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Senescence surveillance: the interplay between the immune system and senescent cells

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I, Branca Isabel Pereira confirm that the work presented in this thesis is original, except where information has been derived from other sources indicated by references in the text.

Signed:

SUMMARY

Aging is the single common risk factor for a variety of diseases including Alzheimer´s, heart disease and cancer. Cellular senescence and the accumulation of senescent cells with age are considered the underlying causes of aging. Due to their potential involvement in age-related diseases, eliminating senescent cells and attenuating the senescence-associated secretory phenotype (SASP) have recently emerged as attractive strategies aiming to reverse or delay the onset of such diseases. Although recent studies indicate that senescent cells may be targeted by the immune system, they are unable to explain why senescent cells accumulate with age and at sites of age-related pathologies. Immune cells also exhibit altered functions with age and it has been suggested that the accumulation of senescent cells might be due to a defective immune surveillance with age. Nevertheless, to our knowledge, no studies have been done to address how immunosenescence affects the surveillance of senescent cells, mainly because these two subjects are often discussed independently.

The overall goal of this work was to investigate the interplay between the immune system and senescent cells. Using an autologous *in vitro* system with skin-explanted fibroblasts and human peripheral lymphocytes isolated from the same donors, the interactions between immune cells and fibroblasts induced to senescence could be investigated. A novel mechanism of immune evasion was identified and dependent on the expression of HLA-E by senescent cells, a non-classical MHC molecule that protects senescent cells from immune clearance via the interaction with the inhibitory Natural Killer (NK) receptor NKG2A, expressed by NK and cytotoxic T cells. Using a monoclonal antibody to block the interaction of HLA-E with NKG2A *in vitro*, we could successfully enhance the clearance of senescent cells by the immune system.

Mechanistically, it was demonstrated that HLA-E expression on senescent cells is regulated by p38MAPK and induced after exposure to inflammatory cytokines, such as IL-6.

Interestingly, it was found that NK cell receptors such as NKG2A and NKG2D were predominantly expressed by a subset of CD8+ T cells with a highly differentiated phenotype, which are expanded with age. To investigate the biological significance of the expression of NK cell receptors by differentiated CD8+ T cells, functional and transcriptomic studies were performed. It was found that highly differentiated CD8+ T cells acquire phenotypic and functional properties resembling NK cells, which are evident at the transcriptomic level by an overexpression of genes involved in NK cell receptor signaling and cytotoxic effector functions. These changes occur in parallel to a repression of genes involved in TCR signaling and TCR-dependent functions, such as proliferation. Collectively, these observations point to a specific program of CD8 T cell differentiation, whereby cells acquire innate-like characteristics as means of maintaining effector functions despite TCR suppression. The mechanisms underlying the acquisition of such unconventional functions, mediated independently of the TCR were investigated revealing a crucial role for the stimulatory NK cell receptor NKG2D in the regulation of innate-like functions mediated by CD8+ T cells.

Overall, the results presented in this thesis contribute to a deeper understanding of the mechanisms underlying the recognition and elimination of senescent cells by the immune system. Moreover by studying the effects of terminal differentiation in the immune system capacity to fight against abnormal cells, the research here developed represent one step forward towards an integrative approach to studying aging.

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This work is dedicated to Maria and Francisco, my new family.

ABBREVIATIONS

4OHT - 4-Hydroxytamoxifen

AF467 - Alexa Fluor 647

APC - allophycocyanin

ATM - apical kinases ataxia telangiectasia mutated

ATR - ATM and RAD-3 related

Blimp-1 - B lymphocyte-induced maturation protein-1

BSA - Bovine Serum Albumin

C - Celsius

CBA - Cytometric Bead Array

CD - Cluster of differentiation

CDK - Cyclin dependent kinase

CDKI - Cyclin-dependent kinase inhibitor

CFSE - 5- (and 6-) carboxyfluorescein succinimidyl ester

CM - Central memory T lymphocyte

CMV – Cytomegaloviruspopulation doublings (cPD)

CTL – Cytotoxic T Lymphocyte

CTLA-4 - Cytotoxic T-lymphocyte-associated protein 4

DAPI - 4'-6-diamidino-2-phenylindole

DDR: DNA damage response

DAMPs - Danger Associated Molecular Patterns

DMEM - Dulbecco‘s modified Eagle medium

DMSO - Simethyl sulphoxide

DNA - Deoxyribonucleic acid

DSB - Double strand break

EBV - Epstein-Barr Virus

EM - Effector memory T lymphocyte

EMRA - Revertant effector memory T lymphocyte

Eomes – Eomesodermin

ER – Estrogen Receptor

FACS - Fluorescence-Activated Cell Sorting

FDR - False-discovery rate

FITC - Fluorescein isothiocyanate

FBS - Fetal bovine serum

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GM-CSF - Granulocyte-macrophage colony-stimulating factor

GROα - Growth regulated oncogene-α

H2AX - H2A histone family, member X

HBSS - Hanks balanced salt solution

HIV - Human immunodeficiency virus

HLA – Human Leucocyte Antigen

HMGB1 - High mobility group box 1 protein

HSV - Herpes simplex virus

hTERT - Human telomerase reverse transcriptase

HUVECs - Human Umbilical Vein Endothelial Cells

IFN - Interferon

Ig - Immunoglobulin

IL - Interleukin

ITAM - Immunoreceptor tyrosine-based activation motif

ITIM - Immunoreceptor tyrosine-based inhibitory motif

KDa - Kilodalton

KIR – Killer Immunoglobulin-like receptor

KLR – Killer cell lectin like receptors

KLRG1 - Killer cell lectin-like receptor subfamily G, member 1

LAT - Linker for T cell activation

LCK - Lymphocyte-specific protein tyrosine kinase

M - Molar (moles per L)

mAb – Monoclonal antibody

MAPK - mitogen-activated protein kinases

MCP1 - Monocyte chemoattractant protein-1

MFI – Mean Fluorescence Intensity

MHC - Major histocompability complex

MICA/B - MHC class I polypeptide-related sequence A and B

MIP – Macrophage inflammatory protein

MMPs - Matrix metalloproteinases

n – nano

NCR – Natural Cytotoxicity Receptor

NFAT - Nuclear factor of activated T-cells

NFκB - nuclear factor kappa-light-chain-enhancer of activated B cells

NKG2D – Natural killer group 2D

NK - Natural Killer

NKR - Natural Killer Receptor

NKT - Natural Killer T cell

PAMPs - Pathogen Associated Molecular Patterns

PBMCs - Peripheral blood mononuclear cells

PBS - Phosphate-buffered saline

PCR – Polymerase Chain Reaction

PD - Population doublings

PD-1 - Programmed death 1

PDL-1 - Programmed death ligand 1

PE - phycoerythrin

PerCP - peridinin-chlorophyll-protein complex

pH - potential hydrogen

PI3K - Phosphatidylinositol-4,5-bisphosphate 3-kinase

PLC-γ 1 - Phospholipase-γ 1

PRR - Pathogen recognition receptors

Rb - retinoblastoma protein

RIPA - Radioimmunoprecipitation

RNA - Ribonucleic acid

rpm - revolutions per minute

RPMI - Roswelli Park Memorial Institute

RT-PCR - Real Time Polymerase Chain Reaction

SASP - Senescence-associated secretory phenotype

SA-β-Gal - Senescence-associated β-Galactosidase

SEB - Staphylococcal Enterotoxin B

siRNA – Small interfering Ribonucleic acid

Syk - Spleen tyrosine kinase

T-bet - T-box expressed in T cells

TCR - T cell receptor

TGFβ - Transforming growth factor β

TNFα -Tumour necrosis factor α

ULBP - UL16-binding protein family

μ – micro

VZV - Varicella-Zoster virus

ZAP70 - Zeta-chain-associated protein kinase 70

LIST OF PUBLICATIONS

Pereira B, Ferreira MG. Sowing the seeds of cancer: telomeres and age-associated tumorigenesis. Curr Opin Oncol. 2013 Jan;25(1):93-8.

Pereira BI, Akbar AN. Convergence of Innate and Adaptive Immunity during Human Aging. Front Immunol. 2016 Nov; 7:445. Doi: 10.3389/fimmu.2016.00445

Pereira BI, Chambers E, Devine O, Patel N, Virasami A, Sebire NJ, Kinsler V, Campisi J, Akbar AN. Senescent cells evade the immune system through the expression of non-classical HLA-E molecules. (manuscript in final preparation).

Pereira BI, Lanna A, Riddell N, Teixeira VH, Janes SM, Henson S, Akbar AN. Reprogramming of senescent CD8+ T cells to innate-like T cells during human aging. (manuscript in final preparation).

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1 INTRODUCTION

1.1 CELLULAR SENESCENCE

Cellular senescence is an important tumour suppressive mechanism that long-lived complex organisms like humans had developed to stop incipient cancer cells from dividing (DeGregori, 2011). Hayflick and colleagues first described cellular senescence as the mechanism that limited the number of divisions a cell could undergo in culture, known as Hayflick limit (Hayflick, 1965). Greider and Harley subsequently demonstrated that the basis for this programmed biological clock was the gradual loss of DNA at the chromosome ends, known as telomeres, with each cell division (Harley et al., 1990). Some years before in 1984, Greider and Blackburn had discovered the enzyme responsible for extending DNA at the chromosome ends, maintaining telomere length after each cell division (Greider and Blackburn, 1985). This enzyme was called telomerase and they later received a Nobel Prize for this discovery. The role of telomeres and telomerase in determining the replicative capacity of a cell was finally confirmed when Shay and Wright demonstrated that senescence could be delayed in primary human fibroblasts if telomerase was reintroduced (Bodnar et al., 1998).

Telomeres are regions of repetitive DNA located at the end of chromosomes, composed of G-rich sequences (in mammals, TTAGGG repeats) (Severino et al., 2000) and associated with a specialized protein complex known as shelterin (Moyzis et al., 1988, Chong et al., 1995). This nucleoprotein complex is important to protect chromosome ends from being recognized as double strand breaks (DSBs), leading to replicative stress (de Lange, 2009). Human telomeres have between 5-15 kilobase pairs in length, however telomere length is variable between cells, tissues and individuals (Samassekou et al., 2010). The inability of DNA polymerases to fully replicate linear DNA molecules leads to a net loss of 100–200 base pairs of telomeric DNA with each cell division, known as the end-replication problem (Levy et al., 1992).

Telomerase is a specialized reverse transcriptase that adds TTAGGG repeats to the end of chromosomes to maintain telomere length, circumventing the end-replication problem (Greider and Blackburn, 1985). However, telomerase is normally only active in specific tissues, including germline and stem cells, as well as in human lymphocytes, being repressed from most other somatic tissues during development (Bodnar et al., 1996, Wright et al., 1996). In the absence of telomerase, telomeres will eventually become critically short triggering a DNA-damage response (DDR), which will induce cellular senescence once a critical telomere length is reached (O'Sullivan and Karlseder, 2010).

1.1.1 CAUSES AND MECHANISMS OF CELLULAR SENESCENCE

Although critical telomere shortening was first described as the cause of cellular senescence, known as replicative senescence (Goldstein, 1990), accumulating evidence indicates that senescence may be driven by other mechanisms independently of telomere shortening. Senescence that occurs before critical telomere shortening is reached is often designated as premature senescence. According to the underlying mechanism, senescence may be classified in telomere-dependent and telomere-independent senescence (Campisi and d'Adda di Fagagna, 2007). Nevertheless, the distinction between telomere-dependent and independent mechanisms may be artificial as recent studies indicate that telomeres may be the source of the senescence signal, irrespective of the cause that induces senescence. In fact, it has been demonstrated that telomeres represent sites of preferential accumulation of DNA-damage, most likely because telomeric DNA damage is irreparable, originating a persistent DNA-damage response that will induce senescence (Hewitt et al., 2012, Fumagalli et al., 2012).

1.1.1.1 TELOMERE-DEPENDENT SENESCENCE

Telomere-dependent senescence represents the forms of senescence associated with telomere shortening or dysfunction. As previously described, critical telomere shortening induces replicative senescence, which is a fundamental process occurring in every somatic cell, limiting the number of cells divisions that a cell can undergo (Campisi, 1997). In cells cultured *in vitro*, replicative senescence refers to the phase when cells stop dividing and reach a plateau in their growth curve – known as the "Hayflick limit (Hayflick and Moorhead, 1961) (**Figure 1.1**).

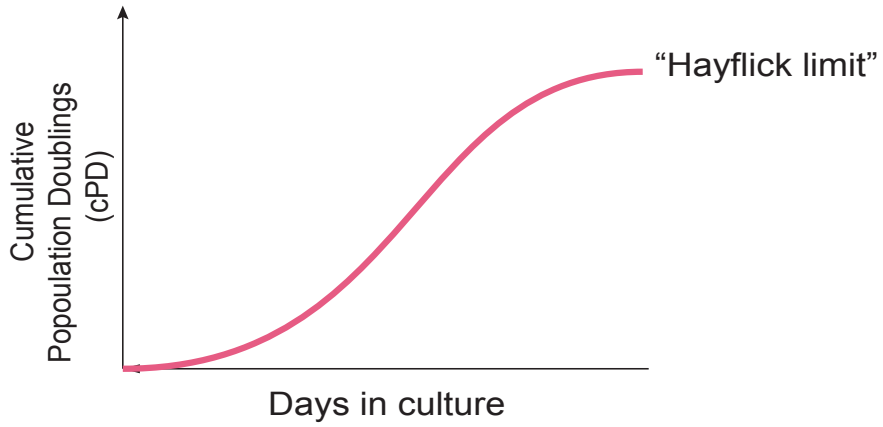

Figure 1.1 – Replicative Senescence.

