK4a} gene expression was increased 4-fold in CD3$^+$ cells and 5-fold in B220$^+$ cells (Figure 2.2A and B). These levels are similar to what has been reported in lymphocytes collected from aged spleens [6]. Absolute cell counts were found diminished on average 50% at 8 weeks post IR for both CD3$^+$ (CD4$^+$ and CD8$^+$) and B220$^+$ cell populations (Figure 2.2C-E). While the injection with GCV efficiently eliminated p16^{INK4a} expressing cells in these populations, only in the CD3$^+$CD4$^+$ fraction were cell counts restored to the level observed in non-irradiated mice.

2.4.3 Impaired proliferation of irradiated T cells is dependent on the splenic environment

We were next interested in the impact of p16^{INK4a} expression may have on the capacity of irradiated splenic T cells to proliferate in response to allogenic stimuli. For this, we first performed an one-way MLR using splenocytes collected from p16-3MR mice treated as described (Figure 2.3A) and freshly irradiated allogenic splenocytes obtained from a CD-1 mouse. Our results showed that the proliferation of splenic T cells, collected from p16-3MR mice irradiated 8-9 weeks earlier, is impaired 40% on average compared to the proliferation of splenic T cells obtained from non-irradiated mice (Figure 2.3B). Interestingly, the proliferation of splenic T-cells collected from irradiated mice treated with GCV was almost fully restored (Figure 2.3B). However, when we repeated this experiment using this time magnetically purified T-cells from p16-3MR splenocytes, we did no observe any impact of the irradiation on the capacity of T-cells to proliferate (Figure

2.3C). In this situation, elimination of p16^{INK4a} expression with GCV did not improve further the proliferation of T-cells (Figure 2.3C).

We then confirmed these results by measuring the capacity of splenic T-cells to proliferate *in vivo* in response to an acute LCMV-Arm infection. On day 7 post viral infection, we found the proliferation of T-cells, as measured by Ki67 expression, to be reduced in the CD8$^+$gp33$^+$ cell subpopulation, gp33 being a commonly recognized epitope of the LCMV-Arm virus (Figure 2.3D). Injection of mice with GCV prior to the viral infection allowed for an increased proliferation of T-cells. A similar impact on the ability of CD8$^+$gp33$^+$ T-cells to secrete granzyme B was also observed in response to the LCMV - Arm (Figure 2.3E). Of note, we did not observe a difference in the viral load in the serum at the time of sacrifice (data not shown).

Overall, these results demonstrate that intrinsic proliferation of splenic T-cells is not impaired by IR. Rather, it is compromised *in vitro* and *in vivo* in the presence of senescent splenocytes. This suggests that in the splenic environment, either cells or their secretome, interferes with T-cell proliferation. To test this hypothesis, we performed a MLR in which the effector cells were CD3$^+$ purified from p16-3MR mice and stimulator cells were freshly irradiated CD-1 splenocytes. Both effector and stimulator cells were then placed in the presence of an "environment" consisted of CD3$^+$ depleted splenocytes isolated from p16-3MR mice treated as described (Figure 2.4A). Using this experimental setup, we confirmed that allogenic proliferation of purified T-cells is compromised when in presence of a previously (8-9 weeks) irradiated splenic environment (Figure 2.4B).

Again, when the splenic environment was collected from mice treated with GCV, the proliferation of T-cells was partially restored (Figure 2.4B).

To define which of the irradiated splenocytes or their secretome is detrimental to T-cell proliferation, we performed a modified MLR in which the effector/stimulator cells were separated from splenocytes by a transwell. In this setting, cellular interactions are inhibited and only the secretome of the splenic environment is able to interfere with proliferation. Using beads couples to anti-CD3 and anti-CD28 antibodies as stimulator, we found that the secretome of irradiated splenocytes alone was sufficient to delay T-cell proliferation (Figure 2.4C and D). This negative impact of the irradiated secretome on T-cell proliferation was also confirmed using allogenic splenocytes as stimulator (Figure 2.4E and F) and was diminished when collected from irradiated mice treated with GCV (Figure 2.4G and H). In this case, although the total fraction of T-cells undergoing proliferation appeared only slightly increased, the proportion of cells undergoing several divisions was greatly increased in the GCV treated group (Figure 2.4H). Overall these results demonstrate that impaired T-cell proliferation is at least partially mediated by the splenic environment through the SASP.

2.4.4 IR impairs macrophages and dendritic cells in the spleen

Macrophages and dendritic cells (DC) play a pivotal role in innate and adaptive immunity by their capacity to phagocyte, process and present antigens. These cells are radioresistant (compared to lymphocytes) as 25-40% of the splenic populations are still alive one week following exposure to IR (Supplementary figure 2.1). Still, long-term (8-9

weeks) after IR, these cells (herein defined as F4/80$^+$ for macrophages and CD11c$^+$ for DC) display elevated levels of p16^{INK4a} expression and a decrease absolute cell counts compared to cells collected from non–irradiated animals (Figure 2.5A-D). In particular, macrophages displayed an over 20-fold increase in p16^{INK4a} expression which was fully abrogated upon GCV injection. In comparison, DC only had a 4-fold increase which was not completely abolished by GCV. The absolute cell count of macrophages was not only restored but even slightly increased following the administration of GCV (Figure 2.5C and D). At the functional level, we noticed that the phagocytic capacity of these cells, as measured by flow cytometry for their ability to uptake a fluorescent substrate, was diminished in cells collected from irradiated mice. Such a decrease in phagocytosis was partially restored in macrophages collected from mice treated with GCV (Figure 2.5E and F). We believe that this defect is intrinsic to the cells as magnetically-purified populations of macrophages and DC were used for this phagocytosis assay (purity >85%). Our results also suggest that a sub-population of cells lost its ability to phagocytose in opposition to a majority of cells with a slightly diminished capability. This is supported by the fact that the mean fluorescence intensity, representing the mean number of particles taken up by each cell, was not reduced (data not shown).

2.5 Discussion

Long-term residual side effects of chemotherapy and radiotherapy is a growing concern for pediatric cancer survivors [286]. For example, decrease immune functions in irradiated animals is similar to what has been observed in the aging population [287]. Identification of

the mechanisms responsible for this phenotype could potentially be pharmacologically exploited to restore the immune system fitness. Here, we provide evidences that there is an increased expression of p16^{INK4a} and the presence of a SASP in the irradiated spleen. We showed that proliferation of splenic T-cells *in vitro* (upon allogenic stimulation) or *in vivo* (in response to an acute LCMV-Arm infection) is compromised in previously (8-9 weeks) irradiated mice. Such defect in T-cell proliferation appeared to be non-autonomous as CD3$^+$ purified cells from irradiated spleens were fully competent in their ability to proliferate in a MLR. These results suggest that increase p16^{INK4a} expression (4-fold) in these cells is not high enough to limit their proliferation or most likely that only a small fraction of the cells express a higher level of p16^{INK4a}. Unfortunately, the intensity of the luminescence/fluorescence signal provided by the 3MR reporter genes does not allow for the quantification of p16^{INK4a} expression at the single cell level. Despite the high relative purity (over 90%) of the CD3$^+$ cell populations used in our studies, we cannot rule out the possibility that the observed increase originates from few contaminating cells expressing high levels of p16^{INK4a} (*i.e.* macrophages).

Another interesting observation is the fact that the irradiated splenic environment was able to limit the proliferation of T-cells in conditions where stimulator cells (CD-1 mouse splenocytes) were irradiated as well. However, in this case, CD-1 splenocytes were freshly irradiated whilst splenocytes isolated from p16-3MR mice were irradiated for as long as 8-9 weeks. Hence, the most plausible explanation for these results is that freshly irradiated CD-1 cells did not have the time to develop a SASP and to increase p16^{INK4a} expression, the latter being delayed several weeks post exposure to IR [99].

Our results also showed that part of the inhibitory effect induced by the irradiated splenic environment is mediated by the SASP. Amongst the several SASP factors we have identified, it is not possible at this point to determine if a specific cytokine is important for the proliferation of T-cells is decreased or if the secretion of an inhibitory molecule is increased. A potential candidate is IL-10, an immunosuppressive cytokine which the expression was found increased 2-fold in irradiated spleens and reduced following administration of GCV. Intriguingly, we found the negative impact of the irradiated splenic environment on the proliferation of purified CD3$^+$ T cells to be more pronounced (50%) than on non-purified splenic T-cells (30-35%, see Figure 2.4B vs. 3B respectively). The explanation for this is likely technical as the addition of a small volume of purified CD3$^+$ T cells in the MLR mix does not dilute the irradiate environment.

Our MLR data performed in absence of cellular interactions between effector cells and the irradiated splenocytes environment showed a significant but smaller decrease in T-cell proliferation compared to when irradiated splenocytes. were in contact with the effector cells (Figure 2.4C and 4E vs Figure 2.4B). As a possible explanation for this difference, we have observed a reduced phagocytosis capacity in macrophages and DC, a defect that was restored in macrophages following the elimination of p16^{INK4a}-positive cells. Whether a decrease in phagocytosis is sufficient to have a negative impact on T-cell proliferation in the context of a MLR is only correlative at this point and remains to be determined. We also observed a slight increase in the number of macrophages following the injection of GCV in irradiated mice. This was unexpected given the high level of p16^{INK4a} expression in this population. We believe the increase may be the results of peripheral macrophages being recruited to the spleen in response to GCV-induced cell

death. Of note, expression of p16^{INK4a} in macrophages was recently shown to be modulated positively and negatively by immune stimuli, suggesting a complex regulation in these cells and more work is needed to better understand this regulation [200]. We also observed a severe reduction in the expression of costimulatory molecules (CD80 and CD86) expressed on irradiated macrophages and DC compared to non-irradiated controls (Supplementary figure 2.2). Again, whether this can translate into impaired T-cell proliferation has to be evaluated. A possibility we have not explored is an increase in the proportion of T-regs in the irradiated spleens. Such an increase in T-regs has been described to impair immune functions in aging mice and humans[288,289]. Finally, the impact of IR on the spleen stromal architecture could also be involved. We have observed a significant increase in p16^{INK4a} expression and a decrease in absolute cell counts upon injection of GCV in sub-populations (gp38$^+$ and CD35$^+$) of splenic stromal cells (Supplementary figure 2.3). Hence, while we showed that the SASP is detrimental to the proliferation of splenic T-cells, the overall impact of IR on the spleen functions is likely multifactorial.

In conclusion, we demonstrated that elimination of p16^{INK4a} expressing cells within the splenic environment using mouse genetics is sufficient to improve some immune functions. It will be interesting to evaluate if the elimination of senescent cells using newly developed senolytic drugs [290] will also increase the fitness of immune cells in mice receiving radiotherapy and/or chemotherapy.

2.6 Materials and Methods

2.6.1 Animals and treatments

p16-3MR mice were kindly donated by Dr. Judith Campisi (Buck Institute) and breed on site according to a Material Transfer Agreement. All *in vivo* manipulations were approved by the Comité Institutionnel des Bonnes Pratiques Animales en Recherche of the CHU Ste-Justine [169]. 12-14 weeks-old p16^{INK4a}-3MR mice were exposed to X-rays at the single sublethal dose of 6.5 Gy (1 Gy/min) using a Faxitron CP-160. GCV was administrated daily by intraperitoneal (*i.p.*) injections for 5 consecutive days at a dose of 25mg/Kg in 1X-PBS (Catalog, G2536, SIGMA).

Mice were injected i.p with 2×10^6 pfu of Lymphocytic Choriomeningitis Virus (LCMV) strain Armstrong (LCMV-Ams) to generate acute infection. Seven days post infection, spleens were harvested from infected mice and filtered through a 70µm-pore-size cell strainer (Falcon, Franklin Lakes, NJ) and centrifuged at 1200rpm for 5 minutes at 4°C. Then, splenocytes were treated with NH_4Cl to remove erythrocytes. For all experiments, dead cells were stained with fixable LIVE/DEAD Aqua (Catalog, L3496, Life Technologies) and were excluded from the analysis. For granzyme B release, splenocytes were restimulated *in vitro* for 4 h with a cognate gp33 peptide (0.1 mM) in the presence of Golgi-Stop (Catalog, 554724, BD). Cells were then fixed and permeabilized using the Cytofix/Cytoperm kit (Catalog, 554722, BD) and were stained for Granzyme B (Clone GRB05, Lifetechnologies). For nuclear staining, splenocytes were processed directly *ex vivo*. Cells were Fc-blocked, and extracellular staining was performed in 50–

100 μL of PBS with 2% (vol/vol) FBS for 20 min on ice before fixation. Cells were fixed with Cytofix/Cytoperm (Catalog, 554722, BD) followed by intracellular Ki67 staining (Clone SolA15, Bioscience).

2.6.2 Bioluminescence

To detect luminescence from the 3MR gene cassette, mice were anesthetized using isoflurane and injected *i.p.* with water-soluble coelenterazine (CTZ) (Catalog, 3031, NanoLight technology™) at a concentration of 1mg/mL in 1X-PBS. Mice were imaged using *In vivo* Epi-Fluorescence & Trans-Fluorescence Imaging System (Labeo Technologies) fourteen minutes post injection. Mice were then euthanized, and their spleens were surgically removed and their bioluminescence levels measured *ex vivo* in a solution of 1mg/mL CTZ.

2.6.3 Gene expression

RNA was extracted from spleens and from isolated CD3$^+$, B220$^+$, gp38$^+$, CD35$^+$, CD11c$^+$ and F4/80$^+$ cell populations using the RNeasy® Mini or Micro Kit (Qiagen, Mississauga, ON). Cells were purified using EasySep™ PE Positive Selection Kit (Catalog, 18551, StemCell Technologies) according to the manufacturer's instructions. RNA was reverse-transcribed using the QuantiTect Reverse 71. Quantitative differences in gene expression was determined by real-time quantitative PCR using SensiMixTM SYBR Low-ROX (Quantace, Taunton, MA) and the MxPro QPCR software (Stratagene, Mississauga, ON).

Values are presented as the ratio of target mRNA to 18S rRNA, obtained using the relative standard curve method of calculation.

2.6.4 Flow cytometry analysis

To obtain the absolute cell count numbers, spleens were processed in 1X-PBS with 2% FBS and mechanically disrupted with the flat portion of a plunger from a 5-mL syringe. Samples were then incubated with collagenase D for 30min (Catalog, 11088866001, Roche). Splenic cell suspension was passed through a 70μm-pore-size cell strainer (Falcon, Franklin Lakes, NJ) and centrifuged at 1200rpm for 5 minutes at 4°C. Splenic cell counts were then determined using Count Bright® Absolute Counting Beads (Catalog, C36950, Termo Fisher) and analyzed using the Becton Dickinson Immunocytometry Systems (BD LSR-Fortessa™). Briefly, red blood cells were lysed by adding 5ml of lyse solution (0.14M NH4CL, 0,02M Tris HCl, pH 7.2). The tubes were incubated at room temperature (RT) for 5 min, and washed twice with 10mL of Roswell Park Memorial Institute medium (RPMI) at 10% of fetal bovine serum (FBS). Cells were then centrifuged, and the pellet re-suspended in 3mL of 1X-PBS from which 10uL of cell suspension was stained with fluorophore-conjugated antibodies from all purchased from Biolegend. F4/80 (clone BM8), CD3 (clone 17A2), CD4 (clone GK1.5), CD8a (clone 53-6.7), CD11b (clone M1/70), CD11c (clone N418), CD35 (clone 7E9), gp38 (clone 8.1.1), CD31 (clone 390), PDGFR (clone APA5), CD45 (clone 30-F11).

2.6.5   In vitro phagocytosis assay

Splenic CD11c$^+$ DCs and F4/80$^+$ macrophages from naïve spleens were purified using EasySep™ PE Positive Selection Kit according to the manufacturer's instructions at a purity of 85%. Purified cells were then used at a concentration of ~1x10^5 cells/ml in RPMI completed with 10%FBS and 1% antibiotics containing Phrodo™ Green Zymosan A particles (Catalog, P35365, Termo Fisher) for 90 min at 37C, 5% CO$_2$. A minimum of 50,000 events were analyzed by Flow cytometry.

2.6.6   Multiplex cytokine analysis

The splenic cell secretome was quantified by Eve Technologies (Calgary, Canada) using the 31-Plex Mouse Cytokine Array / Chemokine Array. Splenocytes samples were processed according to Eve Technologie's recommendations. Briefly, 10x10^6 splenocytes were pellet and washed twice with 1X-PBS, then cells were lysed with radioimmunoprecipitation assay (RIPA) buffer on ice for 10 min (20 mM Tris HCl - {pH 7.5}, 0.5% Tween 20, 150 mM NaCl) with 1% protease inhibitors (**PI**). Lysates were centrifugated at 10 000g for 10min at 4ºC and supernatants were transferred to a new tube and normalized with 1X-PBS to 0.5mg/ml of proteins.

2.6.7   T-cells proliferation assays

T-cell proliferation was evaluated by an allogenic mixed lymphocyte reactions (MLR) assay. Splenocytes or purified CD3$^+$ cells were labelled with CellTrace™ 6-carboxy-succinimidyl-fluorescein-ester dye (CFSE) and used as responder. These cells were

harvested from spleens of p16-3MR mice irradiated 8-9 weeks earlier and treated or not with GCV for 5 consecutive days prior to sacrifice. CD3$^+$ cells were isolated by negative selection using the EasySep Mouse T Cell Isolation Kit (Catalog, 19851, STEMCELL). The purity of CD3$^+$ cells was determined by flow cytometry using a PE-conjugated anti-CD3 antibody (Catalog, 100240, Biolegend). Splenocytes obtained from the outbred CD-1$^®$IGS mouse strain (Charles River, St-Constant, Qc) were used as responders and irradiated at a dose of 30Gy. MLRs were set up with CFSE-labelled 1×10^5 p16-3MR responder cells and 2×10^5 freshly irradiated allogenic CD-1 stimulator splenocytes in round-bottom 96-well plates, at 37°C, 5% CO_2 atmosphere for 3 days. Alternatively, responder and stimulator cells were separated from a splenic "environment" by a Transwell$^®$ system with 8.0μm (Catalog, 3422, Costar). The splenic environment consisted of freshly isolated splenocytes depleted of CD3$^+$ cells CD3$^+$ using a magnetic-bead-mediated positive PE selection kit (Catalog, 19851, STEMCELL). The efficacy of the depletion was over 90% as measured by flow cytometry. Negatively isolated p16-3MR CFSE-labelled CD3$^+$ cells were incubated in a CD3$^+$-depleted environment at a proportion of 1:5. The combination of CFSE-labelled CD3$^+$ cells and CD3$^+$-depleted splenocytes was then incubated with allogenic CD-1 splenocytes at a proportion of 1:2. Finally, the proliferation of p16-3MR CFSE-labelled CD3$^+$ responder cells were also induced using the Dynabeads® mouse T-activator CD3/CD28 system (11456D, Fisher).

2.6.8 Statistical Analysis

GraphPad Prism 7 software was used for statistical analysis; ρ values on multiple comparisons were calculated using One-way analysis of variance (ANOVA) with Bonferroni post Hoc test.[291]

2.7   Figures

Palacio *et al.***,** *Submitted*

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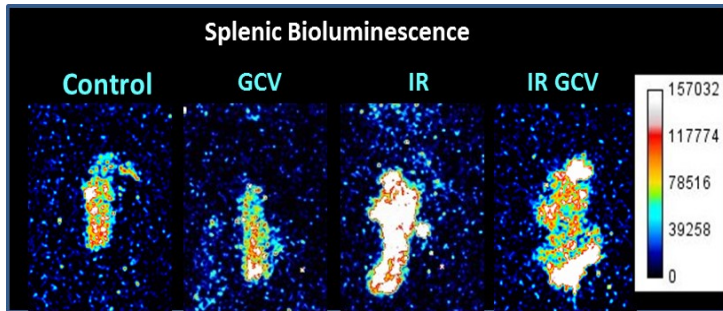
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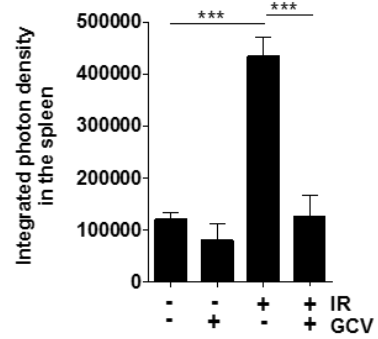
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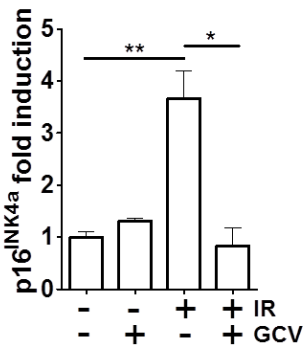
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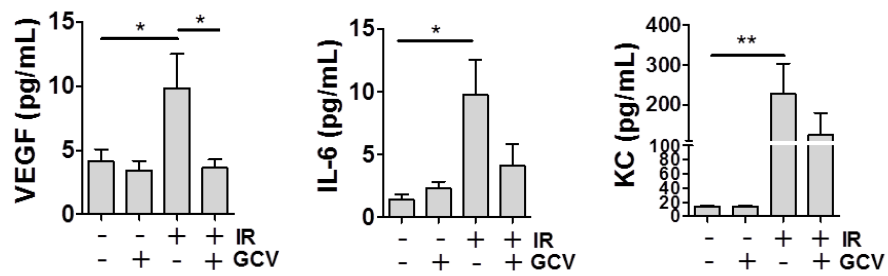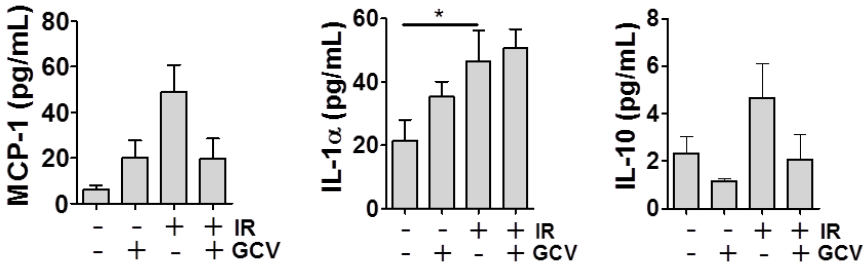
Figure 2.1 Exposure of mice to IR induces p16^{INK4a} expression and a SASP in the spleen.