Cells cultured *in vitro* have a finite proliferative potential: after a period of exponential growth, cells fail to expand and reach a plateau in the growth curve, referred to as the "Hayflick limit".

Not only telomere shortening but also telomere uncapping by the loss of shelterin proteins can induce cellular senescence, through telomere-dependent mechanisms (von Zglinicki et al., 2003). Dysfunctional or uncapped telomeres may lead to chromosome-end fusions and tetraploidization resulting in genomic instability that increases the risk of cancer (Murnane, 2012). As a protective mechanism, cells in risk of transformation activate a DNA-damage response cells and undergo cell death

or cellular senescence to prevent uncontrolled cell proliferation (d'Adda di Fagagna et al., 2003). This mechanism has thus a crucial role in maintaining genome integrity and preventing cancer progression, explaining why it has been evolutionary conserved (Gomes et al., 2011).

1.1.1.2 TELOMERE-INDEPENDENT SENESCENCE

Telomere-independent senescence refers to the forms of senescence that are not driven by telomere dysfunction. Several lines of evidence indicate that DNA damage, usually in the form of double strand breaks (DSBs), is the major cause of senescence (d'Adda di Fagagna, 2008). This may be induced by irradiation, oncogene activation, chromatin changes or oxidative damage.

1.1.1.2.1 IRRADIATION-INDUCED SENESCENCE

Exposure to ionizing radiation is a widely used method of inducing senescence as it originates double strand breaks leading to the activation of the DNA-damage response (DDR) (Bluwstein et al., 2013). Studies have demonstrated that the majority of the DDR foci induced after irradiation of human fibroblasts are efficiently repaired within 24 hours (Rodier et al., 2009, Bluwstein et al., 2013). However, the persistence of a few DDR foci is sufficient to induce a permanent activation of the DNA-damage response and senescence (Fumagalli et al., 2012).

1.1.1.2.2 ONCOGENE-INDUCED SENESCENCE

Oncogene-induced senescence (OIS) is a robust anti-proliferative response that follows oncogene activation and constitutes an efficient barrier to tumour progression (Mooi and Peeper, 2006). Aberrant activation of oncogenes usually results in a biphasic response, characterized by an initial burst of proliferation, followed by a proliferative arrest associated with the activation of the DDR and p53/p16 tumour

suppressor pathways (Bartkova et al., 2006). This response leads to permanent cell cycle arrest and enforces the establishment of cellular senescence.

While the relevance of this mechanism was at first questioned, oncogene-induced senescence has since then been proved to occur *in vivo.* The identification of cells exhibiting senescence markers in human and mouse premalignant lesions (such as human melanocytic nevi, benign prostate hyperplasia amongst other examples) but absent in the malignant counterparts provided strong evidence that oncogene-induced senescence is indeed an important tumour suppressive mechanism *in vivo* (Michaloglou et al., 2005, Braig et al., 2005, Collado et al., 2005, Bartkova et al., 2006, Suram et al., 2012).

1.1.1.2.3 STRESS-INDUCED SENESCENCE

In addition to genotoxic stress, other forms of cellular stress may induce senescence, such as oxidative stress, lack of growth factors or inadequate culture conditions (Ben-Porath and Weinberg, 2005, Campisi and d'Adda di Fagagna, 2007). Chronic signaling by anti-proliferative cytokines such as Interferon-α (Lanna et al., 2013) and β (Moiseeva et al., 2006) and transforming growth factor-β (Vijayachandra et al., 2003) have been shown to induce senescence. Similarly, reactive oxygen species (ROS) can induce direct DNA damage and accelerate telomere erosion, both of which contribute to activation of a DDR and senescence (von Zglinicki, 2002). Recent studies demonstrate that chronic low-grade inflammation might enhance telomere dysfunction by increasing ROS-mediated DNA damage (Jurk et al., 2014). Both ROS and pro-inflammatory cytokines, especially IL-6 and IL-8 are not only generated during senescence but also contribute to stabilize senescence in a positive feedback loop (Passos and Von Zglinicki, 2006, Correia-Melo et al., 2014). The role of ROS in senescence is supported by evidence that treatment with antioxidants delays or prevents senescence (Glade and Meguid, 2015).

1.1.2 PHASES OF THE SENESCENCE RESPONSE

Senescence is carefully regulated by different signaling pathways that act co-ordinately in distinct phases of the senescence program (**Figure 1.2**):

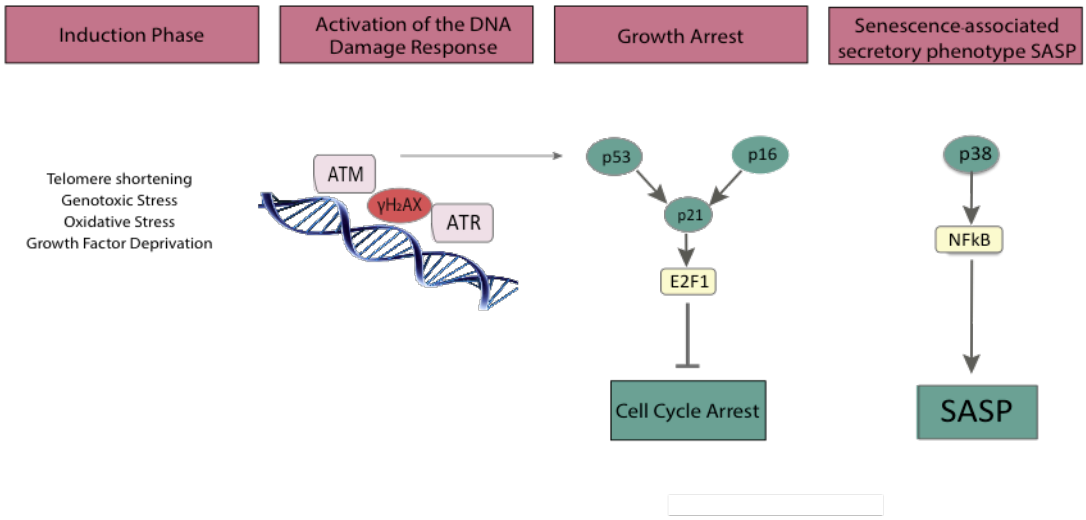

Figure 1.2 – Phases of the senescence response.

The induction phase follows a triggering event such as telomere shortening, DNA-damage, oxidative stress or growth factor deprivation (Akbar and Henson, 2011). The presence of DNA damage foci, marked by the phosphorylation of the histone γH2AX, activates ataxia-telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) signaling cascades, to attempt repair. During this decision period, the majority of the DNA damage foci is repaired within hours, however the persistence of a few irreparable foci is sufficient to determine the next phase: a permanent cell growth arrest, mediated by the activation of the p53 and the $p16^{INK4A}$ pathways (Ben-Porath and Weinberg, 2005, Campisi and d'Adda di Fagagna, 2007). A final phase initiated slowly over days after the initial insult is characterized by the activation of p38MAPK which in turn activates the transcription factor NF-κB leading to global changes in the transcriptome of the cell and to a characteristic secretory phenotype, known as senescence-associated secretory phenotype (SASP) (Freund et al., 2011).

1.1.3 SIGNALING PATHWAYS REGULATING SENESCENCE

1.1.3.1 The DNA damage response

The activation and persistence of a DNA damage response (DDR) is crucial for the commitment to senescence. This response consists of a cascade of events leading to the recruitment of specialized molecules to the sites of DNA damage to attempt repair. At the same time it induces cell cycle arrest (checkpoint function) until the DNA damage is fully repaired. This signaling cascade is orchestrated by two protein kinases, ataxia-telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR), which regulate the phosphorylation and function of downstream effectors, such as the checkpoint kinases Chk1 and 2 and phosphatases (Cdc25) and cyclin-dependent kinases (CDKs) which regulate cell cycle progression (d'Adda di Fagagna, 2008, Jackson and Bartek, 2009).

Phosphorylation of the histone γH2AX at the serine residues (Ser136 and Ser139) is one of the firsts events in this cascade, important for the recruitment of other DNA repair molecules to the sites of damage, in a positive feedback loop (Celeste et al., 2002). Evidence for the activation of the DNA damage response during senescence is demonstrated by the presence of nuclear foci containing phosphorylated γH2AX co-localized with 53-binding protein 1 (53BP1) and MRN-complex proteins (d'Adda di Fagagna et al., 2003, Takai et al., 2003, Herbig et al., 2004, Rodier et al., 2009).

Eventually, this response will converge on the activation of key tumour suppressor proteins, such as p53 and $p16^{INK4A}$ that will induce cell cycle arrest and determine the end fate of the cell: to senesce or to dye by apoptosis. (Ben-Porath and Weinberg, 2005).

1.1.3.2 p53/p21 and p16^{INK4A} tumour suppressor pathways

The tumour suppressor protein p53 has a primordial role in maintaining the genome integrity of a cell after genotoxic stress, being often regarded as the "guardian of the genome" (Lane, 1992). Activation of p53 (both by increased expression or by post-translational modifications) mediates cell-cycle arrest through the activation of p21, a cyclin-dependent kinases (CDK) inhibitor that prevents the phosphorylation and inactivation of the retinoblastoma protein pRB. Active pRB in turn suppresses E2F1 activity, a transcription factor that stimulates the expression of genes that are required for cell-cycle progression (**Figure 1.2**) (Levine, 1997, Sherr and McCormick, 2002).

Similarly, the tumour suppressor p16^{INK4A} is a CDK inhibitor that mediates growth arrest through the activation of pRB and E2F1 suppression (Sherr and Roberts, 1999). p16^{INK4A} is expressed at low levels in normal cells and it may be induced in response to oncogene expression (Serrano et al., 1997), exposure to radiation (Piepkorn, 2000, Ju et al., 2003), oxidative stress (Jenkins et al., 2011) and also to growth factor deprivation or sub-optimal culture conditions (Ramirez et al., 2001). Interestingly, a number of studies have shown that levels of p16^{INK4A} increase with age and its presence has been associated with a decline in tissue function, making it an appealing biomarker of aging (Krishnamurthy et al., 2004, Krishnamurthy et al., 2006, Ressler et al., 2006, Liu et al., 2009).

The contribution of each one of these pathways to senescence may depend on the cell type and probably on the nature of the stimuli that induces it. Whether these pathways act independently or in a redundant way is still not completely clear but it is accepted that there is a significant cross-talk between the two p53/p21 and p16^{INK4A}/Rb pathways, providing an extra layer of protection against tumour development (Yamakoshi et al., 2009).

1.1.3.3 p38MAPK signaling pathway

The senescence program is associated with the activation of yet another very important pathway, the p38 signaling pathway. p38 is a member of the mitogen-activated protein kinase (MAPK) family, which may be activated in response to inflammation, environmental stresses (radiation, oxidative stress, heat shock, ischemia) and also DNA damage (Chang and Karin, 2001). p38MAPK plays an important role in senescence, due to its ability to induce growth arrest, through the activation of both p53 and p16/pRb pathways (Wong et al., 2009). The role of p38MAPK in senescence has been supported by studies where inhibition of p38 activity could delay the senescence response caused by dysfunctional telomeres or reverse the premature senescence phenotype of fibroblasts isolated from patients with progeroid syndromes (Iwasa et al., 2003, Davis et al., 2005). Chronic activation of p38MAPK has been demonstrated in different forms of senescence and also in different type of cells suggesting that p38MAPK involvement in senescence is independent of the stimulus that causes it (Freund et al., 2011, Tivey et al., 2013, Xu et al., 2014). Chronic activation of p38MAPK signaling has been also demonstrated in senescent T cells and implicated in the induction of proliferative arrest and decreased telomerase activity, which could be reverted upon p38 inhibition (Di Mitri et al., 2011, Lanna et al., 2014).

In addition to this, activation of p38MAPK in senescence has been implicated in the regulation of an important feature of senescent cells, known as the senescence-associated secretory phenotype (SASP) (Freund et al., 2011).

1.1.4 SENESCENCE-ASSOCIATED SECRETORY PHENOTYPE (SASP)

When cells become senescent they suffer widespread changes in protein expression and secrete a pletora of factors, including pro-inflammatory cytokines, chemokines, growth factors and proteases. This secretory phenotype, termed the senescence-associated secretory phenotype (SASP) (Coppe et al., 2008) has important consequences to the tissue microenvironment.

A list of the most important SASP factors are summarized in **Table 1.1,** including cytokines such as IL-1, IL-6, IL-8 and TNFα; chemokines, such as the growth regulated oncogene-α and β (GROα, -β also known as CXCL-1, -2), the monocyte chemoattractant protein-1 and 2 (MCP-1, MCP-2) and the macrophage-inflammatory protein-1α (MIP-1α); growth factors, such as the insulin-like growth factor (IGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF); reactive oxygen species and matrix metalloproteinases, amongst others (Davalos et al., 2010). This list of secreted factors is not exhaustive and some factors may differ depending on the type and function of each cell. Nevertheless, the presence of a SASP is highly conserved between species (Coppe et al., 2010b) and occurs in a variety of cell types (Erusalimsky and Kurz, 2005, Salminen et al., 2011).

The SASP was initially thought to result from the persistent activation of the DNA damage response (Rodier et al., 2009), though it was later demonstrated that the major regulator was p38MAPK, which was both necessary and sufficient for its development (Freund et al., 2011). In fact, inhibition of the activity of p38MAPK using chemical inhibitors or by RNA interference significantly reduced the levels of most secreted SASP factors.