(**a**) Experimental design, 12 week-old p16-3MR mice were exposed to 6.5 Gy total body irradiation. Eight to nine weeks later mice were treated or not with GCV for 5 consecutive days to eliminate *p16^{INK4a}*-positive cells. (**b**) One day after the last GCV treatment, mice were injected *i.p.* with coelentarazine (CTZ) and 14 minutes later mice were sacrificed, and spleens surgically removed to quantify the luminescence. Representative photographs for the expression of *p16^{INK4a}* expression in the spleen is shown. (**c**) Shown is the average of integrated photon density ± SEM emitted from p16-3MR mice exposed (+) or not (-) to IR treated (+) or not (-) with GCV. (**d**) Quantification of endogenous *p16^{INK4a}* mRNA levels as determined by qPCR from full spleen lysates. 1*8S* ribosomal RNA was used as an internal control. (**e**) Expression levels of VEGF, IL-6, KC, MCP-1, IL-1α and IL-10 from splenocytes lysates as detected by multiplex array. Shown is the average ± SEM analyzed by One-way ANOVA***$p<0.001$; **$p<0.01$; *$p<0.05$; n=6 mice per group.
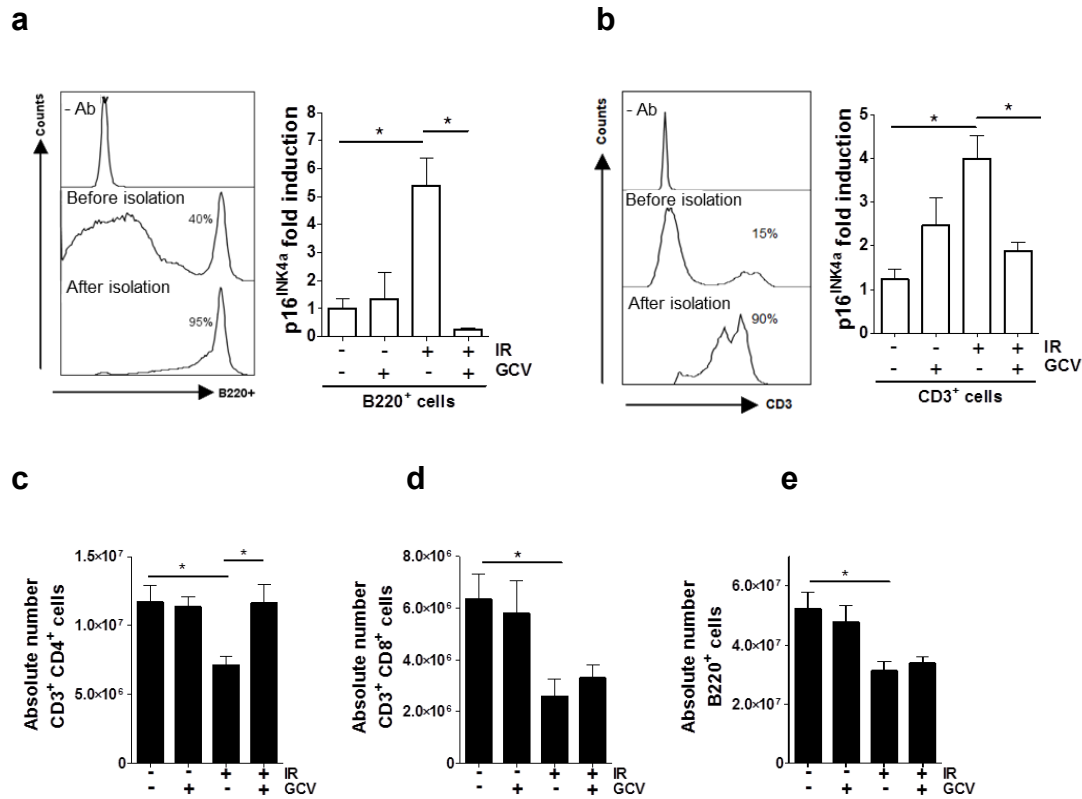
Figure 2.2 Attrition of T and B cell populations in the irradiated spleens

(**a-b**) Representative quantification of the purity for isolated B220+ and CD3+ cell populations respectively (left panels). The right panels show the quantification of *p16^INK4a* mRNA levels by qPCR in these isolated populations. 18S ribosomal RNA was used as an internal control. Quantification by flow cytometry of the absolute cell counts for CD3+CD4+ (**c**), CD3+CD8+ (**d**) and B220+ (**e**) populations per spleen collected from mice treated as indicated. Cell counts were determined one day following the last injection of GCV. Shown is the average and SEM. The *p* value was determined by a One-way ANOVA. *$P<0.05$. n=6 mice per group.

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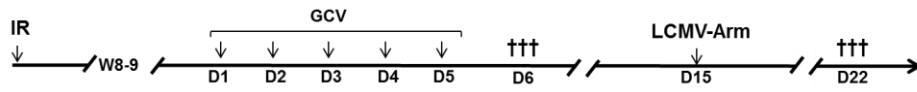
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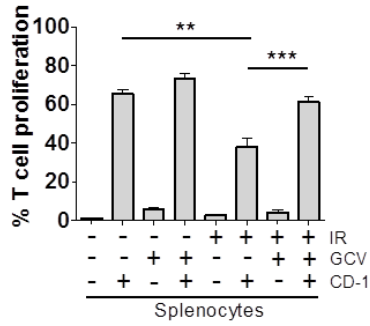
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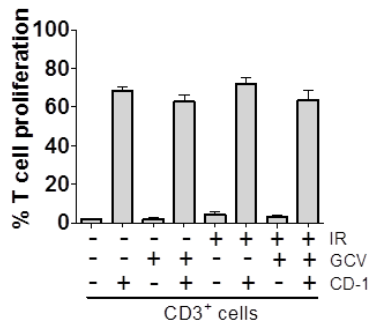
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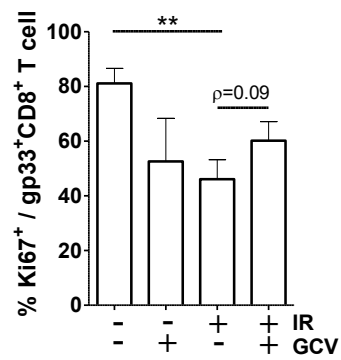
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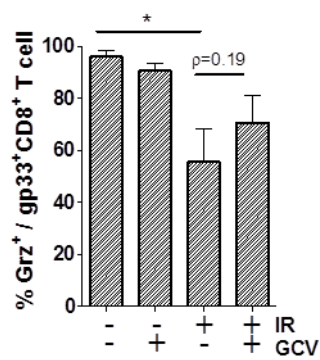
Figure 2.3 IR impairs T cells proliferation *in vitro* and *in vivo*

(**a**) Schematic illustration of the experimental design. 12-week-old p16-3MR mice were exposed to 6.5 Gy total body irradiation and eight to nine weeks later mice were treated or not with GCV for 5 consecutive days. On day 6 (D6) mice were sacrificed and splenocytes were collected and labelled with CFSE. (**b-c**) The proportion of CD3$^+$ cell undergoing proliferation following an allogenic stimulus (MLR) was determined by flow cytometry. In panel b, the proliferation of T-cells was determined from gated CFSE-CD3$^+$ cells from p16-3MR splenocytes responder cells mixed with CD-1 stimulator splenocytes (ratio 1:2). In panel c, the proliferation of T-cells was determined from gated CD3$^+$ from p16-3MR purified CD3$^+$ responder cells mixed with CD-1 stimulator splenocytes (ratio 1:2). (**d**) To quantify the proliferation capability of T-cells *in vivo,* mice were treated as in (a) and exposed to an acute infection of Lymphocytic Choriomeningitis Virus (LCMV) strain Armstrong (LCMV-Arms) on day 15 (D15) and sacrificed seven days after infection (D22). The proportion of gp33$^+$CD8$^+$ T cells undergoing LCMV specific proliferation was determined by flow cytometry as the percentage of cells expressing Ki67. (**e**) The proportion of gp33$^+$ CD8$^+$ T cells expressing granzyme B (Grz) was determined by flow cytometry. For all graphs, the average +/- SEM from n=6-7 mice is shown. The p value was determined by a One-way ANOVA. ***$p<0.001$; **$p<0.01$; *$p<0.05$.

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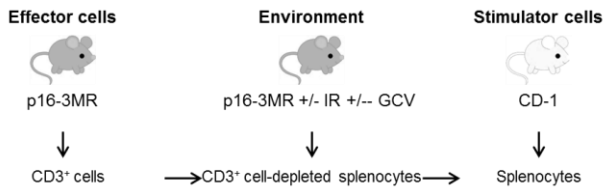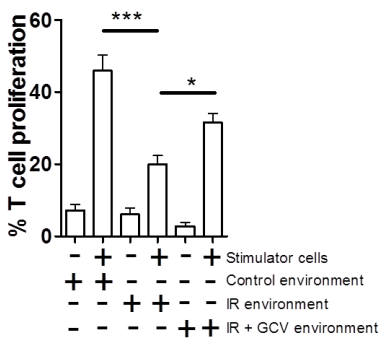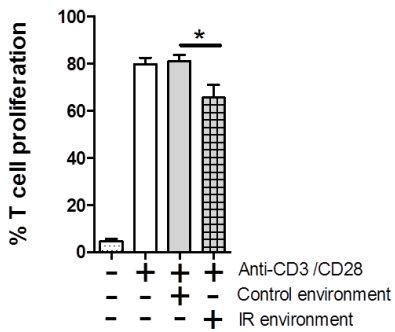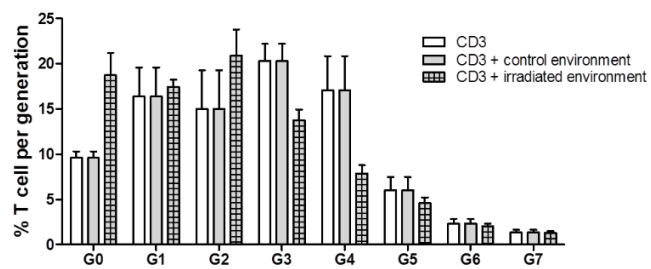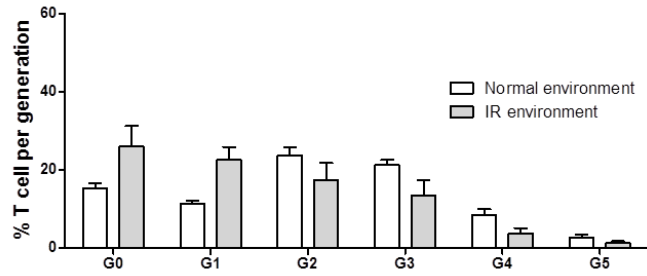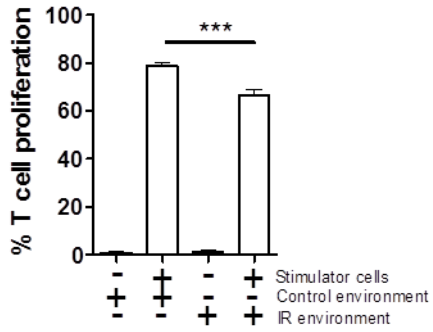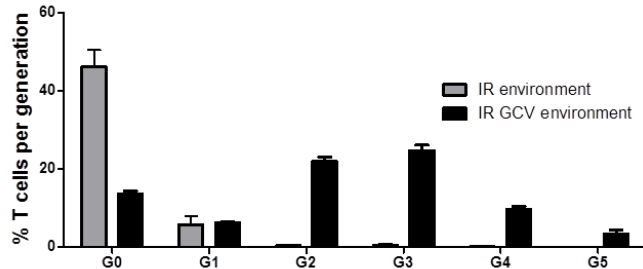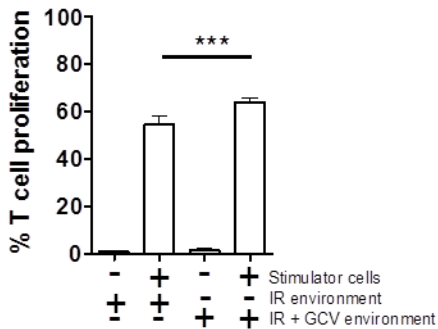
Figure 2.4 The irradiated splenic environment impairs T-cell proliferation

(**a**) Schematic representation of the experimental design. CD3$^+$ effector cells were isolated by negative selection from spleens of p16-3MR mice and labelled with CFSE. The splenic environment corresponds to T-cell depleted splenocytes from p16-3MR mice previously (8-9 weeks) exposed (+) or not (-) to IR and treated or not with GCV. Stimulator cells were freshly irradiated (30 Gy) allogenic splenocytes collected from a CD-1 mouse. (**b**) The proportion of effector CD3$^+$ cell undergoing proliferation in presence of the indicated splenic environment following an allogenic stimulus (CD-1 stimulator). T cells proliferation was determined by flow cytometry (CFSE dilution). (**c**) The proportion of

effector CD3$^+$ cell undergoing proliferation following stimulation with anti-CD3/anti-CD28 coated beads in the lower well of a Transwell plate and the indicated splenic environment in the top well. (**d**) Quantification of the number of CD3$^+$ cells generation from panel c as determined by flow cytometry by gating for each CFSE dilution peaks. (**e**) The proportion of CD3$^+$ cell undergoing proliferation following an allogenic stimulus (CD-1 stimulator) in the lower well of a Transwell plate and the indicated splenic environment in the top well. (**f**) Quantification of the number of CD3$^+$ cells generation from panel e. (**g**) Same as in panel e except for the indicated splenic environment. (**h**) Quantification of the number of CD3$^+$ cells per cell generation from panel g. Shown is the average +/- SEM. Data were analyzed by one-way analysis of variance (ANOVA). ***$p<0.001$; *$p<0.05$

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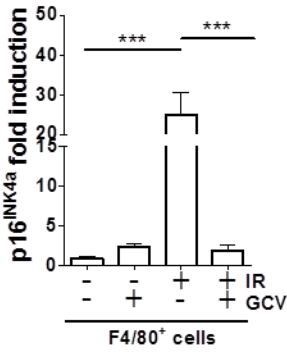
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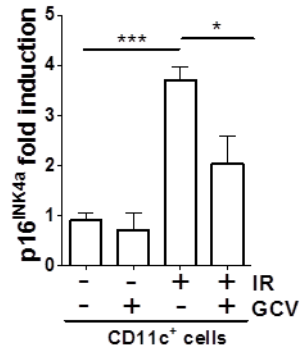
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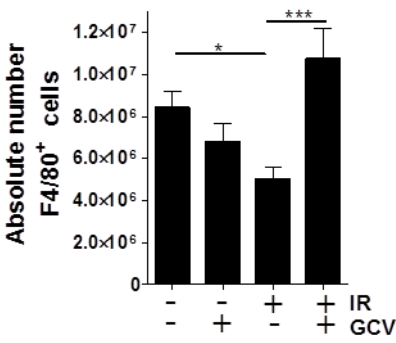
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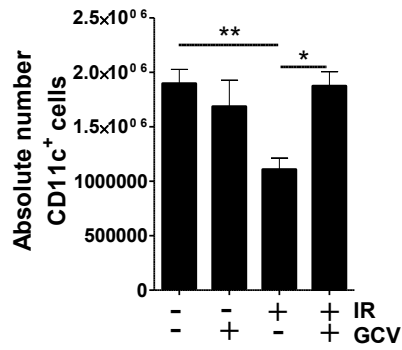
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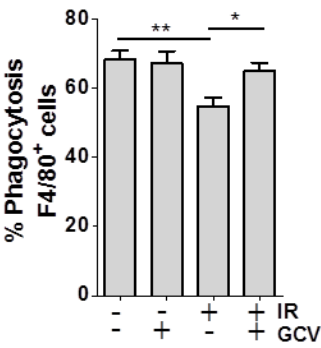
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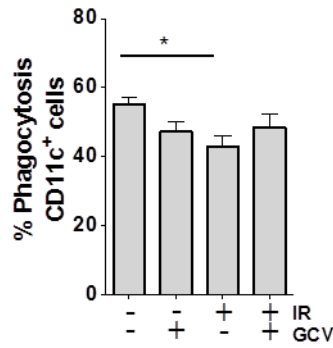
Figure 2.5 Impaired macrophage and DCs counts and function in the splenic environment.

(**a** and **b**) Quantification of $p16^{INK4a}$ mRNA levels as determined by qPCR in isolated F4/80+ and CD11c+ populations, respectively. 18S ribosomal RNA was used as an internal control. (**c** and **d**) Quantification by flow cytometry of the absolute cell counts per spleens for F4/80+ and CD11c+ populations respectively collected from mice treated as indicated. Cell counts were determined one day following the last injection of GCV. (**e** and **f**) Quantification of the proportion of purified F4/80$^+$ macrophages and CD11c$^+$ DCs populations capable of phagocytosis respectively. Shown is the average and SEM. The p value was determined by a One-way ANOVA. ***$p<0.001$; **$p<0.01$; *$p<0.05$. n=6 mice per group.

Supplementary figures

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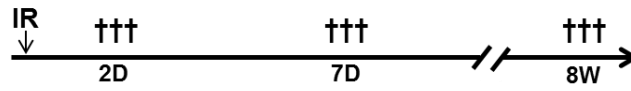
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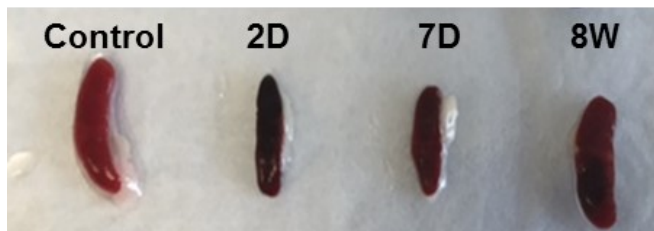
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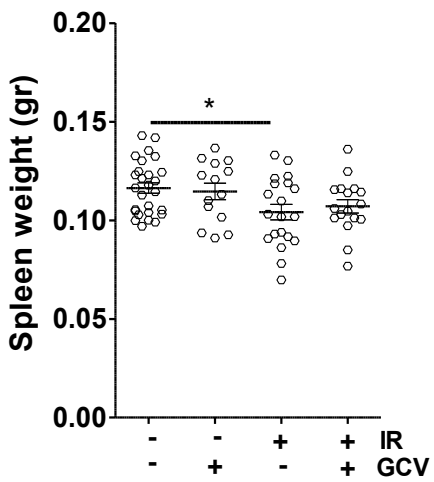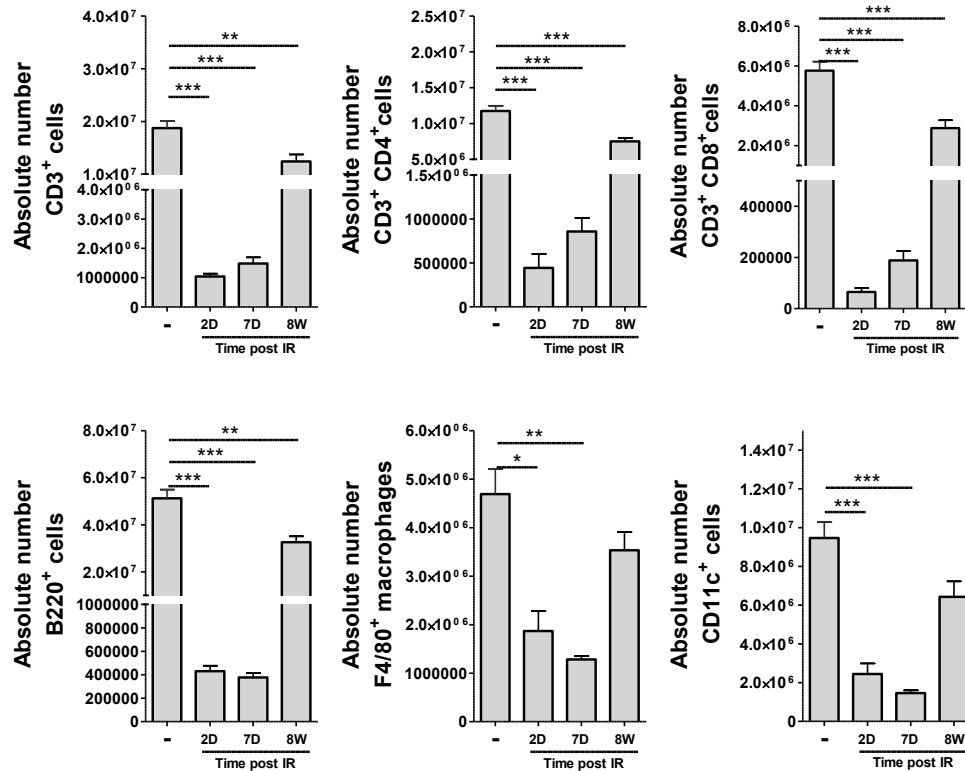
Supplementary figure 2.1 Splenic cell counts following exposure to IR.

(**a**) Experimental design, 12 week-old p16-3MR mice were exposed to 6.5 Gy total body irradiation, two to seven days and 8 weeks later mice were sacrificed. In panel **b**, representative photographs of freshly excised spleens of control mouse and irradiated mice from two and seven days and 8 weeks post exposure. (**c**) Spleen weight expressed in grams (gr) from p16-3MR mice exposed (+) or not (-) to IR treated (+) with GCV. In panel **c**, Quantification by flow cytometry of the absolute cell counts for CD3$^+$, CD3$^+$CD4$^+$, CD3$^+$CD8$^+$, B220$^+$, F4/80$^+$ CD11c$^+$ populations per spleen of controls and irradiated mice. Shown is the average and SEM. The p value was determined by a One-way ANOVA. *$P<0.05$. n=6 mice per group.

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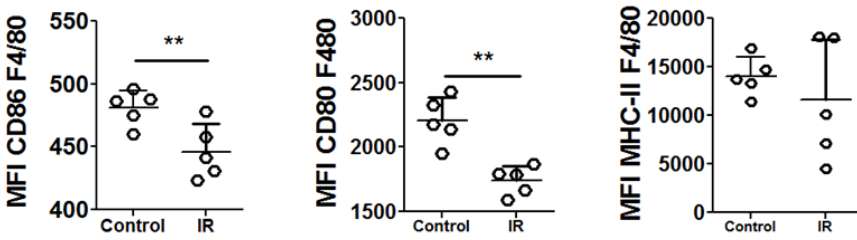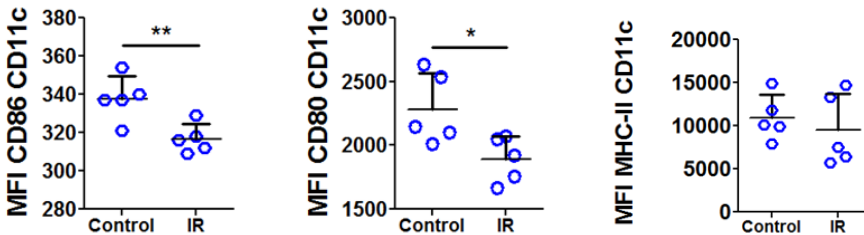
Supplementary Figure 2.2 Irradiation compromised the expression of costimulatory molecules. Quantification of the expression levels of costimulatory molecules CD86 (B7-2), CD80 (B7-1), PD-L1 and MHC-II estimated by the mean fluorescence intensity (MFI) on F4/80$^+$ (**a**) and CD11c$^+$(**b**) cell population. Data are represented as the mean fluorescence intensity (MFI) ± S.D. The data are presented as mean ± SEM; n = at least 4 mice per group ****P* < *.001*.

a b

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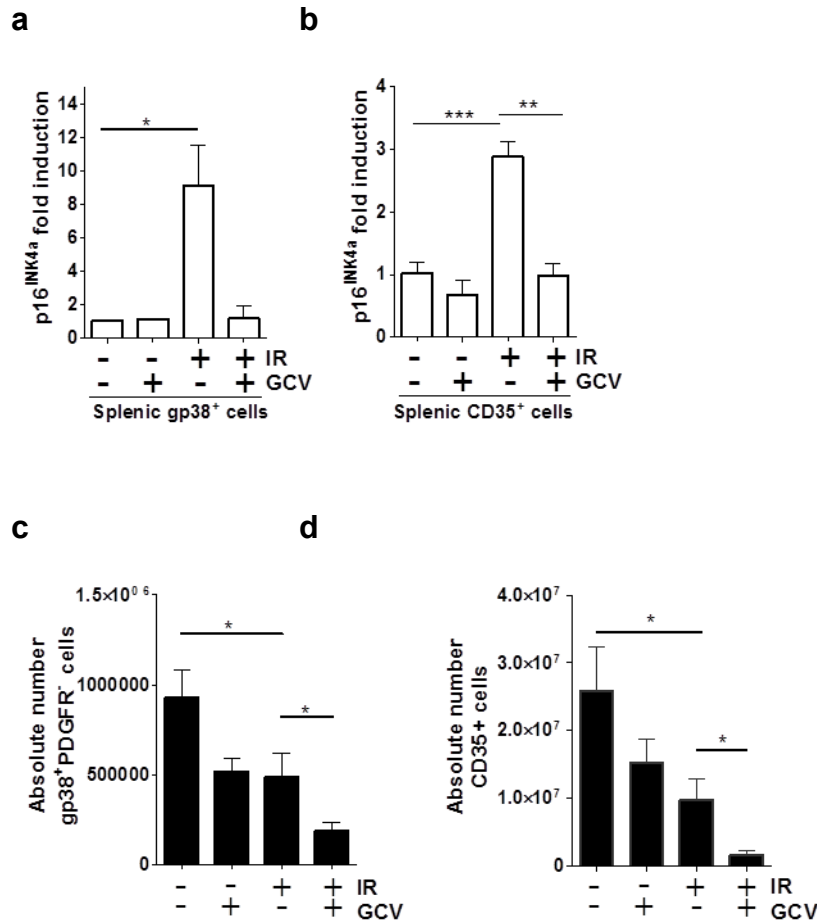
2.3. Exposure to IR impacts splenic stromal cell subpopulations. Quantification of *p16^INK4a* mRNA levels by qPCR in these isolated gp38⁺ (a), and CD35⁺(b) stromal cell populations, respectively. 18S ribosomal RNA was used as an internal control. Quantification by flow cytometry of the absolute cell counts for gp38⁺ PDGFR⁻ (c) and CD35⁺ (d) populations per spleen collected from mice treated as indicated. Shown is the average and SEM. The *p* value was determined by a One-way ANOVA. *$P<0.05$. n=6 mice per group.