SASP factors
Inflammatory cytokines:
IL-6, IL-8, IL-1, TNFα, IL-10, TGFβ
Chemokines:
Growth regulated oncogene-α and β (GROα, GROβ) Monocyte chemoattractant protein-1 and 2 (MCP-1, -2) Macrophage-inflammatory protein-1α (MIP-1α)
Growth Factors:
Granulocyte-macrophage colony-stimulating factor (GM-CSF) Insulin-like growth factor binding proteins and receptors (IGFBPs)
Proteases: Matrix metalloproteinases (MMP-1, MMP-3, MMP-10) Urokinase and tissue-type plasminogen activators (uPA, tPA)
Non-secreted factors:
Reactive oxygen species (ROS), Nitric oxide (NO)

Table 1.1 – Senescence-associated secretory phenotype (SASP) factors

Additionally, it was shown that p38MAPK regulates the SASP through the activation of the transcription factor NF-κB, which is responsible for the increased expression of many SASP genes (Freund et al., 2011). Other studies have demonstrated an important role of mammalian target of rapamycin (mTOR) signaling in regulating the secretome of senescent cells (Herranz et al., 2015, Laberge et al., 2015).

Senescent cells also undergo chromatin reorganization, which is crucial for modulating gene expression, including SASP genes. Recent studies identified epigenetic regulators of the SASP including high mobility group box 2 (HMGB2) (Aird et al., 2016) and bromodomain-containing protein 4 (BRD4)(Tasdemir et al., 2016).

The activity of SASP factors can modify the tissue microenvironment and affect neighbouring cells (Coppe et al., 2010a). Studies indicate that IL-6, IL-8 and GROα can induce senescence in a cell autonomous level, reducing the risk of oncogenic transformation (Acosta et al., 2008), however they can also act at a paracrine level to promote epithelial-to-mesenchymal transition (Krtolica et al., 2001, Parrinello et al., 2005) and tumour progression in neighbouring cells (Nelson et al., 2012, Acosta et al., 2013). Other secreted factors, such as MCP-1 and IL-8 may help recruiting immune cells to clear senescent cells (Xue et al., 2007, Krizhanovsky et al., 2008) and promote tissue repair (Jun and Lau, 2010). However, the activity of SASP factors may also contribute to chronic inflammation and tissue damage and has been often implicated in the pathogenesis of many age-related diseases (Campisi, 2005, Demaria et al., 2014, Franceschi and Campisi, 2014).

1.1.5 MARKERS OF CELLULAR SENESCENCE

One of the most commonly used markers for identifying senescent cells is the activity of senescence-associated β-galactosidase (SA-β-Gal). The expression of this lysosomal enzyme is typically increased in senescent cells, most likely reflecting the increase in lysosomal mass with senescence (Lee et al., 2006, Kurz et al., 2000). The activity of this enzyme, detectable at pH 6.0, is only found in senescent cells being absent in non-senescent, quiescent or terminally differentiated cells, making it a good marker to differentiate these cells. It has been used to identify senescent cells *in vitro* as well as *in vivo*, in culture and in human tissues samples (Dimri et al., 1995). Nevertheless, it has limitations as it has been reported that late-passage and over-confluent cells in culture may stain positively for SA-β-Gal (Yang and Hu, 2005).

Despite the efforts to find a good biomarker of senescence, no single marker is unique or specific enough to be used alone. Most commonly, senescence is confirmed by a combination of markers (Collado and Serrano, 2006). These markers

often represent the most important features or hallmarks of senescence that are delineated below:

- Enlarged and flattened morphology (although only visible in cells in culture);
- Expression of senescence-associated β-galactosidase, detectable at pH 6.0 by histochemical staining;
- Cell cycle arrest or lack of proliferation, generally determined by low levels of Ki67 or bromodioxyuridine (BrdU) labeling;
- Activation of the DNA-damage response with increased expression of DDR proteins and tumour suppressors p53/p21 and $p16^{INK4A}$;
- Formation and persistent expression of nuclear foci containing activated DDR proteins such as ATM, γH2AX, 53BP1 and preferentially located at telomeres;
- Chronic activation of p38MAPK signaling pathway;
- Robust secretory phenotype, with secretion of a plethora of pro-inflammatory cytokines, chemokines, growth factors and metalloproteinases.

1.2 SENESCENCE *IN VIVO*: A ROLE IN AGING AND CANCER

Despite being initially described as a phenomenon occurring in cells in culture, a growing body of evidence indicates that senescent cells can also be found *in vivo* playing a role in the pathogenesis of aging and age-related diseases (Sharpless and Sherr, 2015, van Deursen, 2014). The senescence theory of aging suggests that organismal aging occurs as a result of the accumulation of senescent cells that cannot divide, diminishing the renewal capacity of tissues and/or alter tissue homeostasis due to its high secretory phenotype (Campisi, 2005).

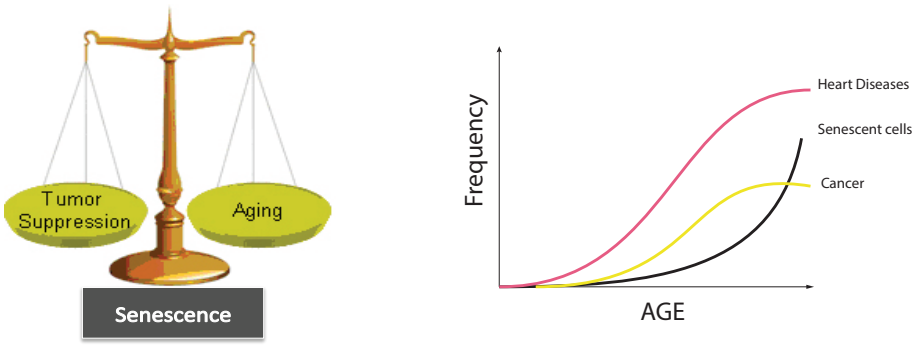

Figure 1.3 – The link between cellular senescence, aging and cancer.

Despite the beneficial effect on tumour suppression, cellular senescence may have deleterious effects, especially later in life. The development of cancer and age-related diseases exponentially increase in late life and it has been associated with the accumulation of senescent cells.

Evidence that senescent cells accumulate with age comes from studies in different organisms, from primates to mice and zebrafish (Herbig et al., 2006, Wang et al., 2009, Henriques et al., 2013). In humans, cells with senescence markers have been detected in high proliferative tissues, such as the skin or the hematopoietic system and the frequency of these cells increases with age (Dimri et al., 1995, van Deursen,

2014). The assumption that the accumulation of senescent cells may be responsible for aging is supported by the identification of senescent cells at sites of age-related pathologies, including atherosclerosis, osteoarthritis, degenerating intervertebral discs and also in liver cirrhosis (Erusalimsky and Kurz, 2005, Price et al., 2002, Roberts et al., 2006, Wiemann et al., 2002).

Despite this, the problem of detecting senescence *in vivo* is inexorably linked with the lack of a robust biomarker of senescence (Sharpless and Sherr, 2015). Although not detectable in all senescent cells, the expression of $p16^{INK4A}$ is the most used marker of aging *in vivo* and many studies have shown an age-dependent accumulation of $p16^{INK4A}$ positive cells, associated with a decline in cell and tissue function (Krishnamurthy et al., 2004, Krishnamurthy et al., 2006, Ressler et al., 2006, Liu et al., 2009, Waaijer et al., 2012). Other studies have shown a correlation between telomere length, age and age-related diseases (Hastie et al., 1990, Samani et al., 2001, Cawthon et al., 2003). However, these studies were only correlative and the causative role of senescence in aging had yet to be proved.

Two recent studies from Baker and colleagues were decisive for the establishment of the role of senescent cells in aging. Using a transgenic mouse model with inducible elimination of $p16^{INK4A}$-positive cells, the authors demonstrated that elimination of senescent cells from early ages extended lifespan and delayed the onset of age-related phenotypes in tissues such as muscle, eye and adipose tissue. Strikingly, elimination of these cells later in life could also halt the progression of pre-established ageing phenotypes. The mechanism behind this was not completely explained, but the authors suggested that the acquisition of a SASP would contribute to age-related tissue dysfunction (Baker et al., 2011, Baker et al., 2016).

Evidence for the existence of a SASP *in vivo* is supported by the age-associated increase in serum levels of IL-6 and TNFα (Bruunsgaard, 2006, Maggio et al., 2006,

Singh and Newman, 2011). However, the exact source of these inflammatory factors is not conclusive and the low-level chronic inflammation associated with aging, known as “inflammaging”, has also been linked with the dysfunction of immune system and immunosenescence (Franceschi et al., 2000). Nevertheless, the most probable scenario is that of multiple and inter-related mechanisms contributing to it.

As cells don´t exist alone but are in constant interaction with each other at a tissue and organismal level, changes in the secretory phenotype of senescent cells may well affect the function of surrounding cells and the tissue microenvironment (Campisi, 2005, Kuilman and Peeper, 2009). This concept of paracrine senescence is now becoming more accepted and recent studies have presented evidence that it also occurs *in vivo* (Nelson et al., 2012, Acosta et al., 2013). Using mouse models of oncogene-induced senescence, Acosta et al observed that senescent cells were often found in clusters, which included different types of cells, mainly stromal fibroblasts, endothelial and immune cells. These findings were confirmed in human specimens of adenomatous polyps of the colon (Acosta et al., 2013).

Although the role of senescence in driving aging may still be controversial, there is an increasing amount of evidence showing that oncogene-induced senescence does occur *in vivo* and represents an important tumour suppressive mechanism. The identification of cells undergoing OIS in both mouse and human premalignant lesions, absent in the malignant counterparts, is a compelling evidence that cellular senescence is indeed an important tumour suppressive mechanism *in vivo* (Michaloglou et al., 2005, Braig et al., 2005, Collado et al., 2005, Suram et al., 2012).

Further supporting the role of senescence in cancer, senescence markers have been identified in tumours after conventional treatment with chemotherapeutic drugs or after ionizing radiation (Chang et al., 1999, Schmitt et al., 2002, Robert H. te Poele, 2002, Efimova et al., 2010). In line with this, the use of targeted therapies to

selectively induce cellular senescence in tumour cells has recently emerged as a promising new weapon for cancer treatment (Collado and Serrano, 2010, Acosta and Gil, 2012, Nardella et al., 2011). Of particular note, several studies indicate that the successful outcome of prosenescence therapies depends on the effective clearance of senescent cells by the immune system (Toso et al., 2014, Soriani et al., 2009) and this immune response designated as senescence immune surveillance has provoked considerable interest not only for cancer therapy but also for the prevention of age-related diseases (Hoenicke and Zender, 2012, Naylor et al., 2013).

1.3 SENESCENCE SURVEILLANCE

The fate of senescent cells in the organism is still unclear. Studies have shown that senescent cells can persist for a long time in tissues in living organisms. As an example, Michaloglou *et al* has shown that melanocytic nevi are formed by senescent melanocyte cells that reside in human skin for decades (Michaloglou et al., 2005). Nevertheless, accumulating evidence now indicates that the immune system is able to recognize senescent cells. An intimate crosstalk between senescent cells and the immune system has been described, involving both the innate and adaptive arms of the immune system.

1.3.1 SURVEILLANCE BY THE INNATE IMMUNE SYSTEM

In a pioneering study, Lowe and colleagues demonstrated that induction of senescence in tumour cells after p53 reactivation triggered an immune response that contributed to tumour regression *in vivo*. In this study, the response was mediated by the innate arm of the immune system, mainly through the activation of natural-killer (NK) cells, macrophages and neutrophils that infiltrated the tumour after p53 restoration (Xue et al., 2007). This was the first demonstration of the interplay

between the senescent cells and the immune system, revealing a new role for senescence in promoting cancer suppression, through the activation of an immune response against senescent cells.

Interestingly, a study by Reimann *et al* revealed that the immune system itself could induce senescence in tumour cells by the secretion of cytokines such as TGF-β by macrophages (Reimann et al., 2010).

The clearance of senescent cells has also been shown to be important for counteracting non-malignant disorders, such as liver fibrosis. Using a mouse model of liver fibrosis, Krizhanovisky *et al* demonstrated that senescence of activated hepatic stellate cells (HSCs) limits the extent of liver fibrosis. They suggested that senescence not only limits the proliferation of HSCs but also facilitates the clearance of these cells from the liver, through the up-regulation of cytokines, adhesion molecules and immune ligands that activate and recruit NK cells. Using *in vitro* and *in vivo* cytotoxicity assays they were able to show that senescent cells (HSCs and fibroblasts) were more sensitive to NK cell-mediated killing than proliferating cells (Krizhanovsky et al., 2008). Later on, the authors demonstrated that senescent cells up-regulated ligands for the stimulatory NK cell receptor NKG2D, particularly MICA/B and ULBP2, which were crucial for triggering an efficient cytotoxic response mediated by NK cells towards senescent cells (Sagiv, 2016).

1.3.2 THE ADAPTIVE IMMUNE SYSTEM AND SENESCENT CELLS

These previous studies offered an insight on how the innate immune system is involved in the clearance of senescent cells. Nevertheless, other studies suggest that the adaptive immune system may also be an important mediator of the immune surveillance of senescent cells.

Using an *in vivo* model of oncogene-induced senescence in the liver, Lars Zender and colleagues demonstrated that premalignant senescent hepatocytes were subjected to immune clearance that was dependent on a response orchestrated by antigen-specific CD4+ T cells but required innate immune cells to execute the killing. Interestingly, the induction of an antigen-specific response against senescent hepatocytes was dependent on the fulfilment of the senescence programme, as hepatocytes that were transduced with a non-oncogenic *Nras* gene and did not undergo senescence were not cleared. These observations imply that the activation of senescence may be required for effective immune surveillance and that it is possible to induce a specific immune response against antigens expressed by premalignant senescent cells. Finally, the authors investigated whether this mechanism would occur *in vivo* by analysing liver samples of patients with chronic hepatitis C alone or co-infected with HCV-HIV. In co-infected patients, with a compromised immune system typically affecting CD4+ T cells, the frequency of senescent (p16+) cells was significantly higher than in mono-infected patients (Kang et al., 2011).

Another study demonstrated how the adaptive immune system, in particular CD4+ T cells were important for the induction of senescence and complete remission of lymphoma and leukemia in mouse models. It was proposed that CD4+ T cells mediated this effect through the secretion of cytokines such as IFNγ and TNFα, IL-15 and MCP-1 that are known to have anti-tumour (and possibly pro-senescence) effects (Rakhra et al., 2010).

Collectively these studies demonstrated a role for both arms of the immune system, in clearing senescent cells (**Figure 1.4**). Intriguingly, none of these studies have implicated CD8+ T cells, which are major mediators of cytolytic responses to infection and cancer, in senescence surveillance.

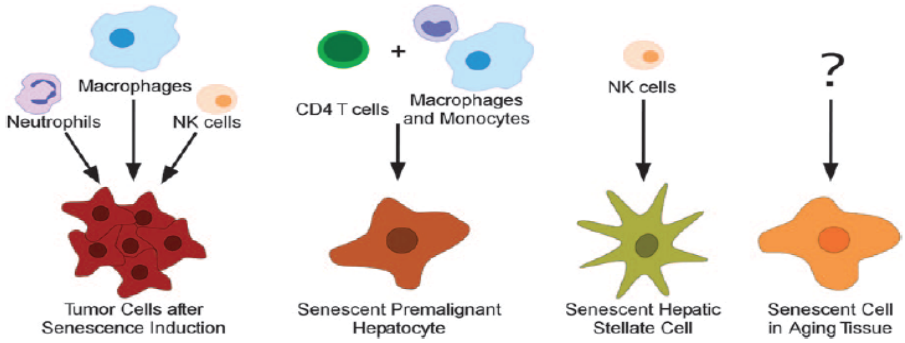

Figure 1.4 – Senescence immune surveillance.

Recent studies indicate a role for the innate immune system (macrophages, neutrophils and NK cells) and adaptive immune system (mainly CD4+ T cells) in mediating responses towards senescent cells in different pathological settings. Whether the immune system is able to mount an immune response towards senescent cells in non-pathological aging tissues still needs to be investigated (adapted from Hoenicke et al. 2004).

The role of other components of the immune system, such as CD8+ T cells in mediating responses towards senescent cells still needs to be investigated.

1.3.3 MECHANIMS OF ALERTING THE IMMUNE SYSTEM BY SENESCENT CELLS

Senescence is associated with striking changes in gene expression and many of the up-regulated genes are involved in the modulation of the immune response (Kuilman et al., 2008, de Magalhaes et al., 2009, Purcell et al., 2014). Alongside the up-regulation of pro-inflammatory cytokines and adhesion molecules typically involved in the recruitment of leukocytes, senescent cells up-regulate the expression of membrane-bound ligands for the activating NK cell receptor NKG2D, rendering these cells sensitive to NK-cell mediated killing (Krizhanovsky et al., 2008, Soriani et al., 2009, Iannello et al., 2013, Sagiv, 2016).

Previously, Gasser and colleagues had demonstrated that genotoxic stress and the activation of the DNA damage response induced the up-regulation of NKG2D ligands in mouse and human fibroblasts exposed to ionizing radiation and other DNA-damaging agents (Gasser et al., 2005). By showing this, the authors demonstrated that the DNA damage response, a fundamental component of the senescence programme, not only serves to arrest the cell cycle and allow DNA repair, but also participates in the communication with the immune system to alert for the presence of potentially harmful cells.

The hypothesis that cells in danger can emit “find me“ signals to alert the immune system has been previously proposed by Matzinger, in particular in response to infection, cancer or stress (Matzinger, 2002). It is now widely accepted that stressed cells secrete alarmins or damage-associated molecular patterns (DAMPs) that are recognized by specific receptors (pattern recognition receptors (PPRs) expressed by dendritic cells, macrophages and other immune cells, priming an immune response that will eventually induce the clearance of these cells (Bianchi, 2007). One of these DAMPs is the high mobility group box 1 protein (HMGB1), which was recently shown to be released by senescent cells (Davalos et al., 2013). HMGB1 can bind several pattern recognition receptors (PPRs), including Toll-like receptor 2 and 4 (TLR-2, -4) as well as receptors for advanced glycosylation end products (RAGE), expressed not only by macrophages and dendritic cells but also by T lymphocytes (Park et al., 2004, Kokkola et al., 2005, Dumitriu et al., 2005). It is plausible that senescent cells use these mechanisms to recruit and activate cells of the immune system.

Thus, it seems that there are mechanisms to alert and mobilize the immune system towards senescent cells. Nonetheless, the immune surveillance of senescent cells is not completely efficient, since senescent cells accumulate with age.

1.3.4 MECHANISMS OF EVADING THE IMMUNE SYSTEM

Not much is known about how senescent cells evade surveillance and clearance by the immune system. In contrast, escape from the immune system is now considered to be one of the hallmarks of cancer (Hanahan and Weinberg, 2011).

Similarly to cancer cells, senescent cells may engage different mechanisms leading to immune escape such as: secretion of anti-inflammatory cytokines (IL-10, TGF-β), induction of regulatory T cells, activation of myeloid suppressor cells or down-regulation of cell surface markers that mediate the interaction with the immune system (Zitvogel et al., 2006).

Down-regulation of the expression of MHC-class I molecules is a well-described strategy that tumours and viruses use to escape CD8+ T cell-mediated responses (Ploegh, 1998, Bubenik, 2004). Modulation of the expression of non-classical MHC class I molecules such as HLA-E and HLA-G, has been described as a strategy used by tumour cells to avoid NK and T cell killing (Derre et al., 2006, de Kruijf et al., 2010, Algarra et al., 2004). Shedding of NKG2D ligands after cleavage by matrix metalloproteinases induces a downmodulation of NKG2D on effector cells, further impairing the immune surveillance of tumour cells (Groh et al., 2002).

Alternatively, tumours may subvert the immune system through changes in the microenvironment, favouring the development of immunosuppressive or tolerogenic immune cells. As an example, overproduction of cytokines such as IL-4, IL-10 and TGF-β are associated with an M2 polarization of macrophages and T_H2 responses as well as with induction of regulatory T cells and activation of myeloid-derived suppressor cells (MDSCs). Furthermore, tumours (or tumour-associated myeloid suppressor cells) may also increase the expression of enzymes such as arginase-1 and indoleamine 2,3- dioxygenase (IDO), which change the availability of important

amino acids in the tumour milieu, blocking proliferation and function of CD8+ T cells (Bronte and Zanovello, 2005, Uyttenhove et al., 2003).

All these mechanisms have been described in cancer cells, however whether senescent cells use similar strategies to evade the immune system remains to be investigated. In addition to this, another factor to be considered is the age-related impairment in immune responses or immunosenescence, which has important consequences for the surveillance of abnormal cells.

1.4 IMMUNOSENESCENCE

Aging is associated with a general decline in immune function, a process generally termed as immunosenescence, which is mostly driven by chronic antigenic stimulation and thymic involution (Vallejo, 2007, Lynch et al., 2009).

These age-dependent alterations in the immune system affect the adaptive immune system and the T cell compartment in particular. T cells are highly susceptible to senescence, not only because they are long-lived but mostly because they undergo massive rounds of proliferation after repeated antigen challenge (Vallejo, 2007). Nevertheless, the effects of aging on the immune system are widespread and recent studies have described alterations in the function of B cells, NK, macrophages and dendritic cells as well as hematopoetic stem cells (Panda et al., 2009, Kuranda et al., 2011, Le Garff-Tavernier et al., 2010).

Clinically, these changes play a significant a role in the increased morbidity and mortality in the elderly and are manifested by a higher susceptibility to infections and cancer, reduced effectiveness of vaccinations and deregulated inflammation (McElhaney et al., 2012).

1.4.1 EFFECTS OF AGING ON THE INNATE IMMUNE SYSTEM

In the innate immune compartment, age-related changes in the phenotype and function of NK cells have been described (Le Garff-Tavernier et al., 2010). These are associated with the accumulation of $CD56^{dim}$ NK cells with a mature phenotype, characterized by the increased expression of maturation markers such as CD57 (Lopez-Verges et al., 2010) and KLRG1 (Huntington et al., 2007, Muller-Durovic et al., 2016). Although the effects of aging on the cytolytic function of NK cells are still controversial, our group recently identified a subset of $CD56^{dim}$ $KLRG1^{high}$ NK cells that is expanded in the elderly, displaying impaired cytotoxicity and proliferation as well as other features of senescence (Muller-Durovic et al., 2016).

Other important changes include the skewing of hematopoiesis towards the myeloid lineages (Beerman et al., 2010, Kuranda et al., 2011) as well as augmented Th17 immune responses (Tesar et al., 2009) that could contribute to the pro-inflammatory milieu characteristic of aging. Indeed, a well-recognized consequence of immunesenescence is a deregulation of the cytokine network towards a pro-inflammatory profile, with increased plasma levels of IL-6 and TNFα found in the elderly that may contribute to the persistent low-grade inflammation termed “inflammaging” (Bruunsgaard et al., 2003, Franceschi et al., 2000).

1.4.2 EFFECTS OF AGING ON THE ADAPTIVE IMMUNE SYSTEM

One of the most recognized age-related changes in the immune system is the involution of the thymus leading to a reduced output of naïve T cells to the periphery. Despite thymic involution, the total number of circulating lymphocytes remains stable throughout life, reflecting the onset of compensatory homeostatic mechanisms that lead to the expansion of memory T cells (Linton and Dorshkind, 2004). Within the peripheral memory T cell pool, there is an accumulation of oligoclonal T cells, specific

for viral infections such as cytomegalovirus (CMV) and Epstein-Barr Virus (EBV) with a differentiated phenotype (Khan et al., 2002, Fletcher et al., 2005, Pawelec and Gouttefangeas, 2006). In combination with the depletion of the peripheral pool of naïve T cells, the accumulation of these terminally differentiated T cells with age skews the immune repertoire and leads to impaired immune responses to new antigens and vaccination in the elderly (Akbar and Fletcher, 2005, McElhaney et al., 2012).

1.4.3 T CELL SENESCENCE: DIFFERENCES AND SIMILARITIES WITH CELLULAR SENESCENCE

Although most of the features of senescence have been described in fibroblasts, similarities and differences between senescence in T cells and fibroblasts are found (summarized in **Table 1.2**). One crucial difference regards the expression of telomerase, which is found on T cells and is absent in fibroblasts. In fact, T lymphocytes are able to reactivate telomerase in response to mitogenic and antigenic stimulation (Hodes et al., 2002). However, T cells lose the capacity to reactivate telomerase with progressive differentiation and thus have a limited replicative capacity, as do all somatic cells (Roth et al., 2003). Repeated antigenic exposure leads to overt proliferation and consequent telomere shortening that will eventually trigger replicative senescence (Fletcher et al., 2005). Telomere-independent mechanisms of senescence may also occur in T cells (Di Mitri et al., 2011, Akbar and Henson, 2011).

Senescence features in fibroblasts:	Senescence features in T cells:
Similarities: Short telomeres Expression of SA-β galactosidase Persistent DNA damage foci (YH2AX, 53BP1) Activation of p53 and pRB - $p16^{INK4a}$ Chronic activation of p38 MAPK Accumulation of senescence cells with age Secretion of growth factors, cytokines, proteases - SASP **Differences:** Permanent cell cycle arrest No telomerase No specific surface markers identified	**Similarities:** Short telomeres Expression of SA-β galactosidase Persistent activation of the DNA damage response (YH2AX) Activation of p53 and pRB - $p16^{INK4a}$ Chronic activation of p38 MAPK Accumulation of differentiated T cells with age Highly functional: increased secretion of TNFα, IFNγ, perforin, granzyme B **Differences:** Decreased proliferative capacity Decreased telomerase activity Altered expression of surface receptors: ↓CD28, ↓CD27,↑CD45RA

Table 1.2 – Similarities and differences between senescence characteristics of fibroblasts and T cells.

Mechanistically, the DNA-damage response activation and signaling through p53-p21 and p16 pathways that regulate growth arrest are identical in both types of cells (Erickson et al., 1998, Scheuring et al., 2002, Mondal et al., 2013, Liu et al., 2011). Evidence of spontaneous phosphorylation of ATM and its downstream target γH2AX in highly differentiated T cells suggests that senescence in T lymphocytes may also depend on the persistent activation of the DNA-damage response (Di Mitri et al., 2011, Lanna et al., 2014, Henson et al., 2014).

Chronic activation of p38MAPK signalling pathway is also a prominent feature of senescence in T cells, which has been implicated in the regulation of telomerase activity and autophagy in T cells. In fact, inhibition of p38MAPK by specific small-molecule inhibitors or RNA interference resulted in enhanced telomerase activity and increased proliferation of senescent T cells (Di Mitri et al., 2011, Lanna et al., 2014) as well as in increased autophagic activity (Henson et al., 2014).

Although it is not yet described whether senescent T cells have a SASP, it is well documented that highly differentiated T cells are very functional and metabolically active, secreting high levels of cytokines such as IFN-γ and TNF-α, as well as IL-6, IL-8, GROα and MIP-1α among other factors (Libri et al., 2011, Henson et al., 2014). As discussed previously, the nature of the SASP may be cell-dependent although a common signature is frequently found.