2.8 References

1   He, S. & Sharpless, N. E. Senescence in Health and Disease. *Cell* **169**, 1000-1011 (2017).

2   He, S. *et al.* Transient CDK4/6 inhibition protects hematopoietic stem cells from chemotherapy-induced exhaustion. *Science translational medicine* **9** (2017).

3   Chen, Z. *et al.* Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* **436**, 725-730 (2005).

4   Cosme-Blanco, W. *et al.* Telomere dysfunction suppresses spontaneous tumorigenesis in vivo by initiating p53-dependent cellular senescence. *EMBO reports* **8**, 497-503 (2007).

5   Feldser, D. M. & Greider, C. W. Short telomeres limit tumor progression in vivo by inducing senescence. *Cancer Cell* **11**, 461-469 (2007).

6   Bartkova, J. *et al.* Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* **444**, 633-637 (2006).

7   Krishnamurthy, J. *et al.* Ink4a/Arf expression is a biomarker of aging. *J Clin Invest* **114**, 1299-1307 (2004).

8   Le, O. N. *et al.* Ionizing radiation-induced long-term expression of senescence markers in mice is independent of p53 and immune status. *Aging Cell* **9**, 398-409 (2010).

9   Kang, T. W. *et al.* Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. *Nature* **479**, 547-551 (2011).

10   Xue, W. *et al.* Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* **445**, 656-660 (2007).

11   Kuilman, T. *et al.* Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell* **133**, 1019-1031 (2008).

12   Coppe, J. P. *et al.* Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol* **6**, 2853-2868 (2008).

13 Oubaha, M. *et al.* Senescence-associated secretory phenotype contributes to pathological angiogenesis in retinopathy. *Science translational medicine* **8**, 362ra144 (2016).

14 Coppe, J. P., Kauser, K., Campisi, J. & Beausejour, C. M. Secretion of vascular endothelial growth factor by primary human fibroblasts at senescence. *J Biol Chem* **281**, 29568-29574 (2006).

15 Krtolica, A., Parrinello, S., Lockett, S., Desprez, P. Y. & Campisi, J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc Natl Acad Sci U S A* **98**, 12072-12077 (2001).

16 Baker, D. J. *et al.* Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* **479**, 232-236 (2011).

17 Baker, D. J. *et al.* Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan. *Nature* **530**, 184-189 (2016).

18 Demaria, M. *et al.* Cellular Senescence Promotes Adverse Effects of Chemotherapy and Cancer Relapse. *Cancer Discov* **7**, 165-176 (2017).

19 Chang, J. *et al.* Clearance of senescent cells by ABT263 rejuvenates aged hematopoietic stem cells in mice. *Nat Med* **22**, 78-83 (2016).

20 Liu, Y. *et al.* Expression of p16(INK4a) prevents cancer and promotes aging in lymphocytes. *Blood* **117**, 3257-3267 (2011).

21 Haynes, L. & Swain, S. L. Aged-related shifts in T cell homeostasis lead to intrinsic T cell defects. *Semin Immunol* **24**, 350-355 (2012).

22 Kogut, I., Scholz, J. L., Cancro, M. P. & Cambier, J. C. B cell maintenance and function in aging. *Semin Immunol* **24**, 342-349 (2012).

23 Nikolich-Zugich, J. Aging of the T cell compartment in mice and humans: from no naive expectations to foggy memories. *J Immunol* **193**, 2622-2629 (2014).

24 You, J., Dong, H., Mann, E. R., Knight, S. C. & Yaqoob, P. Probiotic modulation of dendritic cell function is influenced by ageing. *Immunobiology* **219**, 138-148 (2014).

25 Jiang, S. & Dong, C. A complex issue on CD4(+) T-cell subsets. *Immunological reviews* **252**, 5-11 (2013).

26   Xin, L., Jiang, T. T., Kinder, J. M., Ertelt, J. M. & Way, S. S. Infection susceptibility and immune senescence with advancing age replicated in accelerated aging Lmna(Dhe) mice. *Aging Cell* **14**, 1122-1126 (2015).

27   Pugh, J. L. *et al.* Acute systemic DNA damage in youth does not impair immune defense with aging. *Aging Cell* **15**, 686-693 (2016).

28   Carbonneau, C. L. *et al.* Ionizing radiation-induced expression of INK4a/ARF in murine bone marrow-derived stromal cell populations interferes with bone marrow homeostasis. *Blood* **119**, 717-726 (2012).

29   Demaria, M. *et al.* An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. *Dev Cell* **31**, 722-733 (2014).

30   Palacio, L., Krishnan, V., Le, N. L., Sharpless, N. E. & Beausejour, C. M. Sustained p16INK4a expression is required to prevent IR-induced tumorigenesis in mice. *Oncogene* **36**, 1309-1314 (2017).

31   Lee, A. C. *et al.* Ras proteins induce senescence by altering the intracellular levels of reactive oxygen species. *J Biol Chem* **274**, 7936-7940 (1999).

32   Ariffin, H. *et al.* Young adult survivors of childhood acute lymphoblastic leukemia show evidence of chronic inflammation and cellular aging. *Cancer* **123**, 4207-4214 (2017).

33   Shaw, A. C., Joshi, S., Greenwood, H., Panda, A. & Lord, J. M. Aging of the innate immune system. *Curr Opin Immunol* **22**, 507-513 (2010).

34   Hall, B. M. *et al.* Aging of mice is associated with p16(Ink4a)- and beta-galactosidase-positive macrophage accumulation that can be induced in young mice by senescent cells. *Aging (Albany NY)* **8**, 1294-1315 (2016).

35   Simone, R., Zicca, A. & Saverino, D. The frequency of regulatory CD3+CD8+CD28- CD25+ T lymphocytes in human peripheral blood increases with age. *J Leukoc Biol* **84**, 1454-1461 (2008).

36   Sharma, S., Dominguez, A. L. & Lustgarten, J. High accumulation of T regulatory cells prevents the activation of immune responses in aged animals. *J Immunol* **177**, 8348-8355 (2006).

37   Baar, M. P. *et al.* Targeted Apoptosis of Senescent Cells Restores Tissue Homeostasis in Response to Chemotoxicity and Aging. *Cell* **169**, 132-147 e116 (2017).

38   De Luca, S. *et al.* Accuracy of cut-off value by measurement of third molar index: Study of a Colombian sample. *Forensic science international* **261**, 160 e161-165 (2016).

39   Zindy, F. *et al.* Arf tumor suppressor promoter monitors latent oncogenic signals in vivo. *Proc Natl Acad Sci U S A* **100**, 15930-15935 (2003).

Chapter 3. Sustained p16[INK4a] expression is required to prevent IR-induced tumorigenesis in mice

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3.1 Abstract

Exposure of murine and human tissues to ionizing radiation (IR) induces the expression of p16[INK4a], a tumor suppressor gene and senescence/aging biomarker. Increased *p16[INK4a]* expression is often delayed several weeks post exposure to IR. In this context, it remains unclear if it occurs to suppress aberrant cellular growth of potentially transformed cells or is simply a result of IR-induced loss of tissue homeostasis. To address this question, we used a conditional *p16[INK4a]* null mouse model and determined the impact of *p16[INK4a]* inactivation long-term post exposure to IR. We found that, *in vitro*, bone marrow stromal cells exposed to IR enter DNA replication following *p16[INK4a]* inactivation. However, these cells did not resume growth; instead, they mostly underwent cell cycle arrest in G2. Similarly, delayed inactivation of *p16[INK4a]* in mice several weeks post exposure to IR resulted in increased BrdU incorporation and cancer incidence. In fact, we found that the onset of tumorigenesis was similar whether p16[INK4a] was inactivated before

or after exposure to IR. Overall, our results suggest that IR-induced p16[INK4a] dependent ++growth arrest is reversible in mice and that sustained *p16[INK4a]* expression is necessary to protect against tumorigenesis.

3.2 Keywords

Senescence; p16[INK4a]; ionizing irradiation; tumor suppressor; cell cycle

3.3 Introduction

Exposure to ionizing radiation (IR) leads to an increase in *p16[INK4a]* expression in various murine tissues[99,292]. Similarly, p16[INK4a] expression is also elevated in skin biopsies of leukemia survivors previously exposed to radiation therapy and in T cells collected from breast cancer survivors treated with anthracycline-based chemotherapy [293,294]. Several other inducers of *p16[INK4a]* have also been described such as oncogenic signalling and telomere dysfunction [295]. Most of these inducers seem to have in common the activation of a DNA damage/stress response that in some instances may prelude downstream neoplastic conversion [3,296,297].

Loss of *p16[INK4a]* is observed in many human cancers and predisposes mice to tumorigenesis [298,299]. In fact, p16[INK4a] is a cyclin dependent kinase inhibitor that acts by preventing the phosphorylation of the retinoblastoma (pRb) family proteins and ultimately cell cycle progression [300]. Following IR-induced DNA damage, it is believed that most cells will alt cell cycle progression by a mechanism that entails primarily an ATM/p53/p21

86

cascade [300]. On the other hand, expression of *p16^INK4a* is more complex as it seems to occur in a delayed manner to DNA damage or oncogenic signalling [297,301-303]. For example, normal fibroblasts exposed to IR *in vitro* induce transient but rapid (within hours) upregulation of p53 and p21 protein levels, while *p16^INK4a* expression is not detected until several days later [302,303]. The reason for this delayed increase in p16^INK4a expression following DNA damage is unknown. One hypothesis is that exposure to IR may induce neoplastic stress that later induce p16^INK4a in an indirect manner [297,304]. Alternatively*, p16^INK4a* expression may rise in response to the accumulation of reactive oxygen species or as a bystander effect of IR-induced loss of tissue homeostasis [297,305,306].

 Whether induced following exposure to IR or during normal aging, expression of p16^INK4a seems to occur preferentially into possibly exhausted progenitor and stem cell populations, preventing adequate tissue renewal [292,307-311]. For example, we recently showed increased neurogenesis in the irradiated mouse brain in absence of p16^INK4a expression (Le et al. submitted). Thus, while *p16^INK4a* expression prevents damaged cells from proliferating, it likely also diminishes the regenerative potential of aged/irradiated tissues. In the absence of reliable markers, it remains unknown whether irradiated cells expressing *p16^INK4a* are truly senescent *in vivo* or maintained in check long term. However, we believe that exposure to IR is likely to lead to senescence in most cells either directly through a persistent DNA damage response or by forcing premature exhaustion of cycling progenitor cells.

In this context, the development of strategies that would prevent or limit *p16^INK4a* expression in progenitor/stem cells becomes attractive, as it may allow better tissue

regeneration in cancer survivors. In support of this approach, it was shown that p53/Arf activity is not necessary to protect mice from IR-induced lymphoma [312]. In fact, only transient (as short as six days) p53 and *p19^Arf* expression was sufficient to protect against development of cancer. Whether transient or sustained p16^INK4a expression is necessary to exert a similar tumor suppressive effect remains unknown. Actually, it is unknown if the delayed IR-induced *p16^INK4a* expression occurs to prevent neoplastic progression. We answered this question using a conditional p16^INK4a null mouse model and showed that while the inactivation of p16^INK4a stimulates cell cycle progression in irradiated cells and tissues, its long-term expression is necessary to protect against IR-induced cancer.

3.4 RESULTS AND DISCUSSION

3.4.1 Irradiated bone marrow stromal cells do not resume growth following p16^INK4a deletion

We first examined *in vitro* the role of p16^INK4a in preventing cell cycle progression and proliferation following exposure to IR. We chose to use bone marrow derived stromal cells for our primary cell cultures as we found that these cells do not transform easily *in vitro* when compared to mouse embryonic fibroblasts which grow robustly in presence of a high level *p16^INK4a* expression [137]. Bone marrow stromal cells (defined as Cre p16^L/L) were derived from p16^INK4a specific conditional allele transgenic mice expressing Cre-ERT2 recombinase under the human ubiquitin C (**UBC**) promoter [313]. We found that exposure to 10 Gy resulted in over 90% of the cells to express the senescence-associated β-galactosidase (SAβ-gal) (**Figure 3.1**a and **Supplementary Figure 3.1**a). In contrast,

about 20% of non-irradiated control cells had SAβ-gal staining. As expected, treatment of these cells with 4-hydroxy tamoxifen (4-OHT) on day 5 following exposure to IR efficiently reduced expression of p16INK4a both at the RNA and protein levels (**Figure 3.1**c and **Supplementary Figure 3.1**b). However, while deletion of *p16INK4a* expression did not reduce the proportion of cells staining positive for SAβ-gal, it allowed a fraction of these cells to resume cell cycle and to incorporate BrdU (**Figure 3.1**c). Importantly, no increase in BrdU incorporation was observed in bone marrow stromal cells lacking the Cre recombinase treated with 4-OHT (defined as p16L/L, **Figure 3.1**c). Cell cycle analysis performed five days post exposure to IR, a time at which the senescence phenotype is already initiated, showed that stromal cells are arrested in both G1 and G2 (**Figure 3.1**d). Treatment of these irradiated cell populations with 4-OHT, but not the control vehicle, induced a proportion of cells to progress in S and G2 phases with a greater proportion of cells in G2 being detected at 48 hours post treatment. Finally, we observed no increase in the total cell number up to 96 hours post 4-OHT treatment (**Figure 3.1**e). These results suggest that deletion of p16INK4a in irradiated stromal cell allows for cell cycle re-entry in a significant fraction of cells but that these cells fail to resume growth *in vitro*.

3.4.2 *Increase BrdU incorporation in mice tissues following deletion of p16INK4a*

Murine stromal cells are known to be sensitive to *in vitro* growth conditions and can undergo telomere and p16INK4a independent premature senescence [137] . Therefore, it is not surprising to see about 20% of the early passaged cells (<3) to stain positive for SAβ-gal in absence of IR despite being cultured under low (3%) oxygen concentration (**Figure**

3.1a). In this context, we believe that the absence of cell proliferation following *p16^{INK4a}* inactivation *in vitro* could be the result of a premature stress-induced senescence and thus may not adequately represent the *in vivo* situation. To address this issue, we irradiated Cre p16L/L mice at the sub lethal dose of 2.5 Gy and then waited 8 weeks for *p16^{INK4a}* expression to increase. We had previously performed time course studies and found that a minimum of 6–8 weeks is necessary to observe robust IR-induced *p16^{INK4a}* expression in mouse tissues 16. Treatment of Cre p16^{L/L} mice with tamoxifen for 5 days resulted in efficient (50–80%) recombination and consequent reduction of IR-induced *p16^{INK4a}* expression in both liver and spleen (**Figure 3.2**a and **Supplementary Figure 3.2**). As expected, no decrease in p16^{INK4a} expression was observed in Cre deficient mice injected with tamoxifen **(Figure 3.2**b).

In line with our *in vitro* data, we found that liver cryosections collected from tamoxifen treated *Cre p16^{L/L}* mice showed marked increase in BrdU incorporation (4–8 fold) independently of whether mice were previously exposed or not to IR (**Figure 3.3**a–c). Not surprisingly, we found that liver from irradiated mice had incorporated lower levels of BrdU and that treatment of Cre deficient mice with tamoxifen did not increase BrdU levels. However, these results also showed that p16^{INK4a} expression in relatively young (18 weeks old) non-irradiated mice is sufficient to restrict cell cycle progression in a high proportion of cells in the liver. To confirm these results, we made single cell suspension from control and irradiated livers, and determined BrdU incorporation using flow cytometry. Again, we found that *p16^{INK4a}* inactivation leads to increase BrdU incorporation

(**Figure 3.3**d). Likewise, inactivation of p16^{INK4a} led to a significant increase in BrdU incorporation in the spleen but failed to do so in previously irradiated tissues (**Figure 3.3**d). Further analysis revealed that increase in BrdU incorporation in the spleen was restricted to cells of non-hematopoietic origin (defined as negative for the CD45 marker - **Supplementary Figure 3.3**b). It is unclear at the moment why such a high proportion (25–35%) of non-hematopoietic splenic cell, but not liver cells, incorporated BrdU upon p16^{INK4a} inactivation (**Supplementary Figure 3.3**). Such a high proportion of splenic stromal cells expressing p16^{INK4a} may help explain previous results from our laboratory showing lymphopoiesis is INK4a/ARF dependent [7]. In fact, we have shown that the absence of INK4a/ARF expression leads to a non-cell-autonomous increase in B cells and common lymphoid progenitor cell populations in the spleen [7]. However, whether there is a direct relationship between *p16^{INK4a}* expression in the spleen stroma and altered lymphopoiesis remains to be determined.

3.4.3 *Sustained p16INK4a expression is necessary to limit cancer incidence*

We have shown that *p16^{INK4a}* expression is increased in tissues long-term following exposure to IR and that this limits cell cycle progression. Yet, we don't know if this expression occurs as a tumor suppressive mechanism or simply as a bystander effect to genotoxic stress. Neither do we know if persistent expression of *p16^{INK4a}* is necessary to protect against cancer development or if transient expression would be sufficient to induce an irreversible growth arrest in damaged cells. In light of these possibilities, inhibition of p16^{INK4a} functions after damage could favour tissue regeneration without

increasing the risk of developing cancer. Hence, to test this hypothesis, we injected conditional $p16^{INK4a}$ null mice with tamoxifen for 5 days either before or after exposure to 2.5 Gy irradiation and monitored tumor incidence over one year (a schematic of the different groups used is shown in (**Figure 3.4**a). Inactivation of p16^{INK4a} alone (group B) or exposure to IR alone (group C) was shown to induce cancer in about 60% of mice (**Figure 3.4**b). In contrast, none of the untreated mice (group A) had develop cancer during that time. Inactivation of p16^{INK4a} before exposure to IR (group D) increased cancer incidence with only about half the mice alive 30 weeks post treatment. More importantly, mice that received tamoxifen 8 weeks post exposure to IR (group E), removing sustained $p16^{INK4a}$ expression long after damage induction, displayed a significant increase in cancer incidence with only about half the mice alive 30 weeks post treatment. In fact, inactivation of $p16^{INK4a}$ after exposure to IR, compared to inactivation before IR, seemed to worsen the incidence of cancer one-year post treatment. Analysis of tissues revealed that inactivation of p16^{INK4a} had only a modest impact on the type of cancer occurring with a high proportion of mice in all groups developing mostly (50–84%) lymphomas (**Supplementary Figure 3.4**a). Furthermore, PCR analysis showed that randomly selected tumours derived from all groups had deleted p16^{INK4a}, even in mice not treated with tamoxifen (**Supplementary Figure 3.4**b). More importantly, none of the analyzed tumors seemed to have concomitantly deleted p19^{ARF} gene (**Supplementary Figure 3.4**b). Overall, these results suggest that sustained IR-induced p16^{INK4a} expression is necessary to protect against cancer progression. These results are similar to what was observed following the deletion of p53, suggesting an equivalent role for p16^{INK4a} in maintaining tumorigenic cells in check. Yet, these results are in opposition to a model

where transient expression of p53 (6 days only) was shown to be sufficient to protect mice from IR-induced lymphoma [136] . Reason for such discrepancy is unclear but likely involves variation in the models used (germline vs somatic inactivation). Many reasons may explain why a transient 8 weeks p16[INK4a] response, at the time of damage, failed to protect, if not worsen, cancer progression. First, the simplest explanation would be that p16INK4a-induced senescence/growth arrest is reversible in mice, a phenotype also previously observed in mouse embryo fibroblasts following the inactivation of p53b [247]. Second, it may be possible that the accumulation of p16[INK4a] positive cells, which occurs in group E but not in group D, is detrimental to cancer free survival, especially several weeks following IR. This may be possible if the accumulation of damaged cells in irradiated tissues favours cancer development through, for example, the secretion of inflammatory cytokines [67,314]. However, cytokine arrays performed on serum and spleen lysates collected from mice 8 weeks after IR did not show any meaningful changes compared to age-matched non irradiated animals.

Nonetheless, we speculate that it is possible that variation in certain cytokines, either not measured in these arrays or undetectable at the systemic level, may still have an impact, in the splenic or bone marrow microenvironment (for example). Still, the fact that inactivation of p16[INK4a] 8 weeks after exposure to IR did not somehow delay cancer incidence was very surprising. Third, we cannot rule out the possibility that tumors may

have arisen from irradiated cells that had not yet increase p16^{INK4a} expression prior to tamoxifen treatment, avoiding the need to bypass senescence. However, once again, one would have expected a reduction in cancer incidence in the event that cancer progression is stochastic and not limited to a subtype of cells which have delayed (more than 8 weeks) or do not at all increase p16^{INK4a} expression upon IR. Overall, IR-induced p16^{INK4a} expression is necessary to maintain growth arrest long-term, in at least a subset of oncogenically activated cells. In fact, inactivation of p16^{INK4a} in these cells may have directly lead to cancer progression and the G2 cell cycle arrest we observed in vitro is likely a culture artefact that does not occur *in vivo* (**Figure 3.4**c). We speculate that if a G2 block would have occurred in mice, it would have been expected to at least delay cancer incidence, which it did not. Still, the scenario of a G2 block occurring *in vivo* may be reconcilable with our data if the protective effect of cell cycle block is masked by the protumorigenic inflammatory phenotype. Direct elimination of damaged cells and their secretory phenotype using newly developed mice strains containing a suicide gene under the control of the p16^{INK4a} promoter may help resolve this question [169,296,315]. In conclusion, it will be interesting to determine if there is a link between the development of lymphoma and the proportion of senescent cells observed in the spleen. We believe it is conceivable that senescent splenic stromal cells act in a non-autonomous manner to foster the development of lymphoma, the same way we previously showed they act on lymphopoiesis [7]. Also, given the apparent necessity for sustained p16^{INK4a} expression to protect against cancer progression, we believe it is of utmost importance to identify the inducers of p16^{INK4a} at the molecular level. The identification and subsequent modulation

of these inducers may make it possible to increase the regeneration of irradiated/aged tissues without increasing the risk of developing cancer

3.5 Acknowledgments

3.6 Figures
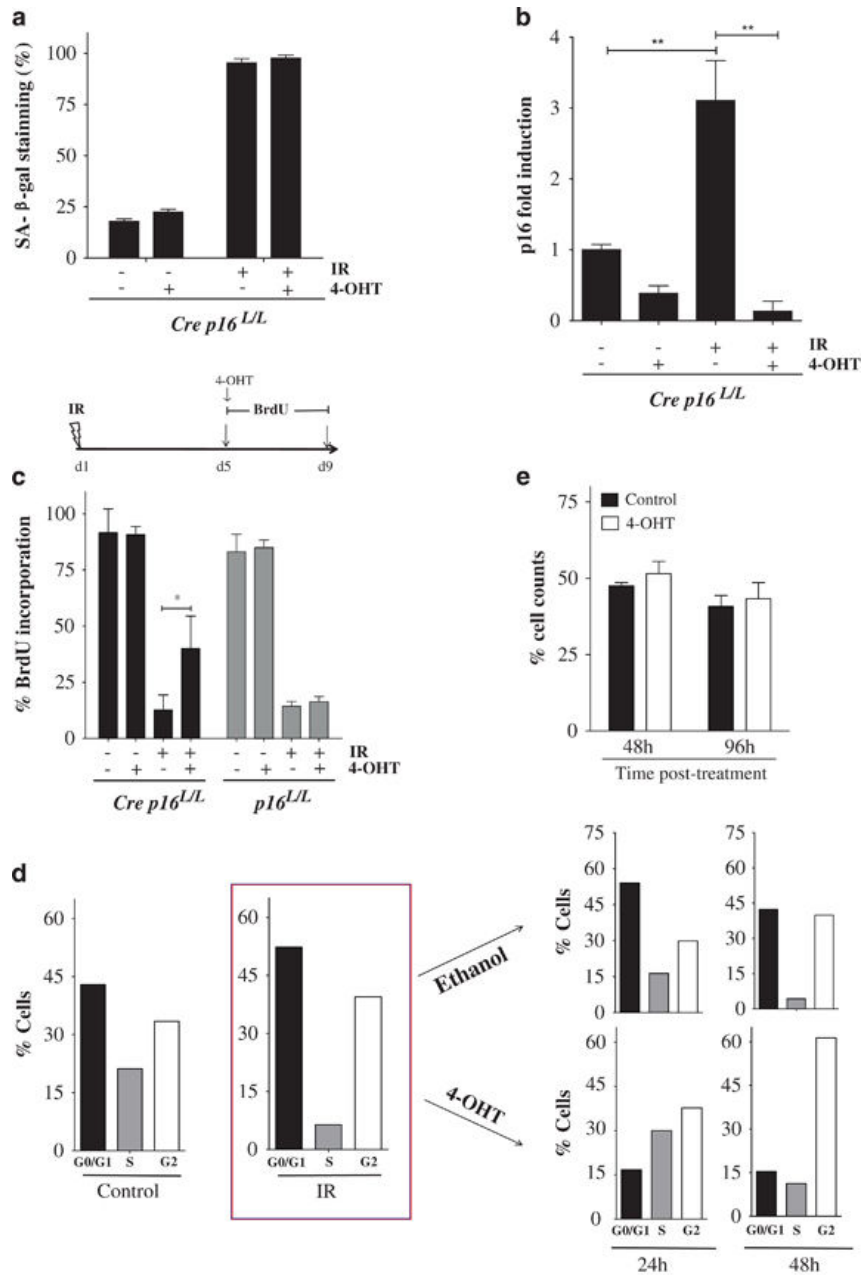
Figure 3.1 Deletion of p16^{INK4a} in irradiated bone marrow stromal cells allows for cell cycle progression but not cell growth.