One important difference in the senescence programme of T cells compared to fibroblasts is the altered expression of surface receptors in T cells that may be used as markers to identify senescent T cells. Hence, T lymphocytes are probably one of the best models to study cellular senescence *in vivo*, not only because they are easily isolated from the blood and analysed *ex vivo,* but also because they can be robustly identified by the differential expression of surface markers.

1.4.4 MARKERS OF T CELL DIFFERENTIATION AND SENESCENCE

Multiple combinations of cell surface markers have been used to categorize T cells into different subsets based on their phenotype and function. The most commonly used markers are the co-stimulatory receptors CD28 and CD27, the lymph-node homing receptor CC-chemokine receptor 7 (CCR7) and the two isoforms of the leukocyte common antigen CD45 (CD45RA and CD45RO) (Appay et al., 2002, Sallusto et al., 2004, Appay et al., 2008).

In the present thesis, CD8+ T cell subsets were stratified according to the relative expression of CD28 and CD27, allowing the distinction of three populations at different stages of differentiation (**Figure 1.5**): an early differentiated subset (CD28+CD27+), an intermediate differentiated population with loss of one of these markers (CD28-CD27+) and a highly differentiated subset that is double negative for both markers (CD28-CD27-) (Appay et al., 2002).

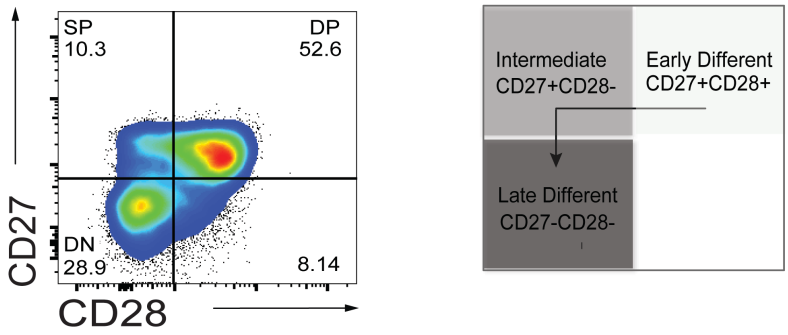

Figure 1.5 – Schematic representation of CD8+ T cell subsets stratified according to CD28 and CD27 expression.

Alternatively, CD8+ T cell subsets can be divided according to the expression of CD27 and CD45RA into naïve (T_{naive} CD27+CD45RA+), central memory (T_{CM}; $CD45RA^{-}CD27^{+}$), effector memory (T_{EM}; $CD45RA^{-}CD27^{-}$) and effector memory T cells that re-express CD45RA (T_{EMRA}; $CD45RA^{+}CD27^{-}$)(Appay et al., 2008). Using this method, re-expression of CD45RA defines the subset of effector memory T cells with characteristics of late differentiation (T_{EMRA}) (Michie et al., 1992, Henson et al., 2012).

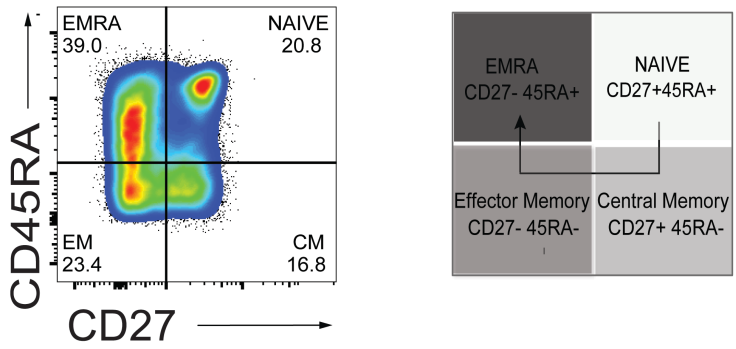

Figure 1.6 - CD8+ T cell subsets stratified according to CD27 and CD45RA expression.

Multiples lines of evidence show that highly differentiated ($CD27^{-}CD28^{-}CD45RA^{+/-}$) T cells exhibit characteristics of senescence:

- Accumulate with age, chronic viral infections and in chronic inflammatory syndromes (Fletcher et al., 2005, Schmidt et al., 1996);
- Have short telomeres (Monteiro et al., 1996, Fletcher et al., 2005);
- Have reduced proliferative capacity (Henson et al., 2009, Di Mitri et al., 2011);
- Lose the capacity to activate telomerase (Plunkett et al., 2007);
- Have persistent activation of the DNA damage response (Di Mitri et al., 2011, Lanna et al., 2014, Henson et al., 2014);
- Have altered signalling pathways, including defective AKT phosphorylation (Plunkett et al., 2007), spontaneous activation of p38MAPK and AMP Kinase activity (Plunkett et al., 2007, Di Mitri et al., 2011, Lanna et al., 2014) and low levels of mTOR (Henson et al., 2014).
- Secret high levels of cytokines such as IFN-γ and TNF-α and, in the case of CD8+ T cells, also express high levels of cytotoxic granules, granzyme B and perforin (Libri et al., 2011, Henson et al., 2014).

In addition to the described features, other characteristics of highly differentiated CD8+ T cells have been recently described, revealing additional aspects of the function of these cells in the organism. As an example, highly differentiated T cells express high levels of CX3CR1, the receptor for fractalkine expressed by inflamed endothelium and LFA-1 (Lymphocyte function-associated antigen 1), an adhesion molecule that binds ICAM-1, facilitating their migration towards inflamed tissues (Faint et al., 2001). Another important feature of terminally differentiated T cells is the acquisition of receptors that are normally attributed to NK cells that will be discussed next.

1.4.5 INCREASED EXPRESSION OF NK CELL RECEPTORS WITH T CELL DIFFERENTIATION

There is mounting evidence that T cells, in particular CD8+ T cells, acquire a variety of NK cell receptors with age and differentiation, including CD16, CD56, NKp30, CD94, members of the NK receptor G2 (NKG2) and killer-cell immunoglobulin-like receptor (KIR) families (Tarazona et al., 2000, Abedin et al., 2005, Strauss-Albee et al., 2014).

Studies in human centenarians have shown an increased proportion of T cells expressing NK cell receptors (NKRs), whereas these cells represented a minor population of circulating lymphocytes in newborns and young healthy individuals (Miyaji et al., 1997, Pittet et al., 2000). The frequency of NKR-expressing T cells not only increases with age but also in conditions associated with chronic immune activation (Tarazona et al., 2000, Abedin et al., 2005, Vallejo et al., 2011).

Killer-cell lectin-like receptor G1 (KLRG1) is an example of NK cell receptor that increases significantly with age in both CD4+ and CD8+ T cells as well as NK cells. The highest percentage of KLRG1+ T cells is seen in the most differentiated subsets, which have lost CD28 and have defective proliferation and therefore KLRG1 is commonly used as a marker of terminal differentiation or maturation (Ouyang et al., 2003, Voehringer et al., 2002, Henson et al., 2009) KLRG1 has been shown to deliver inhibitory signals to T cells inducing defective AKT phosphorylation and blocking proliferation of highly differentiated $CD28^{-}CD27^{-}CD8^{+}$ T cells (Henson et al., 2009). In NK cells, KLRG1 inhibits NK cell function (cytotoxicity, cytokine secretion, proliferation, and telomerase expression) through the activation of AMP kinase (Muller-Durovic et al., 2016).

It has been suggested that the expression of NK receptors on T cells possibly represents another remodelling mechanism that occurs with age to compensate the

loss of co-stimulatory receptors and the contraction of TCR repertoire (Vallejo et al., 2011). However, it may also disclose new functions of the adaptive immune system or re-acquisition of reminiscent innate immune functions, less dependent on the TCR/antigen specificity.

But, how does immunosenescence itself influence the aging process of an organism?

Observations from patients with HIV infection who exhibit features of immunosenescence and are also examples of premature aging syndromes, with increased cardiovascular and other age-associated co-morbidities, suggests that both processes are linked and that immunosenescence may have a detrimental role in the aging process (Le Saux et al., 2012).

It is reasonable to speculate that immunosenescence is one of the main culprits for a defective immunesurveillance against senescent cells, leading to the accumulation of senescent cells with age, contributing to the pathogenesis of age-related diseases. However, to date and to our knowledge no studies have been done to test this: how immunosenescence affects the surveillance of senescent cells and how immunosenescence contributes to aging due to a defective clearance and accumulation of senescent cells.

1.5 HYPOTHESIS AND AIMS

It has been recently demonstrated that the immune system can recognize and clear senescent cells. NK cells, macrophages and CD4+ T cells are amongst the cells that have been shown to participate in the immune surveillance of senescent cells (Xue et al., 2007, Krizhanovsky et al., 2008, Rakhra et al., 2010, Kang et al., 2011). Remarkably a role of CD8+ T cells in the clearance of senescent cells has never been explored. In addition, despite recent observations that senescent cells can be targeted by the immune system, the question remains: why do senescent cells accumulate with age and evade immune clearance?

The aim of this project was to investigate the interplay between the immune system and senescent fibroblasts, with particular attention to CD8+ T cells, and understand why do senescent cells escape CD8+ T cell killing.

The working hypothesis was that senescent cells exhibit changes in the expression of MHC molecules, which will have an impact on the interactions with the immune system. It was also hypothesized that immunosenescence and the age-related defects in the immune responses will contribute to the defective immune surveillance of senescent cells with age.

The specific objectives were as follows:

1. To investigate the expression of classical and non-classical MHC molecules on senescent cells compared to non-senescent and study the mechanisms involved in the regulation of the expression of these molecules upon senescence.

2. To explore the impact of altered MHC expression in the immune surveillance of senescent cells using an autologous co-culture system with primary human fibroblasts and autologous T and NK cells.

3. To investigate the potential benefits of targeting inhibitory receptors to improve immune responses to senescent cells.

4. To assess the effects of immunosenescence in the surveillance of senescent cells.

2 Senescent fibroblasts express ligands to alert the immune system

2.1 ABSTRACT

Recent evidence indicates that senescent cells communicate with the immune system mostly through the secretion of proinflammatory mediators that attract immune cells and induce local immune responses. How senescent cells interact with immune effectors to trigger immune responses is still not completely understood.

In the present chapter, a comprehensive analysis of the expression of Major Histocompatibility Complex (MHC) molecules on the surface of primary human fibroblasts following the induction of senescence was performed using flow cytometry. It was found that senescent cells become immunogenic through the up-regulation of atypical ligands such as the non-classical MHC class I molecules MICA/B and HLA-E, which are absent in non-senescent cells. This pattern of MHC expression was common to different forms of senescence, regardless of the stimuli that cause it. Mechanistically, it was found that the DNA-damage response is involved in the regulation of expression of MICA-B whilst the expression of HLA-E on senescent fibroblasts is mainly regulated by the p38MAPK signaling pathway and induced by the proinflammatory cytokine IL6. These observations suggest a role of inflammation in the induction of HLA-E expression on senescent cells, which could be critical to the regulation of immune responses to senescent cells as it will be further explored in the following chapters.

KEY POINTS

- Senescent cells express non-classical MHC class I molecules, such as MICA/B and HLA-E, not present on non-senescent cells;
- HLA-E expression on senescent cells is regulated by p38MAPK and induced by the proinflammatory cytokine IL6.

2.2 INTRODUCTION

Cellular senescence is associated with important changes in gene expression, which most notably include an overexpression of genes involved in inflammation and modulation of immune responses, suggesting that the communication with the immune system is an important aspect of the senescence program (Kuilman et al., 2008, de Magalhaes et al., 2009, Purcell et al., 2014). Previous studies have shown that senescent cells secrete factors that promote the recruitment of immune effectors and this has been shown to be important for tissue repair and regeneration (Demaria et al., 2014, Yun et al., 2015). Senescent cells may also secrete alarmins or damage-associated molecular patterns (DAMPs), such as the high mobility group box protein (HMGB1) protein, which binds several pattern recognition receptors (PPRs), in dendritic cells, macrophages and other immune cells (Davalos et al., 2013). Moreover, senescent cells up-regulate stress-induced ligands, such as MICA/B and other ligands for NKG2D, rendering them sensitive to NK-cell mediated killing (Krizhanovsky et al., 2008, Soriani et al., 2009, Iannello et al., 2013, Sagiv, 2016).

The modulation of the surface expression of Major Histocompatibility Complex (MHC) molecules is an important mechanism that regulates the interactions of a cell with both the adaptive and innate immune systems. Alterations in the surface expression of MHC molecules leading to immune escape mechanisms have been extensively described in cancer and viral-infected cells (Ploegh, 1998, Zitvogel et al., 2006, Kochan et al., 2013, Halenius et al., 2015). Nevertheless, not much is known about how senescence modifies the expression of MHC molecules and whether these changes may affect the immune surveillance of senescent cells.

The MHC class I region contains the classical human leukocyte antigens (HLA-A, -B, and -C) which are highly polymorphic and ubiquitously expressed at the surface of nearly every nucleated cell in the body. MHC class I molecules are expressed as

heterotrimeric structures involving the HLA class I heavy chain non-covalently associated with the light chain β2-microglobulin (β2m) and a short peptide of 8-10 amino acids (Garcia et al., 1996, Hennecke and Wiley, 2001). These molecules have an essential role in mediating immune responses, sampling both the intracellular and extracellular microenvironment by presenting self- or pathogenic peptides to CD8+ cytotoxic T cells (Neefjes et al., 2011, Neefjes and Ovaa, 2013).