(a) Proportion of cells staining positive for SA-β-galactosidase (SA-β-gal) 9 days post-exposure or not to 10 Gy IR. Where indicated, cells were treated (+) or not (−) with 100 nM

4-Hydroxy Tamoxifen (4-OHT) overnight on day 5 post-IR. Bone marrow stromal cells expressing or not the Cre recombinase (defined as Cre p16L/L or p16L/L, respectively) were isolated has previously described27 from the femur of p16INK4a specific conditional allele transgenic mice. Cells were used at low passage (<3) and cultured in DMEM containing 10% fetal bovine serum under low (3%) oxygen concentration. (b) Differential mRNA expression levels of p16INK4a as determined by qPCR in Cre p16L/L cells treated as described above. Shown is fold increase in p16INK4a expression normalized to 18 S. Student t-test (**P<0.01). q-PCR was performed using SYBR GREEN PCR SensiMixTM low ROX kit (Quantance, San Mateo, CA, USA) using the following primers for p16INK4a and S18 genes F5'AACTCTTTCGGTCGTACCCC3', R5'GCGTGCTTGAGCTGAAGCTA3' and F5'TCAACTTTCGATGGTAGTCGCCGT3', R5'TCCTTGGATGTGGTAGCCGTTTCT3' respectively. (c) Proportion of cells incorporating BrdU (4-day pulse) 5 days after exposure or not to IR as determined by immunostaining (BrdU antibody catalog number 347583, BD Biosciences, San Jose, CA, USA). Inactivation of p16INK4a by 4-OHT was initiated simultaneously with addition of BrdU. Student t-test (*P<0.05). (d) Cell cycle analysis of Cre p16L/L cells before and 5 days post exposure to IR as determined by flow cytometry. Irradiated cells were then treated with 4-OHT or ethanol (vehicle) and cell cycle analyzed again 24 and 48 h later. Shown are results of a representative experiment from n=3 independent cell populations. (e) Cre p16L/L cells were irradiated and 5 day later the cells were treated with 4-OHT or its vehicle. The proportion of viable cells was determined 48 and 96 h later. Data are expressed as mean±s.d. of n=3 independent cell populations.
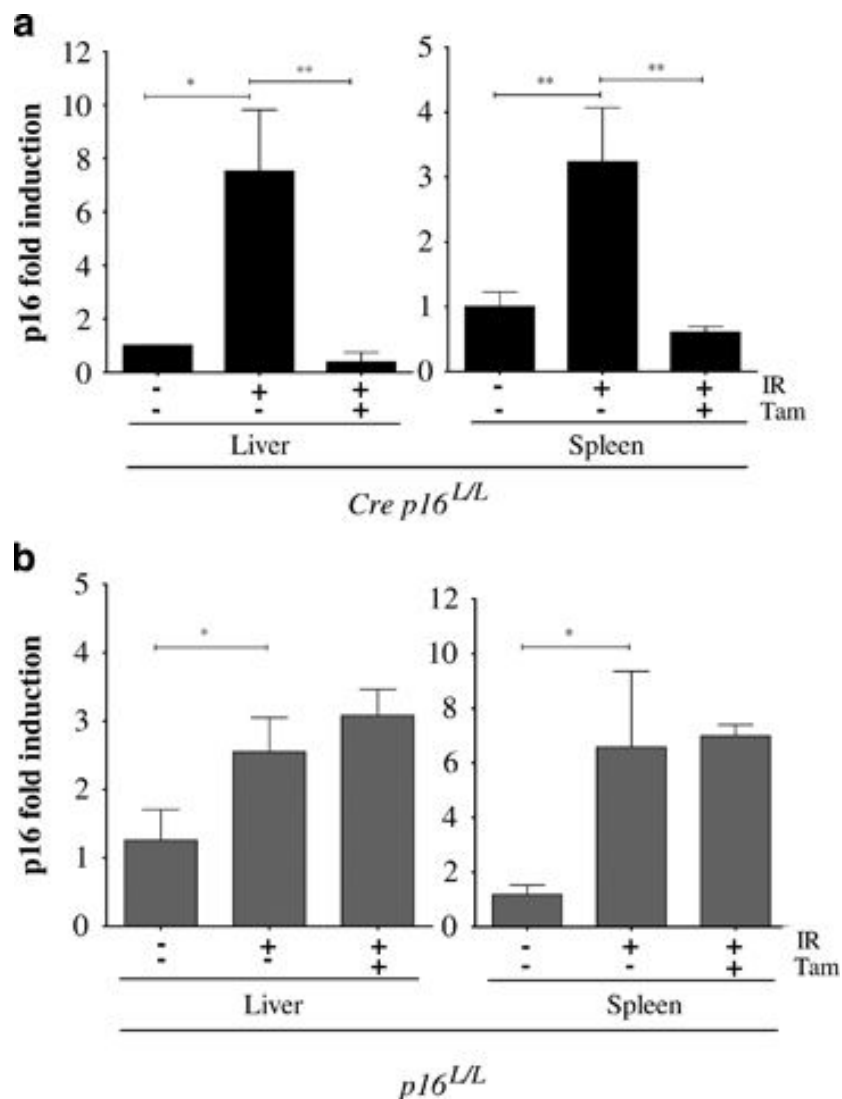
Figure 3.2 Conditional deletion of IR-induced p16^{INK4a} expression in mice.

8–10 weeks old mice were irradiated at the dose of 2.5 Gy (total body irradiation using a Faxitron CP-160 at a rate of 1 Gy/min) and 8 weeks later they were treated (+) or not (−) with Tamoxifen (Tam) at a dose of 200 mg/kg (diluted in a mixture 1:50 of ethanol and corn oil respectively) by gavage for 5 consecutive days to inactivate p16INK4a. Expression of p16INK4a relative to 18S was determined by qPCR on liver and spleen tissues collected from Cre p16L/L (a) or p16L/L (b) mice. n=5 mice per group. Data are expressed as mean±s.d. Student t-test *P<0.05 and **P<0.01.
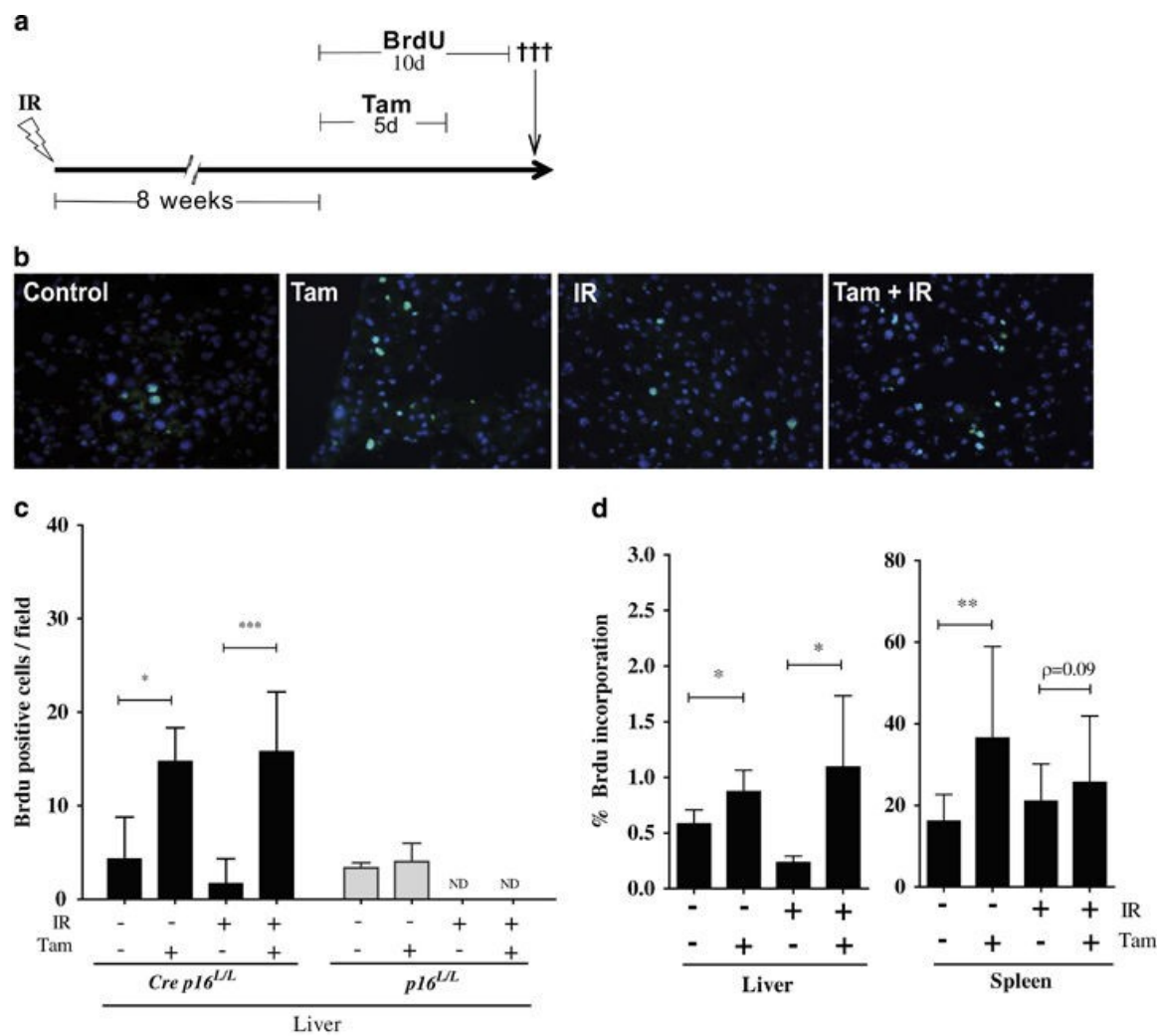
Figure 3.3 Increase BrdU incorporation in irradiated mouse tissues following deletion of p16INK4a

(a) Schematic of the experiment. Cre p16 L/L mice were irradiated or not at a dose of 2.5 Gy (total body irradiation). After 8 weeks, mice were treated or not with Tam by gavage for 5 consecutive days. Beginning with the first Tam injection, mice also received daily intraperitoneally injection of BrdU (50 mg/kg) for a total of 10 consecutive days. (b) Representative images from liver cryosections treated as indicated showing the incorporation of BrdU in green and nuclei in blue (stained with DAPI). The BrdU antibody

used was from BD Biosciences (catalog number 347583). (c) Number of cells incorporating BrdU was determined by counting manually immunostained liver sections collected from both Cre p16 L/L and p16 L/L mice treated as described in a. Data are expressed as mean±s.d. of at least 5 randomly selected fields (× 40) obtained from a minimum of four mice per group. (d) Proportion of cells incorporating BrdU from dissociated liver and spleen tissues collected from Cre p16 L/L mice as determined on single cell suspensions by flow cytometry using the BrdU flow kit (catalog number 559619 from BD Bioscences) and analyzed using a BD-LSRFortesa. Data are expressed as mean±s.d. Dissociated cell samples were collected from a minimum of four mice per group and analyzed individually. Student t-test *P<0.05; **P<0.01 and ***P<0.001.
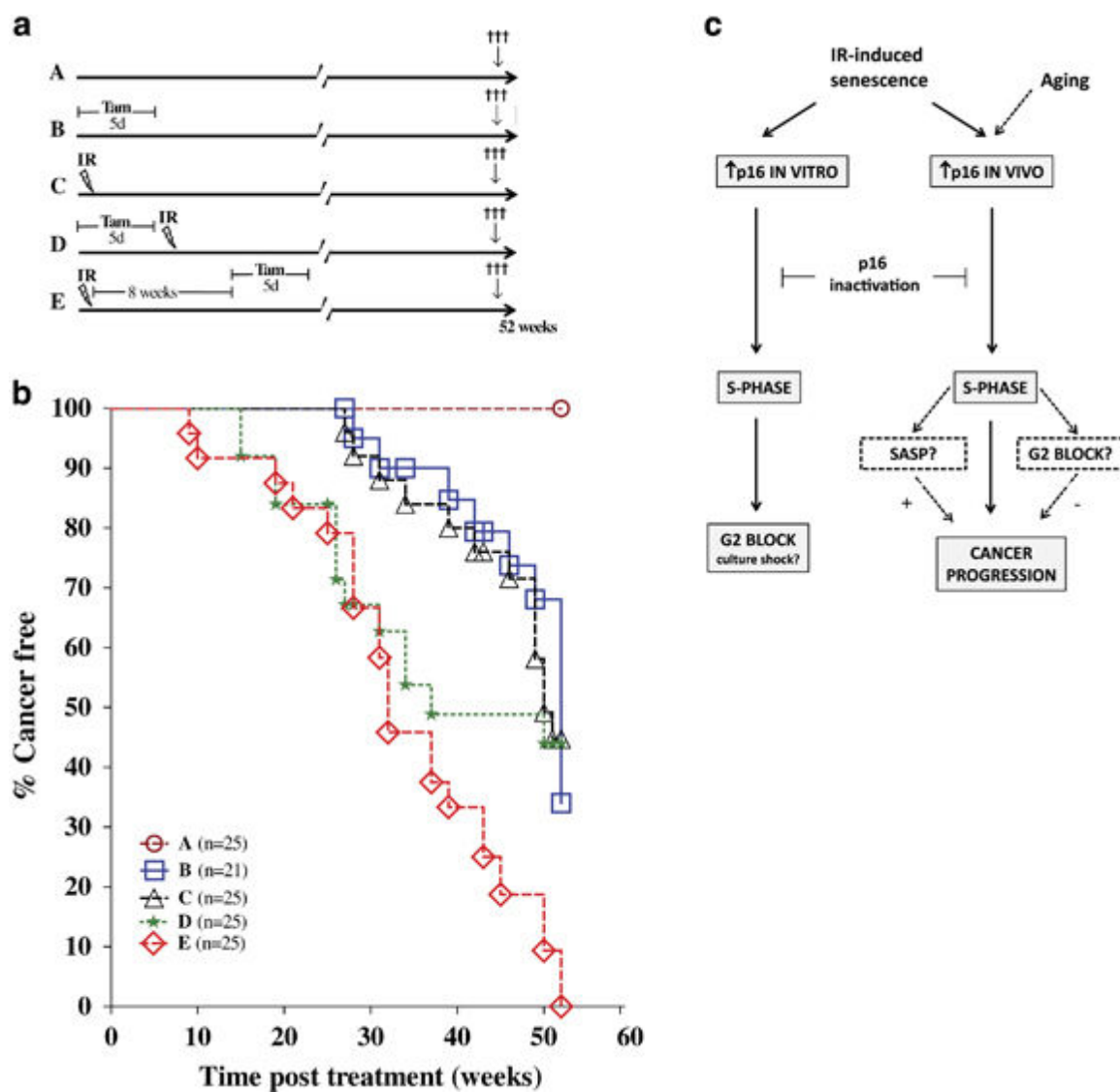
Figure 3.4 Sustained p16^{INK4a} expression is necessary to protect against cancer.

(a) Cre p16 L/L male and female mice of 8–12 weeks of age were randomly distributed in n=21–25 mice per group and killed 52 weeks post treatment. In group A, mice were left untreated. In groups B and C, mice received respectively Tam for 5 days or a single dose of 2.5 Gy total body irradiation. In group D, mice were first treated with Tam for 5 days

and then immediately irradiated as in group C. In group E, mice were first irradiated and 8 weeks later received Tam for 5 days. (b) Kaplan/Meier curves showing cancer free survival of mice treated as described in a. Mice were killed 52 weeks post treatment or once they had reach a distress point in accordance to our institutional animal guideline, whatever happened first. An autopsy was performed at the time of killing and, when possible, tumor type was identified. Groups D and E were not statistically different (Wilcoxon test). Groups B and C were statistically different (P<0.001) from group E but not from group D (P=0.09 and P=0.06 respectively). (c) Schematic describing the expected role played by p16INK4a following exposure to IR. Inactivation of p16INK4a in irradiated cells *in vitro* leads to cell cycle re-entry and subsequent block in G2 that may or may not be dependent on cell culture conditions. Upon inactivation of p16INK4a *in vivo*, following irradiation or normal chronological aging, increase S phase and cancer progression is observed. Whether a G2 block occurs and the extent by which the SASP may contributes to cancer progression is unknown.

A

B
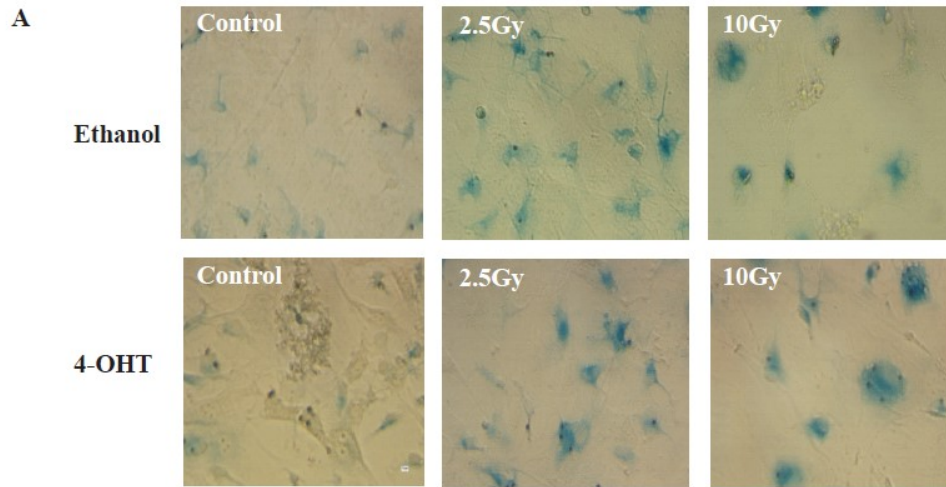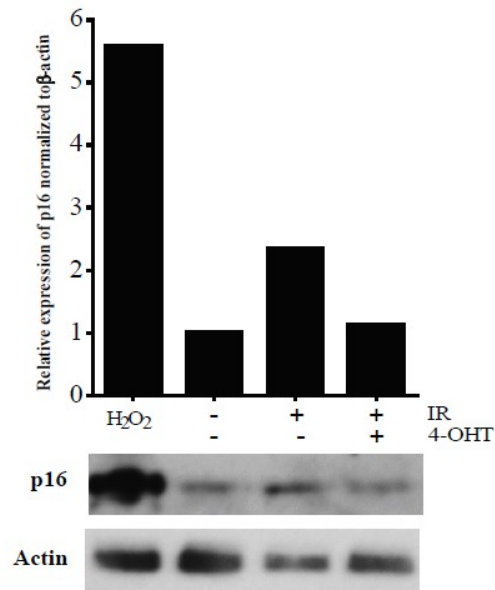
Supplementary Figure 3.1 Deletion of p16^{INK4a} in bone marrow stromal cells in vitro

(a) Representative photographs showing bone marrow derived stromal cells stained for the SA-β-galactosidase (SA-β-gal) marker 9 days post-exposure or not to IR. Where indicated, cells were treated with 4-OHT 5 days following irradiation. (**b**) p16$_{INK4a}$ protein expression was determined by western blotting in stromal cell populations derived from *Cre p16 L/L* mice and treated *in vitro* as indicated. Hydrogen peroxide (H_2O_2) was used as a positive control. Shown is the relative expression of p16$_{INK4a}$ normalized to actin. Anti-p16$_{INK4a}$ mouse (SC-1207, Santa Cruz, CA), anti-actin (MAB1501, Chemicon, USA) and anti-rabbit conjugated to HRP (SC-2054, Santa Cruz) were used. Results from of a representative blot (n=2) is shown.

Palacio *et al.*, 2017
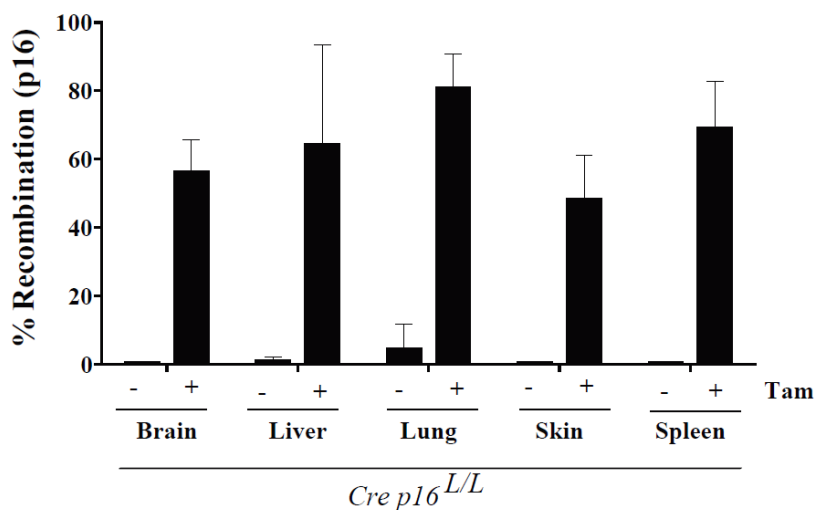
Supplementary Figure 3.2 Efficient p16^{INK4a} exon 1 deletion in various mice tissues.

Shown is the proportion of recombined p16^{INK4a} exon 1 in the DNA of the indicated tissues one day following the injection of Tam (5 days by gavage). Recombination was determined by qPCR using the standard curve method and genomic DNA collected from bone marrow stromal cells successively treated with 4-OHT as a positive control. DNA collected from non-treated cells was used as a dilution to establish the standard curve. Primers used to detect the Floxed allele were F5' TACCACAGTTTGAACAGCGTGA-3' and R5'-AACCAACTTCCTCCTTCCCC-3'.
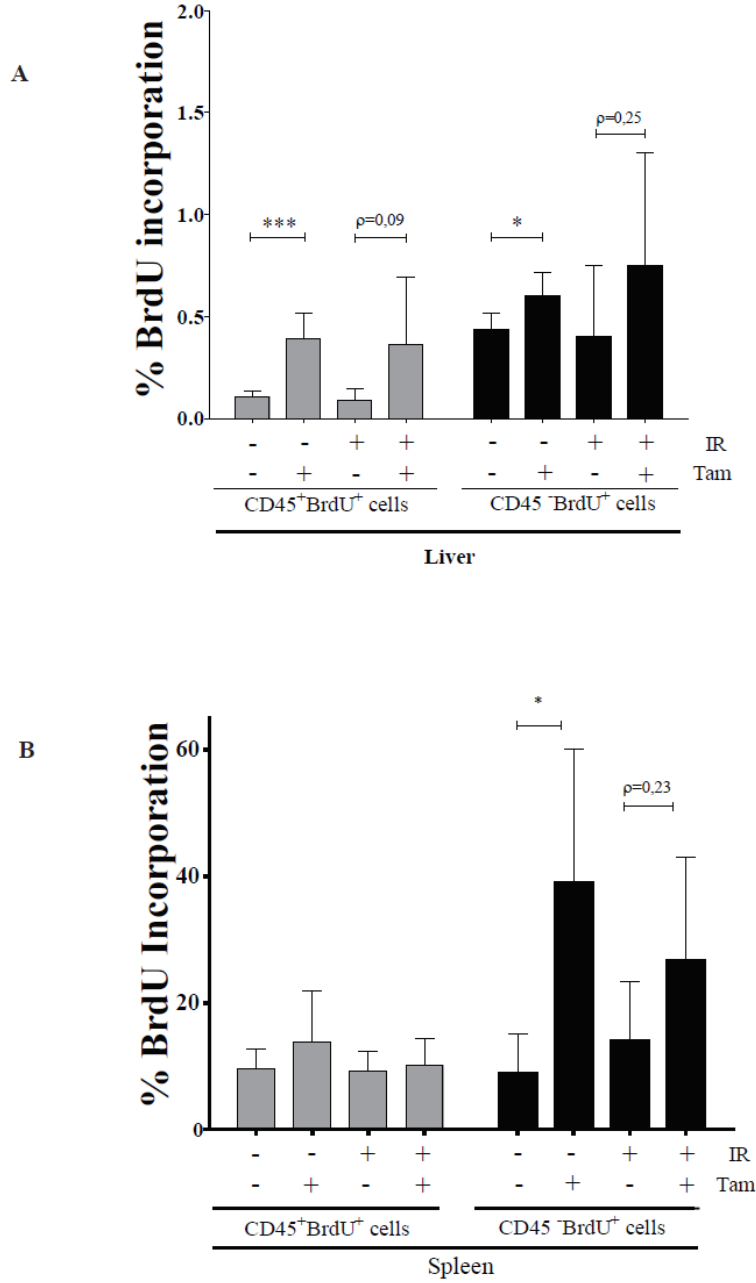
Supplementary Figure 3.3 High level BrdU incorporation in the spleen nonhematopoietic cells following p16[INK4a] inactivation in mice.