The MHC class I group also includes the non-classical MHC class I molecules (HLA-E, -F, and -G), which are structurally similar to the classical class I molecules, but have limited polymorphism and tissue distribution (Wei and Orr, 1990, Gobin and van den Elsen, 2000). Among these, HLA-E is ubiquitously expressed albeit at very low levels at the surface of most cells with higher levels found on leukocytes, endothelial cells and trophoblastic cells (Ulbrecht et al., 1999, Coupel et al., 2007). HLA-E expression has been implicated in the induction of immune tolerance in both physiological (pregnancy) and pathological conditions (tumours or viral infections), by interacting with the inhibitory receptor NKG2A expressed on NK and CTLs (Ishitani et al., 2003, Kochan et al., 2013, Halenius et al., 2015, Parham and Moffett, 2013). Similarly, HLA-G has a very restricted expression on normal cells and aberrant expression at the surface of tumour cells has been associated with immune evasion and poor prognosis. An increasing body of evidence has thus confirmed a role for non-classical MHC class I molecules, in particular HLA-E and HLA-G in viral and tumour escape from NK and CTL-mediated immune responses (Algarra et al., 2004, de Kruijf et al., 2010, Amiot et al., 2011, Zeestraten et al., 2014).

Additional molecules contained in the MHC class I region are MHC class I polypeptide-related sequence A and B (MICA/B) and the UL16-binding protein family (ULBP1-6) (Raulet, 2003, Raulet et al., 2013, Lanier, 2015). These proteins are distant relatives of MHC class I molecules as they do not associate with β2-microglobulin and they are normally not expressed on healthy cells but are often up-

regulated during cellular stress, viral infection and in tumour cells (Nice et al., 2009, Raulet et al., 2013). The expression of these ligands has been implicated in the induction of cytolytic immune responses against tumours and stressed cells through the interaction with the receptor NKG2D expressed on NK and CD8+ T cells (Bauer et al., 1999, Diefenbach et al., 2000, Cosman et al., 2001, Champsaur and Lanier, 2010). Previous studies have demonstrated that senescent cells can also up-regulate the expression of these ligands, therefore becoming more susceptible to NK-cell mediated killing (Krizhanovsky et al., 2008, Sagiv, 2016).

To my knowledge, the expression of MHC molecules on senescent cells has not been thoroughly studied. Therefore, as a first approach to study the immune surveillance of senescent cells, a comprehensive analysis of the surface expression of MHC molecules was performed on primary human fibroblasts following the induction of senescence. The mechanisms involved in the regulation of the expression of MHC class I molecules were also investigated, with particular focus on the regulation of non-classical molecules such as HLA-E, a previously unrecognized immune ligand expressed on senescent cells.

2.3 RESULTS

2.3.1 Establishment of a model of premature senescence in primary human fibroblasts

To investigate the effects of senescence on the expression of MHC molecules, a model of premature senescence was adapted to primary human fibroblasts. Briefly, fibroblasts were derived from the skin of healthy volunteers, using skin explant cultures, as previously described (Takashima, 2001, Vangipuram et al., 2013).

Exposure to ionizing radiation is a widely used method of inducing premature senescence through the activation of the DNA-damage response (Rodier et al., 2009, Bluwstein et al., 2013). Therefore we exposed skin-explanted fibroblasts to 10 Gy of ionizing radiation (Xray), determined on preliminary experiments as the lowest effective dose in inducing senescence without increasing apoptosis. After irradiation, cells were left to recover in culture for 7-14 days, until they develop a senescent phenotype. Cells were harvested at different time points - day 1 (D1), day 7 (D7) and day 14 (D14) post-irradiation - to confirm the expression of senescence markers and to investigate changes on MHC expression, as compared to controls. In every experiment, non-irradiated early-passage fibroblasts (passages 3-9), grown in the same conditions as irradiated cells were used as controls (for a full description of the methodology please refer to **Materials and Methods – Chapter I**).

To confirm that ionizing radiation effectively induced senescence in primary human fibroblasts, a panel of senescence markers was used. The increased activity of senescence-associated β-galactosidase (SA-β-Gal) is a widely used marker of senescence. As expected, there was an increase in the number of cells staining positively for SA-β-Gal activity over time after radiation, compared to controls (**Figure 2.1A,B)**.

Permanent cell growth arrest is a hallmark of senescence. Ki67, a nuclear antigen expressed in active phases of the cell cycle (G1, S, G2 and M phases) and absent in non-proliferating cells, was used as an indirect marker of proliferation. As illustrated in the representative FACS plots (**Figure 2.1C**), Ki67 expression progressively decreased after irradiation. After day 7, the frequency of Ki67+ cells was very low, suggesting that cells have ceased proliferation. The analysis of the growth curves confirmed that, in contrast to controls, irradiated cells do not replicate, corroborating that growth arrest is stable and permanent (**Figure 2.1D**).

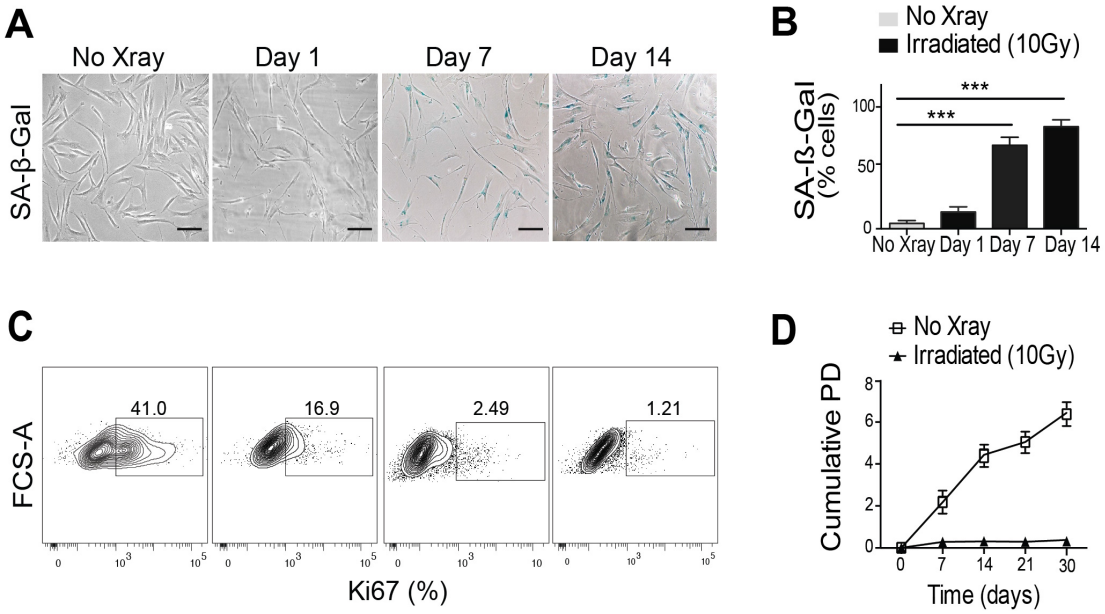

Figure 2.1 - Ionizing radiation effectively induces senescence in primary human fibroblasts.

A. Histochemical staining of irradiated fibroblasts for senescence-associated β-galactosidase (SA-β-Gal). Non-irradiated cells were used as controls (first panel). Cells were observed using a phase-contrast microscope (20x). Scale bar = 100μm. **B**. Summary data obtained as in A) and presented as the percentage of SA-β-Gal+ cells. For each time point at least 100 cells were analysed in 3 independent experiments. **C**. FACS analysis of Ki67 expression at the indicated timepoints after irradiation. Numbers indicate percentages of Ki67+ cells. **D**. Growth curve of irradiated (Δ) and non-irradiated fibroblasts (□). Cumulative population doublings (cPD) were calculated at the indicated timepoints as described in Methods (n=3). Comparison between groups performed with one-way Anova with Dunn´s multiple comparison post-test (***p< 0.001).

Activation of the DNA-damage response (DDR) is another important feature of senescence. The phosphorylation of γH2AX at the serine residue 139 (Ser139) is one of the first events in the DNA-damage response cascade and high levels of phosphorylated γH2AX were detected using phospho-flow cytometry and western blotting, hours after irradiation (**Figure 2.2**).

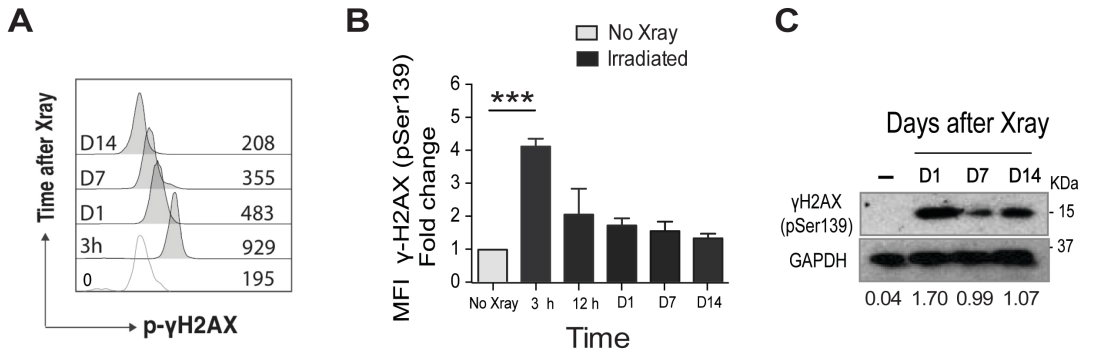

Figure 2.2 - Phosphorylation of the histone γH2AX (Ser139) as an early marker of the activation of the DNA-damage response after irradiation.

A. Time-course analysis of the phosphorylation of γH2AX (Ser139) after irradiation, assessed by phospho-flow cytometry. Numbers indicate mean fluorescence intensity (MFI). **B**. Summary of results obtained as in A), presented as the fold change of γH2AX (Ser139) MFI in irradiated cells relative to non-irradiated controls, set as 1 *(n*=4). Analysis of variance between timepoints performed with repeated measures Anova with Bonferroni correction for multiple comparisons (****p< 0.001)*. **C.** Representative immunoblot of phospho-γH2AX (Ser139) at the indicated timepoints after irradiation. GAPDH expression was used as a protein loading control. Numbers below lanes indicate the relative expression (normalized to GAPDH), determined by ImageJ analysis. Numbers on the right indicate molecular weights (kDa).

The decrease in phosphorylated γH2AX 24 hours post-irradiation, confirms previous studies indicating that the majority of the DDR foci induced after irradiation of human fibroblasts are efficiently repaired within 24 hours (Rodier et al., 2009, Bluwstein et al., 2013). However the persistence of a few irreparable DDR foci is sufficient to induce senescence (Fumagalli et al., 2012). The permanent activation of the DDR was confirmed by immunofluorescence showing the persistence of nuclear foci of phosphorylated γH2AX at day 14 after irradiation (**Figure 2.3, top panel**).

The same cells showed an increased expression of the tumour suppressor $p16^{INK4a}$, another surrogate marker of senescence (**Figure 2.3, lower panel**).

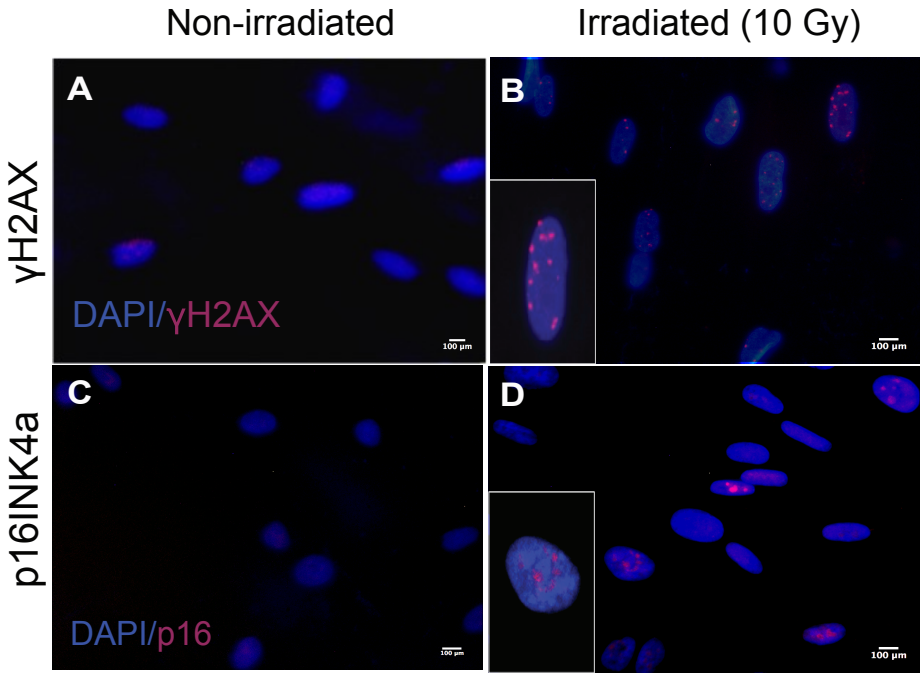

Figure 2.3 – Immunofluorescence staining of γH2AX (Ser139) and $p16^{INK4a}$ in irradiated primary human fibroblasts (Day 14) compared to controls.

Primary human fibroblasts were X- irradiated (10Gy) and analysed for the expression of γH2AX (Ser139) and $p16^{INK4a}$, at day 14 after irradiation (B-D). Non-irradiated cells were used as controls (A-C). Nuclei were counterstained with DAPI (blue). Scale bars, 100 µM.

Collectively these results document that a dose of 10 Gy of ionizing radiation effectively induces senescence in primary human fibroblasts and therefore may be used as a method to induce premature senescence in skin-derived primary human fibroblasts.

In addition to DNA damage, other types of stress are known to induce cellular senescence such as critical telomere shortening, activation of oncogenes or oxidative stress (Sharpless and Sherr, 2015). To validate the observations obtained with the model of irradiation-induced senescence, other models of senescence such as replicative senescence and oncogene-induced senescence were employed.

To experimentally reproduce replicative senescence, primary human fibroblasts were cultured until they reached replicative exhaustion, determined as the point where cells were unable to divide and reached a plateau in their growth rate. As observed in a representative growth curve (**Figure 2.4A**), where cumulative population doublings (cPD) represent a measure of the total number of divisions a cell has undergone since their initial isolation, primary human fibroblasts continuously cultured *in vitro* undergo an initial exponential growth phase and then reach a plateau, at which they exhibit senescence markers such as the positive staining for SA-β-galactosidase (**Figure 2.4B**).

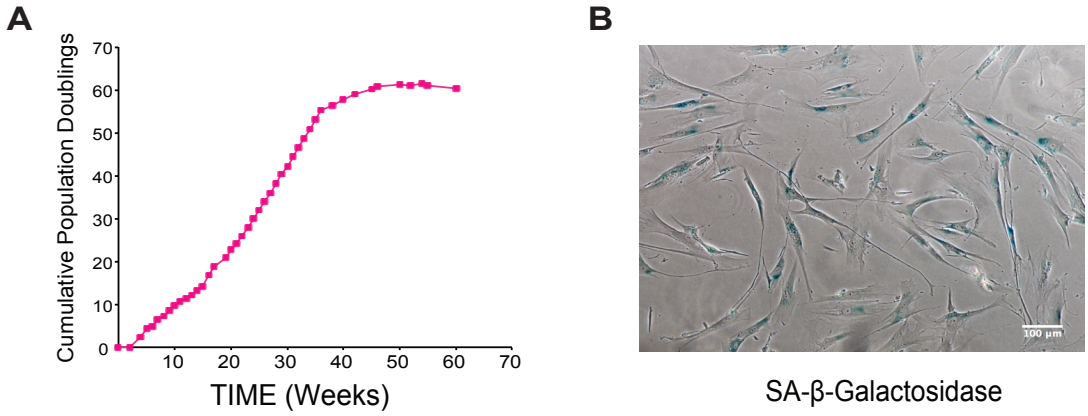

Figure 2.4 - Replicative senescence.

A. Replicative lifespan of primary human fibroblasts passaged until replicative senescence (plateau), confirmed by the presence of SA-β-galactosidase activity (**B**) in cells with cumulative population doublings (cPD) > 60. Scale bar = 100µm.

To study oncogene-induced senescence (OIS), IMR90 human fibroblasts expressing the ER:*H-RAS*^{V12} inducible system were obtained from Dr. Acosta (MRC Institute of Genetics & Molecular Medicine, Edinburgh, UK). These cells have been previously validated as a model of OIS (Acosta et al., 2013). In this model, treatment with 4-hydroxytamoxifen (4OHT) activates the oncogene *H-RAS*^{G12V,} resulting in the induction of a stable cell growth arrest and expression of senescence markers, such as p16^{INK4a} and SA-β-galactosidase, between 4-7 days of treatment (**Figure 2.5**).

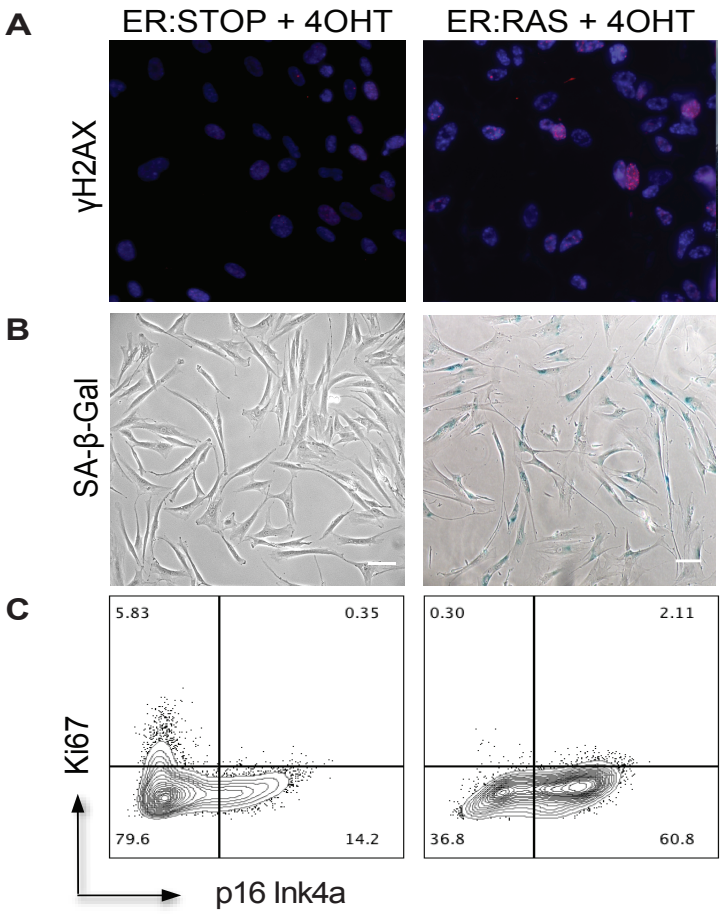

Figure 2.5 – Oncogene-induced senescence.

IMR90 cells infected with a vector expressing *H-RAS*^{G12V} or a control vector (STOP) associated with the tamoxifen-induced ER fusion protein were treated with 200 nM of tamoxifen (4OHT) for 7 days and analysed for the expression of senescence markers. **A)** Immunofluorescence staining of γH2AX (Ser139); **B)** Histochemical staining for SA-β-galactosidase activity; **C)** Flow cytometry analysis of Ki67 and p16^{INK4a} expression demonstrating an increased expression of p16^{INK4a} and a decrease in Ki67 expression after the activation of *H-RAS*^{G12V}. Images are representative stainings of 3 different experiments with similar findings.

2.3.2 Senescent cells express non-classical Major Histocompatibility Complex molecules

To investigate the effects of senescence on MHC expression, a comprehensive analysis of the expression of different MHC molecules was performed, using flow cytometry. The surface expression of classical MHC-class I and II molecules, as well as the non-classical MHC class Ib molecules (HLA-E, -F and –G) and the MHC-related proteins of the MICA/B and ULBP families were monitored on primary human fibroblasts after exposure to ionizing radiation.

Exposure to ionizing radiation induced a significant change in the expression of MHC molecules at the surface of primary human fibroblasts. The most significant changes were observed with HLA-E and MICA/B expression, with a two-fold induction after irradiation relative to non-irradiated cells ($p < 0.05$, **Figure 2.6A**). As expected, the baseline expression of these ligands was very low and virtually no MICA/B or HLA-E-positive cells could be found in normal human fibroblasts. A time-course analysis of cell surface expression of the two ligands revealed a delayed kinetics with a maximal up-regulation observed between 7-14 days after irradiation (**Figure 2.6B and 2.6C**), as they developed features of senescence suggesting that the expression of these immune ligands is stable, associated with the establishment of senescence and not only due to acute DNA damage.

Total MHC class I expression (detected with W6/32 antibody clone that recognizes an epitope shared by HLA-A, -B and –C) and MHC class II expression did not change significantly after the induction of senescence nor did other non-classical MHC class I molecules, such as HLA-G. Regarding ULBP molecules, although an increased expression was observed after irradiation, the fold induction relative to normal cells did not reach significant levels (**Figure 2.6A**).

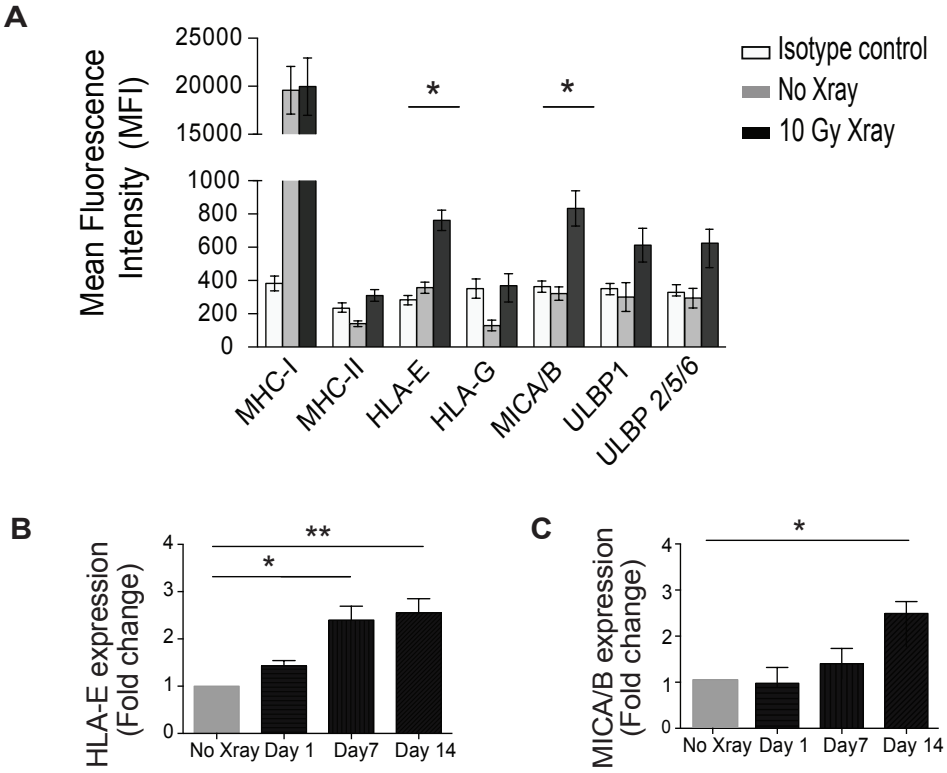

Figure 2.6 – Senescent fibroblasts have an altered expression of MHC molecules, with a significant upregulation of HLA-E and MICA/B relative to normal cells.

Primary human fibroblasts were irradiated with a senescence-inducing dose of X-ray (10Gy) and analysed for the surface expression of the indicated MHC molecules, compared to non-irradiated controls grown in similar culture conditions. **A)** Cumulative data (n=6 donors) of MHC expression on senescent fibroblasts (black bars) at day 14 post-irradiation, presented as the mean fluorescence intensity (MFI) for each of the indicated molecules, compared to non-irradiated controls (grey bars). Isotype controls for each antibody were performed in irradiated cells to account for background fluorescence (white bars). Comparison between irradiated and non-irradiated cells was calculated using Mann-Whitney *U* test (*$p< 0.05$). **B)** Time-course analysis of HLA-E and MICA/B (C) expression post-irradiation, assessed by flow cytometry and presented as the MFI fold change relative to baseline (before irradiation), set as 1 (grey bar). Analysis of variance between timepoints performed with repeated measures Anova with Bonferroni adjustment for multiple comparisons (*$p< 0.05$, **$p< 0.01$).

To assess whether this pattern of MHC expression was common to other forms of senescence, MHC expression was analysed on cells induced to senesce via the activation of *H-RAS*^{G12V} or continuous passaging to replicative senescence. Regardless of the senescence-inducing stimuli, cells undergoing senescence presented a common signature of MHC expression with a consistent induction of HLA-E and MICA/B. This phenotype was also common to other types of cells, such as endothelial cells (HUVECs) (**Figure 2.7**).

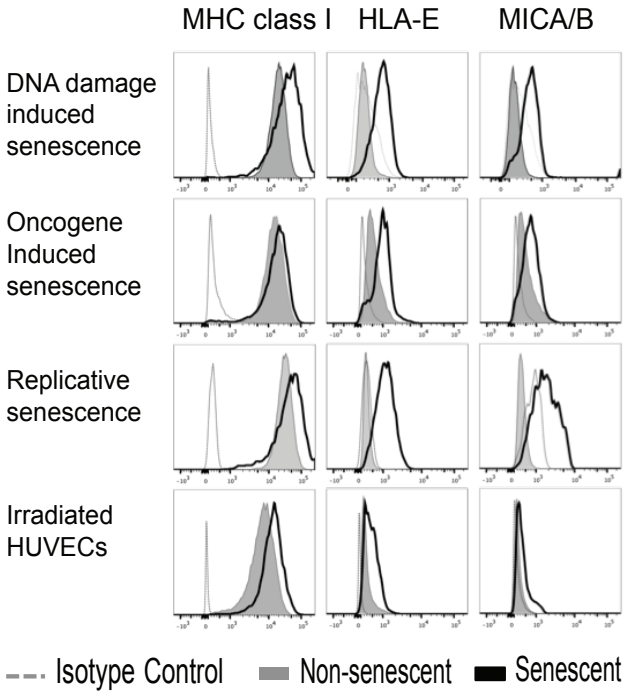

Figure 2.7- Overexpression of MICA/B and HLA-E is a common signature of different forms of senescence.

Representative FACS plots showing a similar pattern of expression of HLA-E and MICA/B in different forms of senescence: senescence was induced in human fibroblasts using irradiation (DNA-damage induced senescence), *H-RAS* activation (oncogene-induced senescence) or continuous passaging (replicative senescence) and the mean fluorescence intensity (MFI) of the represented MHC molecules compared between senescent (black lines), non-senescent (filled histograms) and isotype controls (dashed lines). Human umbilical vein endothelial cells (HUVECs) were irradiated (10 Gy X-ray) and MHC expression analysed by flow cytometry, as described for human fibroblasts.