Cre p16 L/L mice were irradiated or not at a dose of 2.5 Gy (total body irradiation). 8 weeks later, mice were treated or not with Tam by gavage for 5 consecutive days. Beginning with the first Tam injection, mice also received daily intraperitoneally injection of BrdU (50 mg/kg) for a total of 10 consecutive days. Shown is the proportion of cells incorporating BrdU from dissociated liver (a) and spleen (b) tissues as determined on single cell suspensions by flow cytometry (BD-LSRFortesa). In brief, 1x106 cells were stained against the murine CD45 cell surface marker (APC-conjugated, catalogue number 103112 from Biolegend, CA, USA) and then analysed for BrdU incorporation following the manufacturer's instruction (BrdU flow kit catalogue number 559619 from BD Biosciences, CA, USA). Data are expressed as mean ± SD. Dissociated cell samples were collected from a minimum of 4 mice per group and analysed individually. Student t-test * p $\Box$ 0.05 and **p $\Box$ 0.01.

A

Groups	Percentage (%) of lymphoma
A	0
B	75
C	84
D	69
E	50

B

Supplementary Figure 3.4 Characterization of tumor types observed upon p16^{INK4a} inactivation in mice.

(**a**) Proportion of cancer identified as lymphoma in each group. Ingroup A, mice were left untreated. In groups B and C, mice received respectively Tam for 5 days or a single dose of 2.5 Gy total body irradiation. In group D, mice were first treated with Tam for 5 days and then immediately irradiated as in group C. In group E, mice were first irradiated and 8 weeks later received Tam for 5 days. n=21-25 mice per group. Tam was injected at a dose of 200 mg/kg (diluted in a mixture 1:50 of ethanol and corn oil respectively) by gavage for 5 consecutive days (**b**) DNA isolated from randomly selected tumors from each group was analyzed by PCR for the presence of a *p16^{INK4a}* exon 1 floxed allele (305

bp) and the presence of p19 exon 1 (202bp fragment). Of note, tumors analyzed from group A were collected from aged (over 15 months old) mice which eventually developed tumors presumably because of the leakiness of the CRE-ERT2 enzyme. The quality of the DNA extraction was confirmed by amplifying the Lox sequences (261 bp fragment). DNA isolated from a wild-type mouse was used as a negative control for the presence of Lox sequences (amplification of a shorter 194 bp fragment). Primers used were as follow: *p16^INK4a* floxed allele: F5' TACCACAGTTTGAACAGCGTGA-3' and R5'-AACCAACTTCCTCCTTCCCC-3'. p19 exon 1: F5'-TCTCACCTCGCTTGTCACAG-3' and R5'- ATGTTCACGAAAGCCAGAGC-3'. Lox sequences: F5'-GTATGCTATACGAAGTTATTAGGTACTGC-3' and R5'-CTATGTCAGATTTGGCTAGGGAGT-3'.

Cytokines	Serum		Spleen	
	Control	IR	Control	IR
Eotaxin	643,3	616,0	6,16	4,75
G-CSF	185.3	179,0	3,13	2,71
GM-CSF	23,67	29,30	16,57	21,78
IFNy	BD	BD	BD	BD
IL-1a	905,9	722,9	38,08	36,49
IL-1B	37,38	35,68	15,62	5,78
IL-2	7,73	5,72	14,11	19,92
IL-3	5,03	0,36	0,62	2,01
IL-4	0,71	0,65	1,31	1,68
IL-5	18,74	2,36	0,27	0,33
IL-6	6,43	2,99	2,82	7,22
IL-7	BD	BD	5,44	4,76
IL-9	BD	BD	21,08	20,26
IL-10	4,06	2,76	4,38	7,33
IL-12 (p40)	10,94	33,94	10,18	7,98
IL-12 (p70)	17,18	19,35	9,24	4,67
IL-13	83,42	99,40	BD	BD
IL-15	43,13	52.56	11,82	7,61
IL-17	1,89	2,01	0,75	1,77
IP-10	70,44	84,39	26,82	20,68
KC	67,65	59,96	11,03	15,73
LIF	0,66	1,83	BD	BD
LIX	23216	15509	91,16	67,81
MCP-1	18,31	14,18	4,05	23,67
M-CSF	2,93	3,34	4,56	6,105
MIG	61,76	48,20	230,4	242,5
MIP-1a	38,70	29,30	30,01	37,26
MIP-1B	39,25	79,28	25,53	32,16
MIP-2	40,58	59,63	81,11	71,57
RANTES	40,55	51,15	19,02	28,78
TNFa	6,07	7,80	2,07	1,62
VEGF	0,52	0,08	1,66	1,44

Supplementary Table 3.1 Cytokine levels in serum and spleen homogenates collected from mice irradiated or not 8 weeks prior to collection of tissues
Shown is the average n=2 mice. BD= below detection level. Cytokines were measured by Eve Technologies (Calgary, Alberta).

3.7 References

1   Wang, Y., Schulte, B. A., LaRue, A. C., Ogawa, M. & Zhou, D. Total body irradiation selectively induces murine hematopoietic stem cell senescence. *Blood* **107**, 358-366 (2006).

2   Le, O. N. *et al.* Ionizing radiation-induced long-term expression of senescence markers in mice is independent of p53 and immune status. *Aging Cell* **9**, 398-409 (2010).

3   Marcoux, S. *et al.* Expression of the senescence marker p16INK4a in skin biopsies of acute lymphoblastic leukemia survivors: a pilot study. *Radiat Oncol* **8**, 252 (2013).

4   Sanoff, H. K. *et al.* Effect of cytotoxic chemotherapy on markers of molecular age in patients with breast cancer. *J Natl Cancer Inst* **106**, dju057 (2014).

5   Salama, R., Sadaie, M., Hoare, M. & Narita, M. Cellular senescence and its effector programs. *Genes Dev* **28**, 99-114 (2014).

6   Burd, C. E. *et al.* Monitoring tumorigenesis and senescence in vivo with a p16(INK4a)-luciferase model. *Cell* **152**, 340-351 (2013).

7   Sorrentino, J. A. *et al.* p16INK4a reporter mice reveal age-promoting effects of environmental toxicants. *J Clin Invest* **124**, 169-173 (2014).

8   Yamakoshi, K. *et al.* Real-time in vivo imaging of p16Ink4a reveals cross talk with p53. *J Cell Biol* **186**, 393-407 (2009).

9   Sharpless, N. E. *et al.* Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. *Nature* **413**, 86-91 (2001).

10   Sherr, C. J. & Roberts, J. M. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* **13**, 1501-1512 (1999).

11   Zhou, B. B. & Elledge, S. J. The DNA damage response: putting checkpoints in perspective. *Nature* **408**, 433-439 (2000).

12   Robles, S. J. & Adami, G. R. Agents that cause DNA double strand breaks lead to p16INK4a enrichment and the premature senescence of normal fibroblasts. *Oncogene* **16**, 1113-1123 (1998).

13   Stein, G. H., Drullinger, L. F., Soulard, A. & Dulic, V. Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. *Mol Cell Biol* **19**, 2109-2117 (1999).

14   Johmura, Y. *et al.* Necessary and sufficient role for a mitosis skip in senescence induction. *Mol Cell* **55**, 73-84 (2014).

15   Beausejour, C. M. *et al.* Reversal of human cellular senescence: roles of the p53 and p16 pathways. *Embo J* **22**, 4212-4222 (2003).

16   Ito, K. *et al.* Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. *Nature* **431**, 997-1002 (2004).

17   Ito, K. *et al.* Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. *Nat Med* **12**, 446-451 (2006).

18   Janzen, V. *et al.* Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature* **443**, 421-426 (2006).

19   Krishnamurthy, J. *et al.* p16INK4a induces an age-dependent decline in islet regenerative potential. *Nature* **443**, 453-457 (2006).

20   Molofsky, A. V. *et al.* Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing. *Nature* **443**, 448-452 (2006).

21   Sousa-Victor, P. *et al.* Geriatric muscle stem cells switch reversible quiescence into senescence. *Nature* **506**, 316-321 (2014).

22   van Deursen, J. M. The role of senescent cells in ageing. *Nature* **509**, 439-446 (2014).

23   Christophorou, M. A., Ringshausen, I., Finch, A. J., Swigart, L. B. & Evan, G. I. The pathological response to DNA damage does not contribute to p53-mediated tumour suppression. *Nature* **443**, 214-217 (2006).

24   Parrinello, S. *et al.* Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nat Cell Biol* **5**, 741-747 (2003).

25   Ruzankina, Y. *et al.* Deletion of the developmentally essential gene ATR in adult mice leads to age-related phenotypes and stem cell loss. *Cell Stem Cell* **1**, 113-126 (2007).

26   Carbonneau, C. L. *et al.* Ionizing radiation-induced expression of INK4a/ARF in murine bone marrow-derived stromal cell populations interferes with bone marrow homeostasis. *Blood* **119**, 717-726 (2012).

27   Davalli, P., Mitic, T., Caporali, A., Lauriola, A. & D'Arca, D. ROS, Cell Senescence, and Novel Molecular Mechanisms in Aging and Age-Related Diseases. *Oxidative medicine and cellular longevity* **2016**, 3565127 (2016).

28   Voehringer, D., Koschella, M. & Pircher, H. Lack of proliferative capacity of human effector and memory T cells expressing killer cell lectinlike receptor G1 (KLRG1). *Blood* **100**, 3698-3702 (2002).

29   Krtolica, A., Parrinello, S., Lockett, S., Desprez, P. Y. & Campisi, J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc Natl Acad Sci USA* **98**, 12072-12077 (2001).

30   Coppe, J. P. *et al.* Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol* **6**, 2853-2868 (2008).

31   Demaria, M. *et al.* An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. *Dev Cell* **31**, 722-733 (2014).

32   Baker, D. J. *et al.* Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* **479**, 232-236 (2011).

Chapter 4. Discussion, perspectives and conclusions

4.1 General discussion

Ionizing Radiation (IR) is a primary therapeutic intervention in cancer and hematopoietic disorders. Together with surgery and chemotherapy it has helped to increase the life expectancy of patients and, in some cases, has led to complete remission. However, long-after therapy, children encounter alterations in the integrity of tissues/organs associated with aging features [99,292,316,317]. The accumulation of senescent cells plays a pivotal in aging, and is considered an important hallmark of aging [154]. Cellular senescence is a biological process that can be both beneficial and deleterious. The induction of senescence is beneficial for wound healing, limb regeneration (salamanders); however, the accumulation of senescent cells in young individuals is predominantly detrimental.

For example, the SASP has been shown to promote tumor growth and the development of age-associated diseases such as cancer [273-275]. Genetic or pharmacological clearance of senescent cells ameliorates the onset of aging and its associated pathologies in mice [10,13]. Likewise, elimination of IR-induced senescence was shown to alleviate the premature aging phenotype imposed to murine hematopoietic stem cells [12].

4.1.1 IR induces splenic aged-like alterations

In mice, IR induces splenic ablation within 3-7 days with a recovery tendency over the following 3-4 weeks [5,302,303]. Eight weeks after IR exposure mice experience signs of accelerated aging; with tissues experienced a decline in tissue regeneration (spleen and liver). In addition, spleens possessed an decreased absolute numbers of macrophages, lymphocytes, dendritic and stromal cells. We have also shown that eight weeks after IR exposure provoked an accelerated aging as indicated by a delayed upregulation of senescent makers for instance $p16^{INK4a}$ and SASP factors such as IL-6, IL-1α, VEGF and MCP-1.

Experimental observations have shown that during aging lymphocyte cell counts, and functions can experience an accelerated exhaustion led by IR, chemotherapy, transplantation or chronic infections. Chemotherapy and HSC transplantation induce an altered composition of T cells, decreased naïve and memory T cells versus increased effector memory cells and Tregs [316]. Following chemotherapy treatment, breast cancer patients experienced an aggravate senescence and exhaustion of CD8$^+$ T cell sub-populations compare prior to the treatment [4,316]. HSC transplantation leads to decline naïve-T-cell numbers accompanied by accelerated aging of T cells which is associated with signs of telomere shortening, DNA damage response and upregulation of the tumor suppressor $p16^{INK4a}$ gene [294,316,318-321].

4.1.2   IR induces a T cell proliferation impairment in function

T cell activation to proliferate depends on multiple extrinsic signals derived from antigen presenting cells and the environment and the intrinsic signalling response [322]. In the context of antigen presentation, T cell activation resume in T-cell proliferation and differentiation toward effector functions (i.e. Granzyme B and cytokine secretion). CD3$^+$ lymphocytes recognize the antigens present on the surface of the DCs and macrophages in the context of MHC-II presentation. This antigen recognition occurs through the CD3$^+$ T Cell Receptor (TCR), whose three-dimensional structure is complementary to that of the MHC-antigen complexes. T cell activation also requires the action of CD80/CD86 costimulatory molecules on macrophages and DC cells [322].

Aging has a profound impact on the immune system, and it is well-described that cell functions, in secondary lymphoid tissues are compromised. Elderly individuals experienced an increase susceptibility to infectious diseases [277-280]. Importantly, with aging the adaptive response, particularly the proliferation of T-cells and function of dendritic cells is reduced in aged donors [281]. Likewise, deletion of p16^{INK4a} in T-cells specifically was shown to enhance antigen-specific immune responses, suggesting an intrinsic defect of aged T-cells [277,282]. Our results indicated that eight weeks after IR, T-cell proliferation is compromised as observed by allogenic stimulation of T-cells *in vitro* or in response to an acute LCMV-Arm infection *in vivo*. The defect in T-cell proliferation *in vitro* appeared to be non-autonomous as CD3$^+$ purified cells from irradiated spleens were

fully competent in their ability to proliferate in a MLR or by anti-CD28/anti-CD3 conjugated beads.

4.1.3 T cell proliferation is partially limited by non-cell-autonomous mechanisms

The defect in T cell proliferation is partially explained by non-cell-autonomous mechanisms. In other words, eight weeks after IR exposure, splenic environment emanates inhibitory signals that compromised T cell proliferation. Consistently, eight weeks after IR exposure spleens experience an increased expression of SASP factors. Although, it remains elusive to determine a specific cytokine (s) and/or factor (s) that plays an important role for this limited proliferation of T-cells. A potential candidate is IL-10, an immunosuppressive cytokine which expression was found increased 2-fold in irradiated spleens and reduced following administration of GCV.

Considering future factors of SASP that could explain the inhibition of T cell proliferation, PGE$_2$ and exosomes are promising candidates. In aging, old macrophages are associated to increase PGE$_2$ secretion which suppresses T cell-mediated immune functions. Interestingly, T cells from patients showed decreased proliferation in response to PGE$_2$-secreting cancers cells [171,204,323]. Exosomes are also an important component of the secretory program of senescent cells. Exosomes are small vesicles approximately ~30-150nm in diameter originating in the late endosomal compartment from the inward budding of endosomal membranes which generates intracellular multi-vesicular

endosomes (MVEs). IR-induced senescence in tumor cell lines which promotes the secretion of exosomes which in their cargo are loaded with T cell inhibitory molecule, PD-L1 [71]. The interaction between PD-L1 and PD-1 on a T cell triggers inhibitory signalling for T cell activation. [324]. Nevertheless, a deeper and better understanding of multi-level interactions among macrophages, stromal cells, DC, lymphocytes and SASP are executed to impair T cell proliferation.

4.1.4 Macrophages and DC cells

Macrophages and DCs are required to uptake, process and present the antigen (s) to T cells to activate T cell proliferation. In normal aging, macrophages and DCs displayed intrinsic alterations such as diminished chemotaxis, expression of MHC Class II genes, impaired ability to phagocyte, diminished production of ROS and antigen presentation [161,173,178,232,253,255,325]. Likewise, splenic isolated F4/80$^+$ and CD11c$^+$ cells from irradiated mice had a diminished phagocytic capacity of fluorescent particles. The most likely explanation for this observation is that these cells have intrinsic alterations such as recognition and activation of phagocytosis. Compromised phagocyte capacity could result in diminished response to execute T cell activation. We also observed an important reduction in the expression of CD80/CD86 costimulatory molecules. Again, both phagocytosis and antigen presentation can translate into impaired T-cell proliferation has to be evaluated [161,173,178,232,253,255,325].

The fluorescent particles used in this assay of phagocytosis are recognized simultaneously by Toll-like Receptor (TLR) and CD14. TLR are pattern recognition receptors that recognize conserved molecular patterns on microbes and link innate and adaptive immune systems. Normal aging is also associated with a decrease in macrophage and DC function, particularly in the context of TLR activation [259]. Aged splenic macrophages undergo a decreased TLRs expression [259,326]. This impacts the cell function against various infections and may predispose the elderly to various bacterial and yeast infections [259]. A couple of studies have shown that increased extrinsic age-related changes in the environment of IL-10 are associated with phagocytosis impairment by macrophages (F4/80^{+} cells). The increased secretion of this immunosuppressive cytokine IL-10 leads to a weak expression of the phagocytic receptor CD14 [232,327,328]. Likewise, neutrophils from elderly individuals have a reduced phagocytosis capability of opsonised *Escherichia Coli,* and was associated with reduced surface expression of the Fcγ receptor CD16 [329]. Interestingly, eight weeks after 6.5Gy exposure spleens had an increased production of IL-10.

Cudejko *et al*., 2011 suggested that p16[INK4a] is an important modulator of macrophage polarization and show that p16[INK4a]-deficiency skews macrophages towards an IL-4-like phenotype and less capacity to response to LPS [255]. These observations are considered of great relevance considering the ability of macrophages to shape the mediated T-cell responses, and an attenuated proinflammatory response and impaired capacity to clear pathogens.

4.1.5 Splenic stroma cells

Stromal cell function is beyond that merely of a structural scaffold in the secondary lymphoid organs. In the last decade, emerging research has revealed an integral role for these cells in controlling the immune response [242,330,331]. In normal aging, human and murine stromal cells from secondary lymphoid organs one associated with progressive dysregulation and structural changes [332,333]. Park *et al*., (2014) have shown that the splenic stroma composition changed with aging [334]. Our result suggested that long after IR-exposure, CD35$^+$ and gp38$^+$ splenic cell counts are significantly reduced. In addition, these cells possessed presented p16^{INK4a} increased expression. Although we did not show experimental evidences that associated these alterations with T cell function, this might have an important effect in the organization/function of T or B cells. Aw *et al*., (2016) shown that gp38$^+$ FRC network increased the area which accounts the merging of both T and B regions. These cells also presented a reduced secretion of the homeostatic chemokines CCL19 and CCL21 that T cells depend on to properly migrate towards the T zone [236].

4.1.6 p16^{INK4a} limits tissue proliferation and tumour progression

p16^{INK4a} expression is associated with chronological aging and rises in the context of T cells accelerated aging by human-immunodeficiency virus type 1 (HIV-1) infection, smoking, transplantation, physical inactivity, chemotherapy and IR-exposure [105,187,277,294,316,318-321,335-337]. IR is associated with impairment in the regeneration capability probably attributed to induction of senescent markers such as p16^{INK4a} and SASP

[100,186,187]. Long after IR treatment progenitors/stem cells number decline within multiple organs/tissues. Notably, this persistent damage is evidenced in the bone marrow (BM) niche by senescence of hematopoietic progenitors/stem cells and BM stromal cells.

The induction of *p16^{INK4a}* gene expression following exposure to IR or during normal aging seems to occur preferentially and possibly in exhausted progenitors/stem cell populations, preventing adequate tissue renewal [6,178,308,309,338,339]. Elimination of IR-induced p16^{INK4a}-positive cells rejuvenates the hematopoiesis. IR induces BM residual injury manifested by an impaired hematopoiesis, decreased HSC with limited self-renewal capacity and significant decay *per se* in the HSC capacity to produce naïve T-cells [307]. Although the secondary effects endured by peripheral lymphoid organs like the spleen are less well documented. In the spleen, analyzes of stem cell populations ~13-week post-irradiation had the lowest levels of Ly-6$^+$ and CD34$^+$/Ly-6$^+$ stem cells [340]. *p16^{INK4a}* gene expression appears to promote aging by reducing self-renewing capability in BM, brain and pancreas and its elimination ameliorates the regeneration capacity in these tissues [100,307-309,341]. In addition, we found that delayed inactivation of *p16^{INK4a}* eight-week post IR exposure resulted in an augmented BrdU incorporation *in vivo* (liver and spleen) [285].

As a tumor suppressor gene, deletion or inactivation of the p16^{INK4a} gene is observed in many human cancers and predisposes mice to tumorigenesis. In normal aging the thymus experiences an important involution in its mass. This organ plays an

important role in T cell development and loss of p16[INK4a] reduces thymic involution favoring production of naive T cells. Despite, the inactivation of *p16[INK4a]*, T-cell neoplasia is not augmented. In contrast, B lineage-specific ablation of p16[INK4a] gene markedly increased incidence of systemic, high-grade B-cell neoplasm [277]. Not surprisingly, inactivation of *p16[INK4a]* before or after IR treatment leads to an increase of cancer incidence mainly lymphomas. Genetic analysis of some tumors had showed deletion of *p16[INK4a]*, even in mice not treated to provoke deletion of this gene. Overall, these results suggest that sustained IR-induced *p16[INK4a]* expression is necessary to protect against cancer progression.

It is important to consider that the immune system function act as an important anti-cancer mechanism, targeting damaged or stress cell at risk of neoplastic transformation. The adaptive immune response can target tumour-specific antigens. This anti-tumor response relay in patient's own T cells fitness. However, after IR, there is impairment in T cell proliferation that is partially explained by unbalanced SASP secretion. Furthermore, it is important to consider pharmacological strategies such senolytic compounds that limit the deleterious effects of unbalanced senescence produced in cancer patients without risking the appearance of secondary cancer.

4.1.7 Is p16^{INK4a} expression an universal marker of limited proliferation?

Eight weeks after radiation, p16^{INK4a} increases in splenic macrophages and T cell populations a potent cell cycle inhibitor. Our results suggest that increased p16^{INK4a} expression in T lymphocytes is not high enough to limit their proliferation or most likely that only a small fraction of the cells express a higher level of p16^{INK4a} [319,342-344]. Another possible explanation is that after isolation of T cells, there is a small portion of contaminating cells that highly expresses p16^{INK4a}. For example, eight weeks after IR exposure splenic F4/80$^+$ macrophages upregulate delayed high levels of *p16^{INK4a}*.

p16^{INK4a} is not only an important tumour suppressor gene, its activation plays important roles in modulation the immune response mediated by macrophages. In support of this idea, *p16^{INK4a}* expression is upregulated during monocytes differentiation towards macrophages and DCs *in vitro* without negative repercussions in the cell cycle [265]. Macrophages polarize to M1 or M2 state. M1 macrophages are important promoters of inflammation, in contrast M2 macrophages which act in anti-inflammation and encourage tissue repair. Macrophages polarization *in vitro* and *in vivo* requires the upregulation of *p16^{INK4a}* and *p14/p19^{ARF}* [265,345]. Recently, Hall *et al*., (2017) proposed that *p16^{INK4a}/*SA-β-gal positive cells accumulating in normal aging are macrophages and they acquired this phenotype as part of their physiological reprogramming towards anti-inflammatory phenotype [200,212].

4.2 Perspectives

To improve tissue proliferation and cell function without favoring tumor progression we consider senolytic compounds as a promising pharmacological strategy. Senolytic drugs were designed after experimental observations suggested that senescent cells are associated to up-regulation of PI3K/AKT pathways, p53/p21^{Cip1} proteins, serpines and BCL-2/BCL-X$_L$ pro-survival pathway, among others [346]. Two senolytic drugs were reported in 2016, ABT-263 (Navitoclax) and ABT-737. Both senolytics induce anti-senescence by inhibition of the anti-apoptotic target the pro-survival B-cell lymphoma 2 (BCL-2) protein family including the BCL-2, BCL-W and BCL-X$_L$ proteins [347]. In mice, these compounds selectively kill senescent cells making aged tissues young again [12,346]

The forkhead box O family (**FOXO**) of proteins are prominent cell cycle inhibitors by activation of p21^{Cip1}. These proteins are well-known promoters of longevity in mammals. In normal cells, FOXO$_4$ is barely expressed and resides in the nucleus while p53 is mainly restricted to the mitochondria, when p53 is bound to FOXO$_4$ it remains in the nucleus. This observation allowed researchers to design a FOXO$_4$-DRI cell-penetrating peptide which disrupts the p53-DOXO$_4$ interaction and induce senescent cells to undergo apoptosis. Notably, FOXO4-DRI peptide has a strong preference for targeting high-SASP subpopulations of senescent cells without effect on normal cells [290]. In addition to senolytic compounds, it would be important to use complementary treatment to improve cell function.

4.3   Conclusions

Long after IR exposure, splenic environment experienced alterations with increased expression of senescent markers such as p16^{INK4a} and SASP.

The delayed increased p16^{INK4a} expression had impaired the tissue proliferation capability (spleen and liver) and the cell function (particularly within the spleen).

Eight weeks after IR exposure, splenic immune cells had compromised their function. T cell proliferation impairment upon allogenic stimulation *in vitro* was partially explained by a non-cell autonomous mechanism.

The delayed p16^{INK4a} expression following exposure to IR act as tumor suppressive response against damaged or stress cells at risk of neoplastic transformation.

Elimination of p16^{INK4a}-positive cell ameliorated the impaired function of T cell proliferation, and the phagocytic capability of macrophages and DC.

4.4 References

1   Fuster, V. Changing Demographics: A New Approach to Global Health Care Due to the Aging Population. *J Am Coll Cardiol* **69**, 3002-3005, doi:10.1016/j.jacc.2017.05.013 (2017).

2   Lopez-Otin, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. The hallmarks of aging. *Cell* **153**, 1194-1217, doi:10.1016/j.cell.2013.05.039 (2013).

3   Sorrentino, J. A. *et al.* p16INK4a reporter mice reveal age-promoting effects of environmental toxicants. *J Clin Invest* **124**, 169-173 (2014).

4   Onyema, O. O. *et al.* Chemotherapy-induced changes and immunosenescence of CD8+ T-cells in patients with breast cancer. *Anticancer Res* **35**, 1481-1489 (2015).

5   Le, O. N. *et al.* Ionizing radiation-induced long-term expression of senescence markers in mice is independent of p53 and immune status. *Aging Cell* **9**, 398-409 (2010).

6   Krishnamurthy, J. *et al.* Ink4a/Arf expression is a biomarker of aging. *The Journal of clinical investigation* **114**, 1299-1307, doi:10.1172/JCI22475 (2004).

7   Carbonneau, C. L. *et al.* Ionizing radiation-induced expression of INK4a/ARF in murine bone marrow-derived stromal cell populations interferes with bone marrow homeostasis. *Blood* **119**, 717-726 (2012).

8   Campisi, J. & d'Adda di Fagagna, F. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol* **8**, 729-740, doi:10.1038/nrm2233 (2007).

9   Campisi, J. Aging, cellular senescence, and cancer. *Annu Rev Physiol* **75**, 685-705, doi:10.1146/annurev-physiol-030212-183653 (2013).

10   Baker, D. J. *et al.* Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* **479**, 232-236, doi:10.1038/nature10600 (2011).

11   Jeon, O. H. *et al.* Local clearance of senescent cells attenuates the development of post-traumatic osteoarthritis and creates a pro-regenerative environment. *Nat Med* **23**, 775-781, doi:10.1038/nm.4324 (2017).

12   Chang, J. *et al.* Clearance of senescent cells by ABT263 rejuvenates aged hematopoietic stem cells in mice. *Nature medicine* **22**, 78-83, doi:10.1038/nm.4010 (2016).

13   Baker, D. J. *et al.* Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan. *Nature* **530**, 184-189, doi:10.1038/nature16932 (2016).

14   Campisi, J. Cellular senescence as a tumor-suppressor mechanism. *Trends in cell biology* **11**, S27-31 (2001).

15   Rodier, F. & Campisi, J. Four faces of cellular senescence. *J Cell Biol* **192**, 547-556, doi:10.1083/jcb.201009094 (2011).

16   Shay, J. W. & Wright, W. E. Hayflick, his limit, and cellular ageing. *Nat Rev Mol Cell Biol* **1**, 72-76, doi:10.1038/35036093 (2000).

17   Tasdemir, N. & Lowe, S. W. Senescent cells spread the word: non-cell autonomous propagation of cellular senescence. *EMBO J* **32**, 1975-1976, doi:10.1038/emboj.2013.139 (2013).

18   Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. & Lowe, S. W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88**, 593-602 (1997).

19   Serrano, M. *et al.* Role of the INK4a locus in tumor suppression and cell mortality. *Cell* **85**, 27-37 (1996).

20   Di Leonardo, A., Linke, S. P., Clarkin, K. & Wahl, G. M. DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes Dev* **8**, 2540-2551 (1994).

21   Serrano, M., Hannon, G. J. & Beach, D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* **366**, 704-707, doi:10.1038/366704a0 (1993).

22   Ohtani, N. *et al.* Opposing effects of Ets and Id proteins on p16INK4a expression during cellular senescence. *Nature* **409**, 1067-1070, doi:10.1038/35059131 (2001).

23   Narita, M. *et al.* Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* **113**, 703-716 (2003).

24   Bandyopadhyay, D. *et al.* Dynamic assembly of chromatin complexes during cellular senescence: implications for the growth arrest of human melanocytic nevi. *Aging Cell* **6**, 577-591, doi:10.1111/j.1474-9726.2007.00308.x (2007).

25   Sharpless, N. E., Ramsey, M. R., Balasubramanian, P., Castrillon, D. H. & DePinho, R. A. The differential impact of p16(INK4a) or p19(ARF) deficiency on cell growth and tumorigenesis. *Oncogene* **23**, 379-385, doi:10.1038/sj.onc.1207074 (2004).

26   Zindy, F., Quelle, D. E., Roussel, M. F. & Sherr, C. J. Expression of the p16INK4a tumor suppressor versus other INK4 family members during mouse development and aging. *Oncogene* **15**, 203-211, doi:10.1038/sj.onc.1201178 (1997).

27   Nielsen, G. P. *et al.* Immunohistochemical survey of p16INK4A expression in normal human adult and infant tissues. *Lab Invest* **79**, 1137-1143 (1999).

28   Burd, C. E. *et al.* Monitoring tumorigenesis and senescence in vivo with a p16(INK4a)-luciferase model. *Cell* **152**, 340-351, doi:10.1016/j.cell.2012.12.010 (2013).

29   Munoz-Espin, D. & Serrano, M. Cellular senescence: from physiology to pathology. *Nat Rev Mol Cell Biol* **15**, 482-496, doi:10.1038/nrm3823 (2014).

30   Ye, C. *et al.* Radiation-induced cellular senescence results from a slippage of long-term G2 arrested cells into G1 phase. *Cell Cycle* **12**, 1424-1432, doi:10.4161/cc.24528 (2013).

31   Wiebusch, L. & Hagemeier, C. p53- and p21-dependent premature APC/C-Cdh1 activation in G2 is part of the long-term response to genotoxic stress. *Oncogene* **29**, 3477-3489, doi:10.1038/onc.2010.99 (2010).

32   Gire, V. & Dulic, V. Senescence from G2 arrest, revisited. *Cell Cycle* **14**, 297-304, doi:10.1080/15384101.2014.1000134 (2015).

33   Lee, B. Y. *et al.* Senescence-associated beta-galactosidase is lysosomal beta-galactosidase. *Aging Cell* **5**, 187-195, doi:10.1111/j.1474-9726.2006.00199.x (2006).

34   Dimri, G. P. *et al.* A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A* **92**, 9363-9367 (1995).

35 Choi, J. *et al.* Expression of senescence-associated beta-galactosidase in enlarged prostates from men with benign prostatic hyperplasia. *Urology* **56**, 160-166 (2000).

36 Ferlicot, S. *et al.* The role of replicative senescence in chronic allograft nephropathy. *Hum Pathol* **34**, 924-928 (2003).

37 Mishima, K. *et al.* Senescence-associated beta-galactosidase histochemistry for the primate eye. *Invest Ophthalmol Vis Sci* **40**, 1590-1593 (1999).

38 Pendergrass, W. R. *et al.* Cellular proliferation potential during aging and caloric restriction in rhesus monkeys (Macaca mulatta). *J Cell Physiol* **180**, 123-130, doi:10.1002/(SICI)1097-4652(199907)180:1<123::AID-JCP14>3.0.CO;2-W (1999).

39 Kishi, S. *et al.* The identification of zebrafish mutants showing alterations in senescence-associated biomarkers. *PLoS Genet* **4**, e1000152, doi:10.1371/journal.pgen.1000152 (2008).

40 Robbins, E., Levine, E. M. & Eagle, H. Morphologic changes accompanying senescence of cultured human diploid cells. *J Exp Med* **131**, 1211-1222 (1970).

41 Sanchez-Martin, M. M. & Cabezas, J. A. Evaluation of the activities of eight lysosomal hydrolases in sera of humans, rats and pigs of different ages. *Mech Ageing Dev* **99**, 95-107 (1997).

42 Zhang, R. *et al.* Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. *Dev Cell* **8**, 19-30, doi:10.1016/j.devcel.2004.10.019 (2005).

43 Zhang, R., Chen, W. & Adams, P. D. Molecular dissection of formation of senescence-associated heterochromatin foci. *Mol Cell Biol* **27**, 2343-2358, doi:10.1128/MCB.02019-06 (2007).

44 Ye, X. *et al.* Definition of pRB- and p53-dependent and -independent steps in HIRA/ASF1a-mediated formation of senescence-associated heterochromatin foci. *Mol Cell Biol* **27**, 2452-2465, doi:10.1128/MCB.01592-06 (2007).

45 Salama, R., Sadaie, M., Hoare, M. & Narita, M. Cellular senescence and its effector programs. *Genes Dev* **28**, 99-114, doi:10.1101/gad.235184.113 (2014).

46   Rai, T. S. & Adams, P. D. Lessons from senescence: Chromatin maintenance in non-proliferating cells. *Biochim Biophys Acta* **1819**, 322-331, doi:10.1016/j.bbagrm.2011.07.014 (2012).

47   Narita, M. *et al.* A novel role for high-mobility group a proteins in cellular senescence and heterochromatin formation. *Cell* **126**, 503-514, doi:10.1016/j.cell.2006.05.052 (2006).

48   Funayama, R., Saito, M., Tanobe, H. & Ishikawa, F. Loss of linker histone H1 in cellular senescence. *J Cell Biol* **175**, 869-880, doi:10.1083/jcb.200604005 (2006).

49   Beausejour, C. M. *et al.* Reversal of human cellular senescence: roles of the p53 and p16 pathways. *The EMBO journal* **22**, 4212-4222, doi:10.1093/emboj/cdg417 (2003).

50   Takai, H., Smogorzewska, A. & de Lange, T. DNA damage foci at dysfunctional telomeres. *Current biology : CB* **13**, 1549-1556 (2003).

51   Rodier, F. *et al.* DNA-SCARS: distinct nuclear structures that sustain damage-induced senescence growth arrest and inflammatory cytokine secretion. *J Cell Sci* **124**, 68-81, doi:10.1242/jcs.071340 (2011).

52   Munch, S. *et al.* The tumor suppressor PML specifically accumulates at RPA/Rad51-containing DNA damage repair foci but is nonessential for DNA damage-induced fibroblast senescence. *Mol Cell Biol* **34**, 1733-1746, doi:10.1128/MCB.01345-13 (2014).

53   Vernier, M. *et al.* Regulation of E2Fs and senescence by PML nuclear bodies. *Genes Dev* **25**, 41-50, doi:10.1101/gad.1975111 (2011).

54   Zhao, J., Herrera-Diaz, J. & Gross, D. S. Domain-wide displacement of histones by activated heat shock factor occurs independently of Swi/Snf and is not correlated with RNA polymerase II density. *Mol Cell Biol* **25**, 8985-8999, doi:10.1128/MCB.25.20.8985-8999.2005 (2005).

55   Mallette, F. A., Goumard, S., Gaumont-Leclerc, M. F., Moiseeva, O. & Ferbeyre, G. Human fibroblasts require the Rb family of tumor suppressors, but not p53, for PML-induced senescence. *Oncogene* **23**, 91-99, doi:10.1038/sj.onc.1206886 (2004).

56   Mattout-Drubezki, A. & Gruenbaum, Y. Dynamic interactions of nuclear lamina proteins with chromatin and transcriptional machinery. *Cell Mol Life Sci* **60**, 2053-2063, doi:10.1007/s00018-003-3038-3 (2003).

57   Guelen, L. *et al.* Domain organization of human chromosomes revealed by mapping of nuclear lamina interactions. *Nature* **453**, 948-951, doi:10.1038/nature06947 (2008).

58   Rodriguez-Rodero, S. *et al.* Aging genetics and aging. *Aging Dis* **2**, 186-195 (2011).

59   Taimen, P. *et al.* A progeria mutation reveals functions for lamin A in nuclear assembly, architecture, and chromosome organization. *Proc Natl Acad Sci U S A* **106**, 20788-20793, doi:10.1073/pnas.0911895106 (2009).

60   Shin, D. M., Kucia, M. & Ratajczak, M. Z. Nuclear and chromatin reorganization during cell senescence and aging - a mini-review. *Gerontology* **57**, 76-84, doi:10.1159/000281882 (2011).

61   Chandra, T. & Kirschner, K. Chromosome organisation during ageing and senescence. *Curr Opin Cell Biol* **40**, 161-167, doi:10.1016/j.ceb.2016.03.020 (2016).

62   Scaffidi, P. & Misteli, T. Lamin A-dependent misregulation of adult stem cells associated with accelerated ageing. *Nat Cell Biol* **10**, 452-459, doi:10.1038/ncb1708 (2008).

63   Freund, A., Laberge, R. M., Demaria, M. & Campisi, J. Lamin B1 loss is a senescence-associated biomarker. *Mol Biol Cell* **23**, 2066-2075, doi:10.1091/mbc.E11-10-0884 (2012).

64   Sadaie, M. *et al.* Redistribution of the Lamin B1 genomic binding profile affects rearrangement of heterochromatic domains and SAHF formation during senescence. *Genes Dev* **27**, 1800-1808, doi:10.1101/gad.217281.113 (2013).

65   Lenain, C. *et al.* Massive reshaping of genome-nuclear lamina interactions during oncogene-induced senescence. *Genome Res* **27**, 1634-1644, doi:10.1101/gr.225763.117 (2017).

66   Kuilman, T. *et al.* Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell* **133**, 1019-1031, doi:10.1016/j.cell.2008.03.039 (2008).

67   Coppe, J. P. *et al.* Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol* **6**, 2853-2868, doi:10.1371/journal.pbio.0060301 (2008).

68   Acosta, J. C. *et al.* Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell* **133**, 1006-1018, doi:10.1016/j.cell.2008.03.038 (2008).

69   Coppe, J. P., Desprez, P. Y., Krtolica, A. & Campisi, J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol* **5**, 99-118, doi:10.1146/annurev-pathol-121808-102144 (2010).

70   Kulkarni, R. S. & Shinde, R. L. Depression and Its Associated Factors in Older Indians: A Study Based on Study of Global Aging and Adult Health (SAGE)-2007. *J Aging Health* **27**, 622-649, doi:10.1177/0898264314556617 (2015).

71   Lehmann, B. D. *et al.* Senescence-associated exosome release from human prostate cancer cells. *Cancer research* **68**, 7864-7871, doi:10.1158/0008-5472.CAN-07-6538 (2008).

72   Malaquin, N., Carrier-Leclerc, A., Dessureault, M. & Rodier, F. DDR-mediated crosstalk between DNA-damaged cells and their microenvironment. *Front Genet* **6**, 94, doi:10.3389/fgene.2015.00094 (2015).

73   Rodier, F. *et al.* Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nat Cell Biol* **11**, 973-979, doi:10.1038/ncb1909 (2009).

74   Lujambio, A. To clear, or not to clear (senescent cells)? That is the question. *Bioessays* **38 Suppl 1**, S56-64, doi:10.1002/bies.201670910 (2016).

75   Orjalo, A. V., Bhaumik, D., Gengler, B. K., Scott, G. K. & Campisi, J. Cell surface-bound IL-1alpha is an upstream regulator of the senescence-associated IL-6/IL-8 cytokine network. *Proc Natl Acad Sci U S A* **106**, 17031-17036, doi:10.1073/pnas.0905299106 (2009).

76   Acosta, J. C. *et al.* A complex secretory program orchestrated by the inflammasome controls paracrine senescence. *Nat Cell Biol* **15**, 978-990, doi:10.1038/ncb2784 (2013).

77   Ohanna, M. *et al.* Senescent cells develop a PARP-1 and nuclear factor-{kappa}B-associated secretome (PNAS). *Genes Dev* **25**, 1245-1261, doi:10.1101/gad.625811 (2011).

78   (!!! INVALID CITATION !!! 6,60).

79   (!!! INVALID CITATION !!! 9,14,28,61,62).

80   Liu, D. & Hornsby, P. J. Senescent human fibroblasts increase the early growth of xenograft tumors via matrix metalloproteinase secretion. *Cancer Res* **67**, 3117-3126, doi:10.1158/0008-5472.CAN-06-3452 (2007).

81   Bringold, F. & Serrano, M. Tumor suppressors and oncogenes in cellular senescence. *Experimental gerontology* **35**, 317-329 (2000).

82   Lundberg, A. S., Hahn, W. C., Gupta, P. & Weinberg, R. A. Genes involved in senescence and immortalization. *Current opinion in cell biology* **12**, 705-709 (2000).

83   Celeste, A. *et al.* Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. *Nat Cell Biol* **5**, 675-679, doi:10.1038/ncb1004 (2003).

84   el-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W. & Vogelstein, B. Definition of a consensus binding site for p53. *Nat Genet* **1**, 45-49, doi:10.1038/ng0492-45 (1992).

85   Harris, S. L. & Levine, A. J. The p53 pathway: positive and negative feedback loops. *Oncogene* **24**, 2899-2908, doi:10.1038/sj.onc.1208615 (2005).

86   Kruse, J. P. & Gu, W. Modes of p53 regulation. *Cell* **137**, 609-622, doi:10.1016/j.cell.2009.04.050 (2009).

87   Qian, Y. & Chen, X. Senescence regulation by the p53 protein family. *Methods Mol Biol* **965**, 37-61, doi:10.1007/978-1-62703-239-1_3 (2013).

88   Reed, S. M. & Quelle, D. E. p53 Acetylation: Regulation and Consequences. *Cancers (Basel)* **7**, 30-69, doi:10.3390/cancers7010030 (2014).

89   Tang, Y., Zhao, W., Chen, Y., Zhao, Y. & Gu, W. Acetylation is indispensable for p53 activation. *Cell* **133**, 612-626, doi:10.1016/j.cell.2008.03.025 (2008).

90   Li, M. *et al.* Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. *Science* **302**, 1972-1975, doi:10.1126/science.1091362 (2003).

91   Chipuk, J. E. *et al.* Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science* **303**, 1010-1014, doi:10.1126/science.1092734 (2004).

92   Brooks, C. L. & Gu, W. Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. *Curr Opin Cell Biol* **15**, 164-171 (2003).

93   Haupt, Y., Maya, R., Kazaz, A. & Oren, M. Mdm2 promotes the rapid degradation of p53. *Nature* **387**, 296-299, doi:10.1038/387296a0 (1997).

94   Kubbutat, M. H., Jones, S. N. & Vousden, K. H. Regulation of p53 stability by Mdm2. *Nature* **387**, 299-303, doi:10.1038/387299a0 (1997).

95   Li, J., Poi, M. J. & Tsai, M. D. Regulatory mechanisms of tumor suppressor P16(INK4A) and their relevance to cancer. *Biochemistry* **50**, 5566-5582, doi:10.1021/bi200642e (2011).

96   Quelle, D. E., Zindy, F., Ashmun, R. A. & Sherr, C. J. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* **83**, 993-1000 (1995).

97   Lin, Y. C. *et al.* Human p16gamma, a novel transcriptional variant of p16(INK4A), coexpresses with p16(INK4A) in cancer cells and inhibits cell-cycle progression. *Oncogene* **26**, 7017-7027, doi:10.1038/sj.onc.1210507 (2007).

98   Martin, N., Beach, D. & Gil, J. Ageing as developmental decay: insights from p16(INK4a.). *Trends Mol Med* **20**, 667-674, doi:10.1016/j.molmed.2014.09.008 (2014).

99   Le, O. N. *et al.* Ionizing radiation-induced long-term expression of senescence markers in mice is independent of p53 and immune status. *Aging Cell* **9**, 398-409, doi:10.1111/j.1474-9726.2010.00567.x (2010).

100   Carbonneau, C. L. *et al.* Ionizing radiation-induced expression of INK4a/ARF in murine bone marrow-derived stromal cell populations interferes with bone marrow homeostasis. *Blood* **119**, 717-726, doi:10.1182/blood-2011-06-361626 (2012).

101   Kotake, Y., Naemura, M., Murasaki, C., Inoue, Y. & Okamoto, H. Transcriptional Regulation of the p16 Tumor Suppressor Gene. *Anticancer Res* **35**, 4397-4401 (2015).

102   LaPak, K. M. & Burd, C. E. The molecular balancing act of p16(INK4a) in cancer and aging. *Mol Cancer Res* **12**, 167-183, doi:10.1158/1541-7786.MCR-13-0350 (2014).

103   D'Arcangelo, D., Tinaburri, L. & Dellambra, E. The Role of p16INK4a Pathway in Human Epidermal Stem Cell Self-Renewal, Aging and Cancer. *Int J Mol Sci* **18**, doi:10.3390/ijms18071591 (2017).

104   Melendez, B. *et al.* Characterization of the murine p19(ARF) promoter CpG island and its methylation pattern in primary lymphomas. *Carcinogenesis* **21**, 817-821 (2000).

105   Zindy, F. *et al.* Arf tumor suppressor promoter monitors latent oncogenic signals in vivo. *Proc Natl Acad Sci U S A* **100**, 15930-15935, doi:10.1073/pnas.2536808100 (2003).

106   Palmero, I., Pantoja, C. & Serrano, M. p19ARF links the tumour suppressor p53 to Ras. *Nature* **395**, 125-126, doi:10.1038/25870 (1998).

107   Bates, S. *et al.* p14ARF links the tumour suppressors RB and p53. *Nature* **395**, 124-125, doi:10.1038/25867 (1998).

108   Sreeramaneni, R., Chaudhry, A., McMahon, M., Sherr, C. J. & Inoue, K. Ras-Raf-Arf signaling critically depends on the Dmp1 transcription factor. *Mol Cell Biol* **25**, 220-232, doi:10.1128/MCB.25.1.220-232.2005 (2005).

109   (!!! INVALID CITATION !!! [73]).

110   Simboeck, E. & Di Croce, L. p16INK4a in cellular senescence. *Aging* **5**, 590-591, doi:10.18632/aging.100592 (2013).

111   D'Arcangelo, D., Tinaburri, L. & Dellambra, E. The Role of p16(INK4a) Pathway in Human Epidermal Stem Cell Self-Renewal, Aging and Cancer. *Int J Mol Sci* **18**, doi:10.3390/ijms18071591 (2017).

112   Cakouros, D. *et al.* Twist-1 induces Ezh2 recruitment regulating histone methylation along the Ink4A/Arf locus in mesenchymal stem cells. *Mol Cell Biol* **32**, 1433-1441, doi:10.1128/MCB.06315-11 (2012).

113   Hayflick, L. The Limited in Vitro Lifetime of Human Diploid Cell Strains. *Exp Cell Res* **37**, 614-636 (1965).

114   Harley, C. B., Futcher, A. B. & Greider, C. W. Telomeres shorten during ageing of human fibroblasts. *Nature* **345**, 458-460, doi:10.1038/345458a0 (1990).

115   Hayflick, L. & Moorhead, P. S. The serial cultivation of human diploid cell strains. *Experimental cell research* **25**, 585-621 (1961).

116   Olovnikov, A. M. Telomeres, telomerase, and aging: origin of the theory. *Exp Gerontol* **31**, 443-448 (1996).

117   Olovnikov, A. M. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. *J Theor Biol* **41**, 181-190 (1973).

118   Olovnikov, A. M. [Principle of marginotomy in template synthesis of polynucleotides]. *Dokl Akad Nauk SSSR* **201**, 1496-1499 (1971).

119   Watson, J. D. Origin of concatemeric T7 DNA. *Nat New Biol* **239**, 197-201 (1972).

120   Greider, C. W. & Blackburn, E. H. The telomere terminal transferase of Tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity. *Cell* **51**, 887-898 (1987).

121   Greenberg, R. A., Allsopp, R. C., Chin, L., Morin, G. B. & DePinho, R. A. Expression of mouse telomerase reverse transcriptase during development, differentiation and proliferation. *Oncogene* **16**, 1723-1730, doi:10.1038/sj.onc.1201933 (1998).

122   Kim, N. W. *et al.* Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011-2015 (1994).

123   von Zglinicki, T. Oxidative stress shortens telomeres. *Trends Biochem Sci* **27**, 339-344 (2002).

124   von Zglinicki, T. Role of oxidative stress in telomere length regulation and replicative senescence. *Ann N Y Acad Sci* **908**, 99-110 (2000).

125   Cong, Y. S., Wright, W. E. & Shay, J. W. Human telomerase and its regulation. *Microbiol Mol Biol Rev* **66**, 407-425, table of contents (2002).

126   Valenzuela, M. T. *et al.* A comparison of p53 and p16 expression in human tumor cells treated with hyperthermia or ionizing radiation. *Int J Cancer* **72**, 307-312 (1997).

127   Chung, H. Y., Kim, H. J., Kim, K. W., Choi, J. S. & Yu, B. P. Molecular inflammation hypothesis of aging based on the anti-aging mechanism of calorie restriction. *Microsc Res Tech* **59**, 264-272, doi:10.1002/jemt.10203 (2002).

128   Vasto, S. *et al.* Inflammatory networks in ageing, age-related diseases and longevity. *Mech Ageing Dev* **128**, 83-91, doi:10.1016/j.mad.2006.11.015 (2007).

129   Rogers, S. C., Zhang, X., Azhar, G., Luo, S. & Wei, J. Y. Exposure to high or low glucose levels accelerates the appearance of markers of endothelial cell senescence and induces dysregulation of nitric oxide synthase. *J Gerontol A Biol Sci Med Sci* **68**, 1469-1481, doi:10.1093/gerona/glt033 (2013).

130   Kojima, H., Kunimoto, H., Inoue, T. & Nakajima, K. The STAT3-IGFBP5 axis is critical for IL-6/gp130-induced premature senescence in human fibroblasts. *Cell Cycle* **11**, 730-739, doi:10.4161/cc.11.4.19172 (2012).

131   Bartkova, J. *et al.* Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* **444**, 633-637, doi:10.1038/nature05268 (2006).

132   Coppe, J. P. *et al.* Tumor suppressor and aging biomarker p16(INK4a) induces cellular senescence without the associated inflammatory secretory phenotype. *J Biol Chem* **286**, 36396-36403, doi:10.1074/jbc.M111.257071 (2011).

133   (!!! INVALID CITATION !!! [25]).

134   Demaria, M., Desprez, P. Y., Campisi, J. & Velarde, M. C. Cell Autonomous and Non-Autonomous Effects of Senescent Cells in the Skin. *J Invest Dermatol* **135**, 1722-1726, doi:10.1038/jid.2015.108 (2015).

135   Nita, M. & Grzybowski, A. The Role of the Reactive Oxygen Species and Oxidative Stress in the Pathomechanism of the Age-Related Ocular Diseases and Other Pathologies of the Anterior and Posterior Eye Segments in Adults. *Oxidative medicine and cellular longevity* **2016**, 3164734, doi:10.1155/2016/3164734 (2016).

136   Davalli, P., Mitic, T., Caporali, A., Lauriola, A. & D'Arca, D. ROS, Cell Senescence, and Novel Molecular Mechanisms in Aging and Age-Related Diseases. *Oxidative medicine and cellular longevity* **2016**, 3565127, doi:10.1155/2016/3565127 (2016).

137   Parrinello, S. *et al.* Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nat Cell Biol* **5**, 741-747 (2003).

138   Ray, P. D., Huang, B. W. & Tsuji, Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular signalling* **24**, 981-990, doi:10.1016/j.cellsig.2012.01.008 (2012).

139   Chandrasekaran, A., Idelchik, M. & Melendez, J. A. Redox control of senescence and age-related disease. *Redox Biol* **11**, 91-102, doi:10.1016/j.redox.2016.11.005 (2017).

140   Packer, L. & Fuehr, K. Low oxygen concentration extends the lifespan of cultured human diploid cells. *Nature* **267**, 423-425 (1977).

141   Wiemann, S. U. *et al.* Hepatocyte telomere shortening and senescence are general markers of human liver cirrhosis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **16**, 935-942, doi:10.1096/fj.01-0977com (2002).

142   Parrinello, S. *et al.* Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nat Cell Biol* **5**, 741-747, doi:10.1038/ncb1024 (2003).

143   Busuttil, R. A., Rubio, M., Dolle, M. E., Campisi, J. & Vijg, J. Oxygen accelerates the accumulation of mutations during the senescence and immortalization of murine cells in culture. *Aging cell* **2**, 287-294 (2003).

144   Harman, D. The free radical theory of aging. *Antioxidants & redox signaling* **5**, 557-561, doi:10.1089/152308603770310202 (2003).

145   Deckbar, D., Jeggo, P. A. & Lobrich, M. Understanding the limitations of radiation-induced cell cycle checkpoints. *Crit Rev Biochem Mol Biol* **46**, 271-283, doi:10.3109/10409238.2011.575764 (2011).

146   Wahl, G. M., Linke, S. P., Paulson, T. G. & Huang, L. C. Maintaining genetic stability through TP53 mediated checkpoint control. *Cancer Surv* **29**, 183-219 (1997).

147   Di Micco, R. *et al.* Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature* **444**, 638-642, doi:10.1038/nature05327 (2006).

148   Courtois-Cox, S., Jones, S. L. & Cichowski, K. Many roads lead to oncogene-induced senescence. *Oncogene* **27**, 2801-2809, doi:10.1038/sj.onc.1210950 (2008).

149   Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674, doi:10.1016/j.cell.2011.02.013 (2011).

150   Malumbres, M. *et al.* Cellular response to oncogenic ras involves induction of the Cdk4 and Cdk6 inhibitor p15(INK4b). *Mol Cell Biol* **20**, 2915-2925 (2000).

151   Kilbey, A., Terry, A., Cameron, E. R. & Neil, J. C. Oncogene-induced senescence: an essential role for Runx. *Cell Cycle* **7**, 2333-2340, doi:10.4161/cc.6368 (2008).

152   Mallette, F. A., Gaumont-Leclerc, M. F. & Ferbeyre, G. The DNA damage signaling pathway is a critical mediator of oncogene-induced senescence. *Genes Dev* **21**, 43-48, doi:10.1101/gad.1487307 (2007).

153   Michaloglou, C. *et al.* BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* **436**, 720-724, doi:10.1038/nature03890 (2005).

154   Zhuang, D. *et al.* C-MYC overexpression is required for continuous suppression of oncogene-induced senescence in melanoma cells. *Oncogene* **27**, 6623-6634, doi:10.1038/onc.2008.258 (2008).

155   Lin, A. W. *et al.* Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes Dev* **12**, 3008-3019 (1998).

156   Zhu, J., Woods, D., McMahon, M. & Bishop, J. M. Senescence of human fibroblasts induced by oncogenic Raf. *Genes Dev* **12**, 2997-3007 (1998).

157   Hemann, M. T. & Narita, M. Oncogenes and senescence: breaking down in the fast lane. *Genes & development* **21**, 1-5, doi:10.1101/gad.1514207 (2007).

158   Lee, A. C. *et al.* Ras proteins induce senescence by altering the intracellular levels of reactive oxygen species. *J Biol Chem* **274**, 7936-7940 (1999).

159   Wynn, T. A., Chawla, A. & Pollard, J. W. Macrophage biology in development, homeostasis and disease. *Nature* **496**, 445-455, doi:10.1038/nature12034 (2013).

160   De La Fuente, M. Changes in the macrophage function with aging. *Comp Biochem Physiol A Comp Physiol* **81**, 935-938 (1985).

161   Aprahamian, T., Takemura, Y., Goukassian, D. & Walsh, K. Ageing is associated with diminished apoptotic cell clearance in vivo. *Clin Exp Immunol* **152**, 448-455, doi:10.1111/j.1365-2249.2008.03658.x (2008).

162   Hubackova, S. *et al.* Regulation of the PML tumor suppressor in drug-induced senescence of human normal and cancer cells by JAK/STAT-mediated signaling. *Cell Cycle* **9**, 3085-3099, doi:10.4161/cc.9.15.12521 (2010).

163   Pearson, M. *et al.* PML regulates p53 acetylation and premature senescence induced by oncogenic Ras. *Nature* **406**, 207-210, doi:10.1038/35018127 (2000).

164   Mevorach, D. *et al.* What do we mean when we write "senescence," "apoptosis," "necrosis," or "clearance of dying cells"? *Ann N Y Acad Sci* **1209**, 1-9, doi:10.1111/j.1749-6632.2010.05774.x (2010).

165   Linehan, E. & Fitzgerald, D. C. Ageing and the immune system: focus on macrophages. *Eur J Microbiol Immunol (Bp)* **5**, 14-24, doi:10.1556/EUJMI-D-14-00035 (2015).

166   Kim, W. Y. & Sharpless, N. E. VHL inactivation: a new road to senescence. *Cancer Cell* **13**, 295-297, doi:10.1016/j.ccr.2008.03.012 (2008).

167   Young, A. P. *et al.* VHL loss actuates a HIF-independent senescence programme mediated by Rb and p400. *Nat Cell Biol* **10**, 361-369, doi:10.1038/ncb1699 (2008).

168   Campisi, J. Aging and cancer: the double-edged sword of replicative senescence. *Journal of the American Geriatrics Society* **45**, 482-488 (1997).

169   Demaria, M. *et al.* An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. *Dev Cell* **31**, 722-733, doi:10.1016/j.devcel.2014.11.012 (2014).

170   Munoz-Espin, D. *et al.* Programmed cell senescence during mammalian embryonic development. *Cell* **155**, 1104-1118, doi:10.1016/j.cell.2013.10.019 (2013).

171   Storer, M. & Keyes, W. M. Developing senescence to remodel the embryo. *Commun Integr Biol* **7**, doi:10.4161/cib.29098 (2014).

172   Storer, M. *et al.* Senescence is a developmental mechanism that contributes to embryonic growth and patterning. *Cell* **155**, 1119-1130, doi:10.1016/j.cell.2013.10.041 (2013).

173   Oishi, Y. & Manabe, I. Macrophages in age-related chronic inflammatory diseases. *NPJ Aging Mech Dis* **2**, 16018, doi:10.1038/npjamd.2016.18 (2016).

174   Vicente, R., Mausset-Bonnefont, A. L., Jorgensen, C., Louis-Plence, P. & Brondello, J. M. Cellular senescence impact on immune cell fate and function. *Aging Cell* **15**, 400-406, doi:10.1111/acel.12455 (2016).

175   Braumuller, H. *et al.* T-helper-1-cell cytokines drive cancer into senescence. *Nature* **494**, 361-365, doi:10.1038/nature11824 (2013).

176   Kang, T. W. *et al.* Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. *Nature* **479**, 547-551, doi:10.1038/nature10599 (2011).

177   Lujambio, A. *et al.* Non-cell-autonomous tumor suppression by p53. *Cell* **153**, 449-460, doi:10.1016/j.cell.2013.03.020 (2013).

178   Krizhanovsky, V. *et al.* Senescence of activated stellate cells limits liver fibrosis. *Cell* **134**, 657-667, doi:10.1016/j.cell.2008.06.049 (2008).

179 Raj, N. & Attardi, L. D. Tumor suppression: p53 alters immune surveillance to restrain liver cancer. *Current biology : CB* **23**, R527-530, doi:10.1016/j.cub.2013.04.076 (2013).

180 Xue, W. *et al.* Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* **445**, 656-660, doi:10.1038/nature05529 (2007).

181 Little, M. P. Risks associated with ionizing radiation. *British medical bulletin* **68**, 259-275 (2003).

182 Gridley, D. S., Rizvi, A., Luo-Owen, X., Makinde, A. Y. & Pecaut, M. J. Low dose, low dose rate photon radiation modifies leukocyte distribution and gene expression in CD4(+) T cells. *J Radiat Res* **50**, 139-150 (2009).

183 Pecaut, M. J., Nelson, G. A. & Gridley, D. S. Dose and dose rate effects of whole-body gamma-irradiation: I. Lymphocytes and lymphoid organs. *In Vivo* **15**, 195-208 (2001).

184 Manda, K., Glasow, A., Paape, D. & Hildebrandt, G. Effects of ionizing radiation on the immune system with special emphasis on the interaction of dendritic and T cells. *Front Oncol* **2**, 102, doi:10.3389/fonc.2012.00102 (2012).

185 Donnelly, E. H. *et al.* Acute radiation syndrome: assessment and management. *South Med J* **103**, 541-546, doi:10.1097/SMJ.0b013e3181ddd571 (2010).

186 Shao, L. *et al.* Total body irradiation causes long-term mouse BM injury via induction of HSC premature senescence in an Ink4a- and Arf-independent manner. *Blood* **123**, 3105-3115, doi:10.1182/blood-2013-07-515619 (2014).

187 Wang, Y., Schulte, B. A., LaRue, A. C., Ogawa, M. & Zhou, D. Total body irradiation selectively induces murine hematopoietic stem cell senescence. *Blood* **107**, 358-366, doi:10.1182/blood-2005-04-1418 (2006).

188 Hayashi, T. *et al.* Long-term effects of radiation dose on inflammatory markers in atomic bomb survivors. *Am J Med* **118**, 83-86, doi:10.1016/j.amjmed.2004.06.045 (2005).

189 Yamaoka, M. *et al.* Decreases in percentages of naive CD4 and CD8 T cells and increases in percentages of memory CD8 T-cell subsets in the peripheral blood lymphocyte populations of A-bomb survivors. *Radiat Res* **161**, 290-298 (2004).

190   Richardson, R. B. Ionizing radiation and aging: rejuvenating an old idea. *Aging (Albany NY)* **1**, 887-902, doi:10.18632/aging.100081 (2009).

191   Hayashi, T. *et al.* Evaluation of systemic markers of inflammation in atomic-bomb survivors with special reference to radiation and age effects. *FASEB J* **26**, 4765-4773, doi:10.1096/fj.12-215228 (2012).

192   Hashimoto, M. *et al.* Elimination of p19ARF-expressing cells enhances pulmonary function in mice. *JCI insight* **1**, e87732, doi:10.1172/jci.insight.87732 (2016).

193   Childs, B. G. *et al.* Senescent intimal foam cells are deleterious at all stages of atherosclerosis. *Science* **354**, 472-477, doi:10.1126/science.aaf6659 (2016).

194   Kirkland, J. L. & Tchkonia, T. Clinical strategies and animal models for developing senolytic agents. *Exp Gerontol* **68**, 19-25, doi:10.1016/j.exger.2014.10.012 (2015).

195   Ogrodnik, M. *et al.* Cellular senescence drives age-dependent hepatic steatosis. *Nat Commun* **8**, 15691, doi:10.1038/ncomms15691 (2017).

196   Palmer, A. K. *et al.* Cellular Senescence in Type 2 Diabetes: A Therapeutic Opportunity. *Diabetes* **64**, 2289-2298, doi:10.2337/db14-1820 (2015).

197   Schafer, M. J. *et al.* Cellular senescence mediates fibrotic pulmonary disease. *Nat Commun* **8**, 14532, doi:10.1038/ncomms14532 (2017).

198   Roos, C. M. *et al.* Chronic senolytic treatment alleviates established vasomotor dysfunction in aged or atherosclerotic mice. *Aging Cell* **15**, 973-977, doi:10.1111/acel.12458 (2016).

199   Xu, M. *et al.* JAK inhibition alleviates the cellular senescence-associated secretory phenotype and frailty in old age. *Proc Natl Acad Sci U S A* **112**, E6301-6310, doi:10.1073/pnas.1515386112 (2015).

200   Hall, B. M. *et al.* Aging of mice is associated with p16(Ink4a)- and beta-galactosidase-positive macrophage accumulation that can be induced in young mice by senescent cells. *Aging* **8**, 1294-1315, doi:10.18632/aging.100991 (2016).

201   Isobe, K. I., Nishio, N. & Hasegawa, T. Immunological aspects of age-related diseases. *World J Biol Chem* **8**, 129-137, doi:10.4331/wjbc.v8.i2.129 (2017).

202   Lu, S. Y. *et al.* Ripe areca nut extract induces G1 phase arrests and senescence-associated phenotypes in normal human oral keratinocyte. *Carcinogenesis* **27**, 1273-1284, doi:10.1093/carcin/bgi357 (2006).

203   Huang, N. N., Wang, D. J. & Heppel, L. A. Stimulation of aged human lung fibroblasts by extracellular ATP via suppression of arachidonate metabolism. *J Biol Chem* **268**, 10789-10795 (1993).

204   Beharka, A. A., Wu, D., Han, S. N. & Meydani, S. N. Macrophage prostaglandin production contributes to the age-associated decrease in T cell function which is reversed by the dietary antioxidant vitamin E. *Mech Ageing Dev* **93**, 59-77 (1997).

205   Chou, J. P., Ramirez, C. M., Ryba, D. M., Koduri, M. P. & Effros, R. B. Prostaglandin E2 promotes features of replicative senescence in chronically activated human CD8+ T cells. *PLoS One* **9**, e99432, doi:10.1371/journal.pone.0099432 (2014).

206   Chen, H. *et al.* Elevated COX2 expression and PGE2 production by downregulation of RXRalpha in senescent macrophages. *Biochem Biophys Res Commun* **440**, 157-162, doi:10.1016/j.bbrc.2013.09.047 (2013).

207   Hayek, M. G. *et al.* Enhanced expression of inducible cyclooxygenase with age in murine macrophages. *J Immunol* **159**, 2445-2451 (1997).

208   Claycombe, K. J. *et al.* Ceramide mediates age-associated increase in macrophage cyclooxygenase-2 expression. *J Biol Chem* **277**, 30784-30791, doi:10.1074/jbc.M204463200 (2002).

209   Wu, D. & Meydani, S. N. Mechanism of age-associated up-regulation in macrophage PGE2 synthesis. *Brain Behav Immun* **18**, 487-494, doi:10.1016/j.bbi.2004.05.003 (2004).

210   Demaria, M. *et al.* An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. *Dev Cell* **31**, 722-733 (2014).

211   Yun, M. H., Davaapil, H. & Brockes, J. P. Recurrent turnover of senescent cells during regeneration of a complex structure. *Elife* **4**, doi:10.7554/eLife.05505 (2015).

212 Hall, B. M. *et al.* p16(Ink4a) and senescence-associated beta-galactosidase can be induced in macrophages as part of a reversible response to physiological stimuli. *Aging (Albany NY)* **9**, 1867-1884, doi:10.18632/aging.101268 (2017).

213 Mebius, R. E. & Kraal, G. Structure and function of the spleen. *Nature reviews. Immunology* **5**, 606-616, doi:10.1038/nri1669 (2005).

214 Steiniger, B. S. Human spleen microanatomy: why mice do not suffice. *Immunology* **145**, 334-346, doi:10.1111/imm.12469 (2015).

215 Cesta, M. F. Normal structure, function, and histology of the spleen. *Toxicol Pathol* **34**, 455-465, doi:10.1080/01926230600867743 (2006).

216 Bronte, V. & Pittet, M. J. The spleen in local and systemic regulation of immunity. *Immunity* **39**, 806-818, doi:10.1016/j.immuni.2013.10.010 (2013).

217 Steiniger, B., Timphus, E. M. & Barth, P. J. The splenic marginal zone in humans and rodents: an enigmatic compartment and its inhabitants. *Histochem Cell Biol* **126**, 641-648, doi:10.1007/s00418-006-0210-5 (2006).

218 Arcaini, L., Rossi, D. & Paulli, M. Splenic marginal zone lymphoma: from genetics to management. *Blood* **127**, 2072-2081, doi:10.1182/blood-2015-11-624312 (2016).

219 Kraal, G. & Mebius, R. New insights into the cell biology of the marginal zone of the spleen. *International review of cytology* **250**, 175-215, doi:10.1016/S0074-7696(06)50005-1 (2006).

220 Snook, T. Studies on the Perifollicular Region of the Rat's Spleen. *The Anatomical record* **148**, 149-159 (1964).

221 Veninga, H. *et al.* Antigen targeting reveals splenic CD169+ macrophages as promoters of germinal center B-cell responses. *European journal of immunology* **45**, 747-757, doi:10.1002/eji.201444983 (2015).

222 Del Portillo, H. A. *et al.* The role of the spleen in malaria. *Cell Microbiol* **14**, 343-355, doi:10.1111/j.1462-5822.2011.01741.x (2012).

223 Hey, Y. Y. & O'Neill, H. C. Murine spleen contains a diversity of myeloid and dendritic cells distinct in antigen presenting function. *J Cell Mol Med* **16**, 2611-2619, doi:10.1111/j.1582-4934.2012.01608.x (2012).

224   Katakai, T. Marginal reticular cells: a stromal subset directly descended from the lymphoid tissue organizer. *Frontiers in immunology* **3**, 200, doi:10.3389/fimmu.2012.00200 (2012).

225   Cerutti, A., Cols, M. & Puga, I. Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. *Nat Rev Immunol* **13**, 118-132, doi:10.1038/nri3383 (2013).

226   Luther, S. A., Tang, H. L., Hyman, P. L., Farr, A. G. & Cyster, J. G. Coexpression of the chemokines ELC and SLC by T zone stromal cells and deletion of the ELC gene in the plt/plt mouse. *Proc Natl Acad Sci U S A* **97**, 12694-12699, doi:10.1073/pnas.97.23.12694 (2000).

227   Masters, A. R., Haynes, L., Su, D. M. & Palmer, D. B. Immune senescence: significance of the stromal microenvironment. *Clinical and experimental immunology* **187**, 6-15, doi:10.1111/cei.12851 (2017).

228   Cremasco, V. *et al.* B cell homeostasis and follicle confines are governed by fibroblastic reticular cells. *Nature immunology* **15**, 973-981, doi:10.1038/ni.2965 (2014).

229   Wang, X. *et al.* Follicular dendritic cells help establish follicle identity and promote B cell retention in germinal centers. *The Journal of experimental medicine* **208**, 2497-2510, doi:10.1084/jem.20111449 (2011).

230   Nolte, M. A. *et al.* A conduit system distributes chemokines and small blood-borne molecules through the splenic white pulp. *The Journal of experimental medicine* **198**, 505-512, doi:10.1084/jem.20021801 (2003).

231   Austyn, J. M. & Gordon, S. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur J Immunol* **11**, 805-815, doi:10.1002/eji.1830111013 (1981).

232   Linehan, E. *et al.* Aging impairs peritoneal but not bone marrow-derived macrophage phagocytosis. *Aging Cell* **13**, 699-708, doi:10.1111/acel.12223 (2014).

233   Aw, D., Silva, A. B. & Palmer, D. B. Immunosenescence: emerging challenges for an ageing population. *Immunology* **120**, 435-446, doi:10.1111/j.1365-2567.2007.02555.x (2007).

234   Richner, J. M. *et al.* Age-Dependent Cell Trafficking Defects in Draining Lymph Nodes Impair Adaptive Immunity and Control of West Nile Virus Infection. *PLoS Pathog* **11**, e1005027, doi:10.1371/journal.ppat.1005027 (2015).

235   Turner, V. M. & Mabbott, N. A. Influence of ageing on the microarchitecture of the spleen and lymph nodes. *Biogerontology* **18**, 723-738, doi:10.1007/s10522-017-9707-7 (2017).

236   Aw, D. *et al.* Disorganization of the splenic microanatomy in ageing mice. *Immunology* **148**, 92-101, doi:10.1111/imm.12590 (2016).

237   Turner, V. M. & Mabbott, N. A. Influence of ageing on the microarchitecture of the spleen and lymph nodes. *Biogerontology*, doi:10.1007/s10522-017-9707-7 (2017).

238   Turner, V. M. & Mabbott, N. A. Ageing adversely affects the migration and function of marginal zone B cells. *Immunology* **151**, 349-362, doi:10.1111/imm.12737 (2017).

239   Birjandi, S. Z., Ippolito, J. A., Ramadorai, A. K. & Witte, P. L. Alterations in marginal zone macrophages and marginal zone B cells in old mice. *J Immunol* **186**, 3441-3451, doi:10.4049/jimmunol.1001271 (2011).

240   Brown, K. L., Gossner, A., Mok, S. & Mabbott, N. A. The effects of host age on the transport of complement-bound complexes to the spleen and the pathogenesis of intravenous scrapie infection. *J Virol* **86**, 25-35, doi:10.1128/JVI.05581-11 (2012).

241   Brown, K. L. & Mabbott, N. A. Evidence of subclinical prion disease in aged mice following exposure to bovine spongiform encephalopathy. *J Gen Virol* **95**, 231-243, doi:10.1099/vir.0.058958-0 (2014).

242   Lefebvre, J. S. *et al.* The aged microenvironment contributes to the age-related functional defects of CD4 T cells in mice. *Aging Cell* **11**, 732-740, doi:10.1111/j.1474-9726.2012.00836.x (2012).

243   Aydar, Y., Balogh, P., Tew, J. G. & Szakal, A. K. Age-related depression of FDC accessory functions and CD21 ligand-mediated repair of co-stimulation. *Eur J*

Immunol **32**, 2817-2826, doi:10.1002/1521-4141(2002010)32:10<2817::AID-IMMU2817>3.0.CO;2-Z (2002).

244   Lever, M., Maini, P. K., van der Merwe, P. A. & Dushek, O. Phenotypic models of T cell activation. *Nat Rev Immunol* **14**, 619-629, doi:10.1038/nri3728 (2014).

245   Mahoney, K. M., Rennert, P. D. & Freeman, G. J. Combination cancer immunotherapy and new immunomodulatory targets. *Nat Rev Drug Discov* **14**, 561-584, doi:10.1038/nrd4591 (2015).

246   Salam, N. *et al.* T cell ageing: effects of age on development, survival & function. *The Indian journal of medical research* **138**, 595-608 (2013).

247   Voehringer, D., Koschella, M. & Pircher, H. Lack of proliferative capacity of human effector and memory T cells expressing killer cell lectinlike receptor G1 (KLRG1). *Blood* **100**, 3698-3702, doi:10.1182/blood-2002-02-0657 (2002).

248   Goronzy, J. J., Li, G., Yu, M. & Weyand, C. M. Signaling pathways in aged T cells - a reflection of T cell differentiation, cell senescence and host environment. *Semin Immunol* **24**, 365-372, doi:10.1016/j.smim.2012.04.003 (2012).

249   Lages, C. S., Lewkowich, I., Sproles, A., Wills-Karp, M. & Chougnet, C. Partial restoration of T-cell function in aged mice by in vitro blockade of the PD-1/ PD-L1 pathway. *Aging Cell* **9**, 785-798, doi:10.1111/j.1474-9726.2010.00611.x (2010).

250   Tu, W. & Rao, S. Mechanisms Underlying T Cell Immunosenescence: Aging and Cytomegalovirus Infection. *Front Microbiol* **7**, 2111, doi:10.3389/fmicb.2016.02111 (2016).

251   Abedin, S., Michel, J. J., Lemster, B. & Vallejo, A. N. Diversity of NKR expression in aging T cells and in T cells of the aged: the new frontier into the exploration of protective immunity in the elderly. *Exp Gerontol* **40**, 537-548, doi:10.1016/j.exger.2005.04.012 (2005).

252   Hermes, M., Albrecht, C., Schrod, A., Brameier, M. & Walter, L. Expression patterns of killer cell immunoglobulin-like receptors (KIR) of NK-cell and T-cell subsets in Old World monkeys. *PLoS One* **8**, e64936, doi:10.1371/journal.pone.0064936 (2013).

253 Swift, M. E., Kleinman, H. K. & DiPietro, L. A. Impaired wound repair and delayed angiogenesis in aged mice. *Lab Invest* **79**, 1479-1487 (1999).

254 Solana, R. *et al.* Innate immunosenescence: effect of aging on cells and receptors of the innate immune system in humans. *Semin Immunol* **24**, 331-341, doi:10.1016/j.smim.2012.04.008 (2012).

255 Hearps, A. C. *et al.* Aging is associated with chronic innate immune activation and dysregulation of monocyte phenotype and function. *Aging Cell* **11**, 867-875, doi:10.1111/j.1474-9726.2012.00851.x (2012).

256 Coates, P. J., Rundle, J. K., Lorimore, S. A. & Wright, E. G. Indirect macrophage responses to ionizing radiation: implications for genotype-dependent bystander signaling. *Cancer Res* **68**, 450-456, doi:10.1158/0008-5472.CAN-07-3050 (2008).

257 Frasca, D. & Blomberg, B. B. Inflammaging decreases adaptive and innate immune responses in mice and humans. *Biogerontology* **17**, 7-19, doi:10.1007/s10522-015-9578-8 (2016).

258 Takeuchi, O. & Akira, S. Pattern recognition receptors and inflammation. *Cell* **140**, 805-820, doi:10.1016/j.cell.2010.01.022 (2010).

259 Renshaw, M. *et al.* Cutting edge: impaired Toll-like receptor expression and function in aging. *J Immunol* **169**, 4697-4701 (2002).

260 Boehmer, E. D., Meehan, M. J., Cutro, B. T. & Kovacs, E. J. Aging negatively skews macrophage TLR2- and TLR4-mediated pro-inflammatory responses without affecting the IL-2-stimulated pathway. *Mech Ageing Dev* **126**, 1305-1313, doi:10.1016/j.mad.2005.07.009 (2005).

261 Gomez, C. R., Goral, J., Ramirez, L., Kopf, M. & Kovacs, E. J. Aberrant acute-phase response in aged interleukin-6 knockout mice. *Shock* **25**, 581-585, doi:10.1097/01.shk.000029553.39081.ec (2006).

262 Linton, P. J. & Dorshkind, K. Age-related changes in lymphocyte development and function. *Nature immunology* **5**, 133-139, doi:10.1038/ni1033 (2004).

263 Wu, D. *et al.* Age-associated increase in PGE2 synthesis and COX activity in murine macrophages is reversed by vitamin E. *Am J Physiol* **275**, C661-668 (1998).

264 Franceschi, C. *et al.* Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann N Y Acad Sci* **908**, 244-254 (2000).

265 Cudejko, C. *et al.* p16INK4a deficiency promotes IL-4-induced polarization and inhibits proinflammatory signaling in macrophages. *Blood* **118**, 2556-2566, doi:10.1182/blood-2010-10-313106 (2011).

266 van Duin, D. *et al.* Prevaccine determination of the expression of costimulatory B7 molecules in activated monocytes predicts influenza vaccine responses in young and older adults. *J Infect Dis* **195**, 1590-1597, doi:10.1086/516788 (2007).

267 Weiskopf, D., Weinberger, B. & Grubeck-Loebenstein, B. The aging of the immune system. *Transpl Int* **22**, 1041-1050, doi:10.1111/j.1432-2277.2009.00927.x (2009).

268 He, S. & Sharpless, N. E. Senescence in Health and Disease. *Cell* **169**, 1000-1011, doi:10.1016/j.cell.2017.05.015 (2017).

269 He, S. *et al.* Transient CDK4/6 inhibition protects hematopoietic stem cells from chemotherapy-induced exhaustion. *Science translational medicine* **9**, doi:10.1126/scitranslmed.aal3986 (2017).

270 Chen, Z. *et al.* Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* **436**, 725-730, doi:10.1038/nature03918 (2005).

271 Cosme-Blanco, W. *et al.* Telomere dysfunction suppresses spontaneous tumorigenesis in vivo by initiating p53-dependent cellular senescence. *EMBO reports* **8**, 497-503, doi:10.1038/sj.embor.7400937 (2007).

272 Feldser, D. M. & Greider, C. W. Short telomeres limit tumor progression in vivo by inducing senescence. *Cancer Cell* **11**, 461-469, doi:10.1016/j.ccr.2007.02.026 (2007).

273 Oubaha, M. *et al.* Senescence-associated secretory phenotype contributes to pathological angiogenesis in retinopathy. *Science translational medicine* **8**, 362ra144, doi:10.1126/scitranslmed.aaf9440 (2016).

274 Coppe, J. P., Kauser, K., Campisi, J. & Beausejour, C. M. Secretion of vascular endothelial growth factor by primary human fibroblasts at senescence. *J Biol Chem* **281**, 29568-29574, doi:10.1074/jbc.M603307200 (2006).

275   Krtolica, A., Parrinello, S., Lockett, S., Desprez, P. Y. & Campisi, J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc Natl Acad Sci U S A* **98**, 12072-12077, doi:10.1073/pnas.211053698 (2001).

276   Demaria, M. *et al.* Cellular Senescence Promotes Adverse Effects of Chemotherapy and Cancer Relapse. *Cancer Discov* **7**, 165-176, doi:10.1158/2159-8290.CD-16-0241 (2017).

277   Liu, Y. *et al.* Expression of p16(INK4a) prevents cancer and promotes aging in lymphocytes. *Blood* **117**, 3257-3267, doi:10.1182/blood-2010-09-304402 (2011).

278   Haynes, L. & Swain, S. L. Aged-related shifts in T cell homeostasis lead to intrinsic T cell defects. *Semin Immunol* **24**, 350-355, doi:10.1016/j.smim.2012.04.001 (2012).

279   Kogut, I., Scholz, J. L., Cancro, M. P. & Cambier, J. C. B cell maintenance and function in aging. *Semin Immunol* **24**, 342-349, doi:10.1016/j.smim.2012.04.004 (2012).

280   Nikolich-Zugich, J. Aging of the T cell compartment in mice and humans: from no naive expectations to foggy memories. *J Immunol* **193**, 2622-2629, doi:10.4049/jimmunol.1401174 (2014).

281   You, J., Dong, H., Mann, E. R., Knight, S. C. & Yaqoob, P. Probiotic modulation of dendritic cell function is influenced by ageing. *Immunobiology* **219**, 138-148, doi:10.1016/j.imbio.2013.08.012 (2014).

282   Jiang, S. & Dong, C. A complex issue on CD4(+) T-cell subsets. *Immunological reviews* **252**, 5-11, doi:10.1111/imr.12041 (2013).

283   Xin, L., Jiang, T. T., Kinder, J. M., Ertelt, J. M. & Way, S. S. Infection susceptibility and immune senescence with advancing age replicated in accelerated aging Lmna(Dhe) mice. *Aging Cell* **14**, 1122-1126, doi:10.1111/acel.12385 (2015).

284   Pugh, J. L. *et al.* Acute systemic DNA damage in youth does not impair immune defense with aging. *Aging Cell* **15**, 686-693, doi:10.1111/acel.12478 (2016).

285 Palacio, L., Krishnan, V., Le, N. L., Sharpless, N. E. & Beausejour, C. M. Sustained p16INK4a expression is required to prevent IR-induced tumorigenesis in mice. *Oncogene* **36**, 1309-1314, doi:10.1038/onc.2016.298 (2017).

286 Ariffin, H. *et al.* Young adult survivors of childhood acute lymphoblastic leukemia show evidence of chronic inflammation and cellular aging. *Cancer* **123**, 4207-4214, doi:10.1002/cncr.30857 (2017).

287 Shaw, A. C., Joshi, S., Greenwood, H., Panda, A. & Lord, J. M. Aging of the innate immune system. *Curr Opin Immunol* **22**, 507-513, doi:10.1016/j.coi.2010.05.003 (2010).

288 Simone, R., Zicca, A. & Saverino, D. The frequency of regulatory CD3+CD8+CD28- CD25+ T lymphocytes in human peripheral blood increases with age. *J Leukoc Biol* **84**, 1454-1461, doi:10.1189/jlb.0907627 (2008).

289 Sharma, S., Dominguez, A. L. & Lustgarten, J. High accumulation of T regulatory cells prevents the activation of immune responses in aged animals. *J Immunol* **177**, 8348-8355 (2006).

290 Baar, M. P. *et al.* Targeted Apoptosis of Senescent Cells Restores Tissue Homeostasis in Response to Chemotoxicity and Aging. *Cell* **169**, 132-147 e116, doi:10.1016/j.cell.2017.02.031 (2017).

291 De Luca, S. *et al.* Accuracy of cut-off value by measurement of third molar index: Study of a Colombian sample. *Forensic science international* **261**, 160 e161-165, doi:10.1016/j.forsciint.2016.01.026 (2016).

292 Wang, Y., Schulte, B. A., LaRue, A. C., Ogawa, M. & Zhou, D. Total body irradiation selectively induces murine hematopoietic stem cell senescence. *Blood* **107**, 358-366 (2006).

293 Marcoux, S. *et al.* Expression of the senescence marker p16INK4a in skin biopsies of acute lymphoblastic leukemia survivors: a pilot study. *Radiat Oncol* **8**, 252 (2013).

294 Sanoff, H. K. *et al.* Effect of cytotoxic chemotherapy on markers of molecular age in patients with breast cancer. *J Natl Cancer Inst* **106**, dju057 (2014).

295 Salama, R., Sadaie, M., Hoare, M. & Narita, M. Cellular senescence and its effector programs. *Genes Dev* **28**, 99-114 (2014).

296 Burd, C. E. *et al.* Monitoring tumorigenesis and senescence in vivo with a p16(INK4a)-luciferase model. *Cell* **152**, 340-351 (2013).

297 Yamakoshi, K. *et al.* Real-time in vivo imaging of p16Ink4a reveals cross talk with p53. *J Cell Biol* **186**, 393-407 (2009).

298 Sharpless, N. E. *et al.* Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. *Nature* **413**, 86-91 (2001).

299 Sherr, C. J. & Roberts, J. M. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* **13**, 1501-1512 (1999).

300 Zhou, B. B. & Elledge, S. J. The DNA damage response: putting checkpoints in perspective. *Nature* **408**, 433-439 (2000).

301 Robles, S. J. & Adami, G. R. Agents that cause DNA double strand breaks lead to p16INK4a enrichment and the premature senescence of normal fibroblasts. *Oncogene* **16**, 1113-1123 (1998).

302 Stein, G. H., Drullinger, L. F., Soulard, A. & Dulic, V. Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. *Mol Cell Biol* **19**, 2109-2117 (1999).

303 Johmura, Y. *et al.* Necessary and sufficient role for a mitosis skip in senescence induction. *Mol Cell* **55**, 73-84 (2014).

304 Beausejour, C. M. *et al.* Reversal of human cellular senescence: roles of the p53 and p16 pathways. *Embo J* **22**, 4212-4222 (2003).

305 Ito, K. *et al.* Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. *Nature* **431**, 997-1002 (2004).

306 Ito, K. *et al.* Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. *Nat Med* **12**, 446-451 (2006).

307 Janzen, V. *et al.* Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature* **443**, 421-426 (2006).

308 Krishnamurthy, J. *et al.* p16INK4a induces an age-dependent decline in islet regenerative potential. *Nature* **443**, 453-457 (2006).

309   Molofsky, A. V. *et al.* Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing. *Nature* **443**, 448-452 (2006).

310   Sousa-Victor, P. *et al.* Geriatric muscle stem cells switch reversible quiescence into senescence. *Nature* **506**, 316-321 (2014).

311   van Deursen, J. M. The role of senescent cells in ageing. *Nature* **509**, 439-446 (2014).

312   Christophorou, M. A., Ringshausen, I., Finch, A. J., Swigart, L. B. & Evan, G. I. The pathological response to DNA damage does not contribute to p53-mediated tumour suppression. *Nature* **443**, 214-217 (2006).

313   Ruzankina, Y. *et al.* Deletion of the developmentally essential gene ATR in adult mice leads to age-related phenotypes and stem cell loss. *Cell Stem Cell* **1**, 113-126 (2007).

314   Krtolica, A., Parrinello, S., Lockett, S., Desprez, P. Y. & Campisi, J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc Natl Acad Sci USA* **98**, 12072-12077 (2001).

315   Baker, D. J. *et al.* Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* **479**, 232-236 (2011).

316   Wood, W. A. *et al.* Chemotherapy and Stem Cell Transplantation Increase p16INK4a Expression, a Biomarker of T-cell Aging. *EBioMedicine* **11**, 227-238, doi:10.1016/j.ebiom.2016.08.029 (2016).

317   Meng, A., Wang, Y., Van Zant, G. & Zhou, D. Ionizing radiation and busulfan induce premature senescence in murine bone marrow hematopoietic cells. *Cancer Res* **63**, 5414-5419 (2003).

318   Lichterfeld, M. *et al.* Shelterin dysfunction and p16(INK4a)-mediated growth inhibition in HIV-1-specific CD8 T cells. *J Virol* **86**, 5533-5540, doi:10.1128/JVI.00196-12 (2012).

319   Migliaccio, M., Raj, K., Menzel, O. & Rufer, N. Mechanisms that limit the in vitro proliferative potential of human CD8+ T lymphocytes. *J Immunol* **174**, 3335-3343 (2005).

320 Rosko, A. *et al.* Autologous hematopoietic stem cell transplant induces the molecular aging of T-cells in multiple myeloma. *Bone Marrow Transplant* **50**, 1379-1381, doi:10.1038/bmt.2015.143 (2015).

321 Pathai, S. *et al.* Accelerated biological ageing in HIV-infected individuals in South Africa: a case-control study. *AIDS* **27**, 2375-2384, doi:10.1097/QAD.0b013e328363bf7f (2013).

322 Zhou, Y. *et al.* Ionizing radiation selectively reduces skin regulatory T cells and alters immune function. *PLoS One* **9**, e100800, doi:10.1371/journal.pone.0100800 (2014).

323 William T.Shearer, S. Z., James M. Reuben, Bang-Ning Lee, Janet S.Butel Predictors of immune function in space flight. *Acta Astronautica* **60**, 247-253 (2007).

324 Barach, Y. S., Lee, J. S. & Zang, X. T cell coinhibition in prostate cancer: new immune evasion pathways and emerging therapeutics. *Trends Mol Med* **17**, 47-55, doi:10.1016/j.molmed.2010.09.006 (2011).

325 Sene, A. *et al.* Impaired cholesterol efflux in senescent macrophages promotes age-related macular degeneration. *Cell Metab* **17**, 549-561, doi:10.1016/j.cmet.2013.03.009 (2013).

326 Swift, M. E., Burns, A. L., Gray, K. L. & DiPietro, L. A. Age-related alterations in the inflammatory response to dermal injury. *J Invest Dermatol* **117**, 1027-1035, doi:10.1046/j.0022-202x.2001.01539.x (2001).

327 Popi, A. F., Lopes, J. D. & Mariano, M. Interleukin-10 secreted by B-1 cells modulates the phagocytic activity of murine macrophages in vitro. *Immunology* **113**, 348-354, doi:10.1111/j.1365-2567.2004.01969.x (2004).

328 Kim, O. H. *et al.* Impaired phagocytosis of apoptotic cells causes accumulation of bone marrow-derived macrophages in aged mice. *BMB reports* **50**, 43-48 (2017).

329 Butcher, S. K. *et al.* Senescence in innate immune responses: reduced neutrophil phagocytic capacity and CD16 expression in elderly humans. *J Leukoc Biol* **70**, 881-886 (2001).

330   Fletcher, A. L., Acton, S. E. & Knoblich, K. Lymph node fibroblastic reticular cells in health and disease. *Nat Rev Immunol* **15**, 350-361, doi:10.1038/nri3846 (2015).

331   Chang, J. E. & Turley, S. J. Stromal infrastructure of the lymph node and coordination of immunity. *Trends in immunology* **36**, 30-39, doi:10.1016/j.it.2014.11.003 (2015).

332   Al'fonsova, E. V. [Functional morphology of conjunctive tissue stroma of spleen in the age aspect]. *Advances in gerontology = Uspekhi gerontologii* **25**, 415-421 (2012).

333   Rodrigues, C. J., Sacchetti, J. C. & Rodrigues, A. J., Jr. Age-related changes in the elastic fiber network of the human splenic capsule. *Lymphology* **32**, 64-69 (1999).

334   Park, J. *et al.* Splenic stromal cells from aged mice produce higher levels of IL-6 compared to young mice. *Mediators Inflamm* **2014**, 826987, doi:10.1155/2014/826987 (2014).

335   Liu, G., Park, Y. J., Tsuruta, Y., Lorne, E. & Abraham, E. p53 Attenuates lipopolysaccharide-induced NF-kappaB activation and acute lung injury. *J Immunol* **182**, 5063-5071, doi:10.4049/jimmunol.0803526 (2009).

336   Haynes, L. & Maue, A. C. Effects of aging on T cell function. *Curr Opin Immunol* **21**, 414-417, doi:10.1016/j.coi.2009.05.009 (2009).

337   Sorrentino, J. A. *et al.* p16INK4a reporter mice reveal age-promoting effects of environmental toxicants. *J Clin Invest* **124**, 169-173, doi:10.1172/JCI70960 (2014).

338   Sanoff, H. K. *et al.* Effect of cytotoxic chemotherapy on markers of molecular age in patients with breast cancer. *J Natl Cancer Inst* **106**, dju057, doi:10.1093/jnci/dju057 (2014).

339   Krimpenfort, P. & Berns, A. Rejuvenation by Therapeutic Elimination of Senescent Cells. *Cell* **169**, 3-5, doi:10.1016/j.cell.2017.03.014 (2017).

340   Gridley, D. S., Pecaut, M. J. & Nelson, G. A. Total-body irradiation with high-LET particles: acute and chronic effects on the immune system. *Am J Physiol Regul Integr Comp Physiol* **282**, R677-688, doi:10.1152/ajpregu.00435.2001 (2002).

341   Kim, W. Y. & Sharpless, N. E. The regulation of INK4/ARF in cancer and aging. *Cell* **127**, 265-275, doi:10.1016/j.cell.2006.10.003 (2006).

342   Ribeiro, S. P. *et al.* p16INK4a Expression and Immunologic Aging in Chronic HIV Infection. *PLoS One* **11**, e0166759, doi:10.1371/journal.pone.0166759 (2016).

343   Nelson, J. A. *et al.* Expression of p16(INK4a) as a biomarker of T-cell aging in HIV-infected patients prior to and during antiretroviral therapy. *Aging Cell* **11**, 916-918, doi:10.1111/j.1474-9726.2012.00856.x (2012).

344   Migliaccio, M., Alves, P. M., Romero, P. & Rufer, N. Distinct mechanisms control human naive and antigen-experienced CD8+ T lymphocyte proliferation. *J Immunol* **176**, 2173-2182 (2006).

345   Fuentes, L. *et al.* Downregulation of the tumour suppressor p16INK4A contributes to the polarisation of human macrophages toward an adipose tissue macrophage (ATM)-like phenotype. *Diabetologia* **54**, 3150-3156, doi:10.1007/s00125-011-2324-0 (2011).

346   Zhu, Y. *et al.* Identification of a novel senolytic agent, navitoclax, targeting the Bcl-2 family of anti-apoptotic factors. *Aging cell* **15**, 428-435, doi:10.1111/acel.12445 (2016).

347   Yosef, R. *et al.* Directed elimination of senescent cells by inhibition of BCL-W and BCL-XL. *Nature communications* **7**, 11190, doi:10.1038/ncomms11190 (2016).