Taken together, these results indicate that senescent cells have the means of communicating with the immune system by up-regulating non-classical MHC class I molecules that are not expressed under basal conditions. The expression of MICA/B and other NKG2D ligands by senescent cells has been described in recent papers (Iannello et al., 2013, Sagiv, 2016). To the best of my knowledge, the up-regulation of HLA-E expression in the context of senescence has never been reported before.

2.3.3 HLA-E positive cells exhibit features of senescent cells

To confirm whether cells expressing HLA-E were in fact senescent cells, flow cytometry and immunofluorescence microscopy were used to analyse the co-expression of HLA-E with senescence-associated markers. As illustrated in the representative flow cytometry plots in **Figure 2.8A**, HLA-E positive cells rarely expressed Ki67 yet the expression of the tumour suppressor $p16^{Ink4a}$ was frequently found (**Figure 2.8B)**, indicating that the majority of HLA-E expressing cells were in cell cycle arrest.

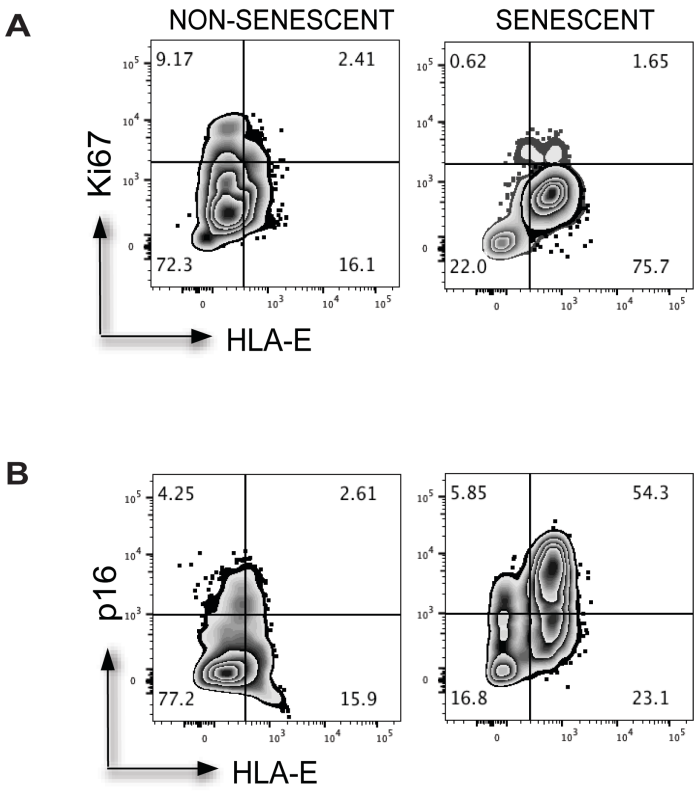

Figure 2.8 – HLA-E expressing cells exhibit high levels of p16Ink4a and low expression of Ki67.
Flow cytometry analysis of the co-expression of HLA-E with Ki67 (**A**), and HLA-E with $p16^{Ink4a}$ (**B**) in senescent cells compared to non-senescent. Numbers indicate percentages of cells per quadrant. Images are representative of two experiments (one performed with irradiated-senescent fibroblasts and other with IMR90 ER:RAS+4OHT compared to controls).

Immunofluorescence microscopy confirmed the expression of HLA-E at the surface of senescent cells that concomitantly presented nuclear DNA damage foci of phosphorylated γH2AX, whereas none of these were found in controls (**Figure 2.9A).** The same cells stained positively for the presence of senescence-associated β-galactosidase using histochemistry (**Figure 2.9A, left panel**), further indicating that HLA-E expressing cells exhibit the markers of senescent cells.

Importantly, the kinetics of HLA-E expression followed the pattern observed for other senescence-associated markers, in particular the activation of p38MAPK which has been previously associated with senescence (Freund et al., 2011, Tivey et al., 2013, Xu et al., 2014). In fact, as shown in the representative immunoblot (**Figure 2.9B**), the expression of HLA-E follows a similar pattern to that of p38MAPK phosphorylation (at Thr180/Tyr182), with a slow increase after radiation, reaching a peak at day 7 and maintained at day 14. By contrast, the phosphorylation of γH2AX follows a different pattern with a striking increase immediately after irradiation and slow recovery thereafter, suggesting that HLA-E up-regulation is not associated with the DNA-damage response.

In summary, from these data it may concluded that HLA-E positive cells exhibit markers of senescence, suggesting that HLA-E expression is also part of the senescence phenotype in fibroblasts.

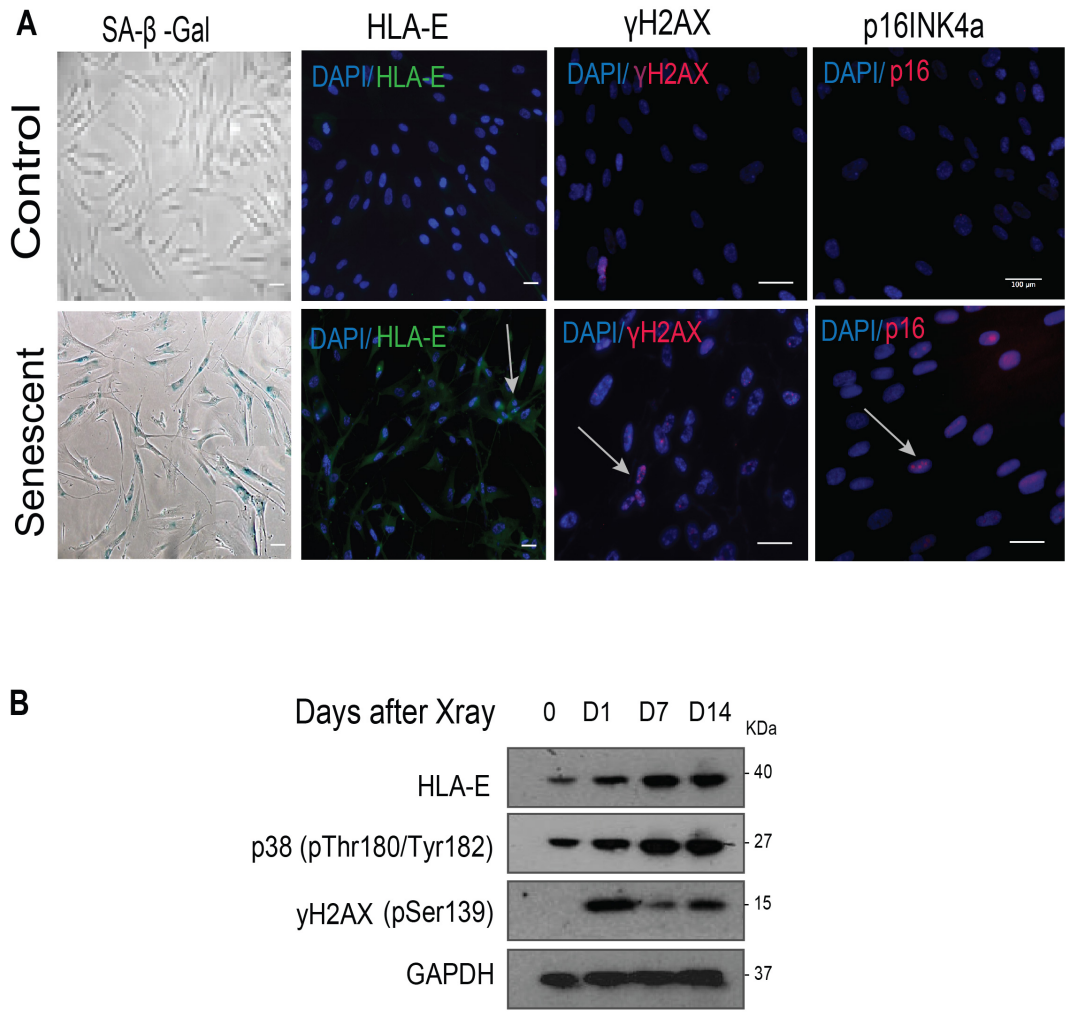

Figure 2.9 – HLA-E expressing cells express senescence-associated markers.

A) Immunofluorescence staining of irradiated primary human fibroblasts (day 14 after Xray 10 Gy) compared to non-irradiated controls for the expression of HLA-E, γH2AX (pSer139) and $p16^{INK4a}$. The same cells stained for senescence associated-β-galactosidase (SA-β-Gal) using histochemistry (left panel). **B)** Western blot analysis performed on lysates from cells harvested at the indicated time points after irradiation showing similar kinetics for HLA-E expression and the phosphorylation of p38MAPK. Phosphorylated γH2AX (ser139) is also shown but following different kinetics. GAPDH was used a loading control. Numbers indicate molecular weights (kDa) of blotted proteins. Representative immunoblot of three different experiments.

2.3.4 HLA-E expression on senescent cells is regulated by p38MAPK

To understand the mechanisms regulating HLA-E expression on senescent cells we investigated two major signaling pathways involved in senescence: the DNA-damage response (DDR) and the p38MAPK-signaling pathway.

The DDR has been previously implicated in the regulation of MICA/B expression on human and mouse fibroblasts exposed to genotoxic stress (Gasser et al., 2005, Soriani et al., 2009, Cerboni et al., 2014). Therefore, it was investigated whether the DDR was also involved in the modulation of HLA-E expression after irradiation. To this end, cells were pretreated with the specific ATM inhibitor KU-55933 (10μM), 12 hours before irradiation and continuously thereafter, over a period of 7 days. MHC expression was monitored by flow cytometry at day 1, 4 and 7 post-irradiation, compared to vehicle-treated cells and non-irradiated controls. Consistent with previous reports, inhibition of ATM significantly decreased MICA/B expression after irradiation (**Figure 2.10A**, $p < 0.05$), corroborating the role of the DDR in maintaining MICA/B expression on senescent cells.

Notably, the expression of HLA-E on senescent fibroblasts was not substantially altered after ATM inhibition (**Figure 2.10B**). These results were further confirmed with the use of an alternative small molecule inhibitor of both ATM and ATR (CGK733, 10μM), suggesting that canonical DDR signaling is not necessary for the senescence-associated expression of HLA-E (data not shown).

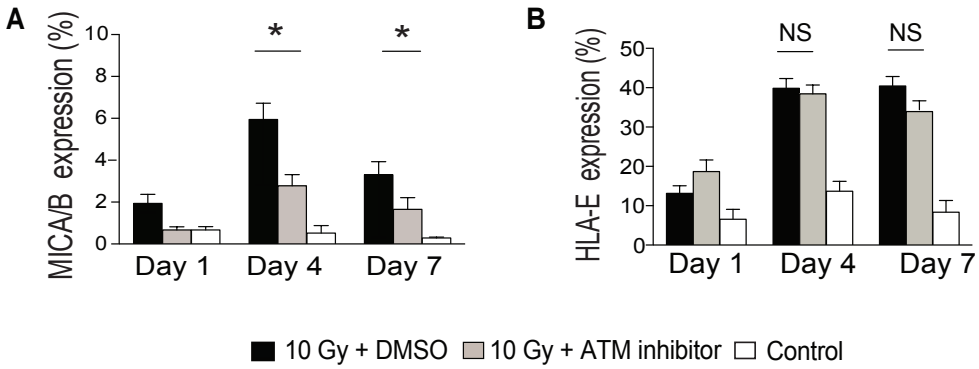

Figure 2.10 – ATM inhibition prevents MICA/B upregulation but not HLA-E expression after radiation.

Primary human fibroblasts were treated with 10μM of the ATM inhibitor KU55933 12 hours before irradiation and continuously thereafter, over a period of 7 days. Cells were harvested at the indicated time points and compared to DMSO-treated and non-irradiated controls for the surface expression of HLA-E and MICA/B by flow cytometry. Summary data (n=4) of the effect of ATM inhibition on MICA/B (**A**) and HLA-E expression (**B**), presented as the percentage of cells expressing the indicated ligands within the different conditions. Comparison between treated and vehicle-treated groups performed with the Mann-Whitney *U* test (*$p < 0.05$).

Sustained activation of p38MAPK is a hallmark of senescence (Freund et al., 2011) and the parallel kinetics of HLA-E expression and p38MAPK activation (**Figure 2.9B**) prompted us to study the role of p38MAPK signaling in the regulation of HLA-E expression on senescent cells. To investigate this, cells were treated with BIRB796, a small molecule that specifically inhibits p38MAPK at a dose of 0.5μM, as previously described (Di Mitri et al., 2011), before and after irradiation for 7 days. Similarly, cells were harvested at day 1, 4 and 7 post-irradiation to monitor HLA-E expression by western blotting (**Fig 2.11A**) and flow cytometry (**Figure 2.11C**). As indicated by the levels of phosphorylated heat-shock protein 27 (at serine 78), a downstream target of p38MAPK (Zarubin and Han, 2005), treatment with BIRB796 effectively blocked the activation of p38MAPK after irradiation (**Figure 2.11A**). Strikingly, treatment with this inhibitor also prevented the up-regulation of HLA-E after irradiation, suggesting that activation of p38MAPK is required for HLA-E expression (**Figure 2.11A**).

Similar observations were obtained in the oncogene-induced senescence (OIS) model, where treatment with BIRB796 significantly inhibited HLA-E up-regulation after the induction of senescence upon oncogene activation (**Figure 2.11B**).

The role of p38MAPK signaling in the regulation of HLA-E expression was further corroborated by flow cytometry monitoring HLA-E expression on cells pretreated with the small molecule SB203580, which inhibits p38MAPK through a different mechanism to BIRB796, thus excluding off-target effects of the drugs (**Figure 2.11C-D**). Interestingly, we did not find a significant effect of p38MAPK inhibition on MICA/B expression (**Figure 2.11E**), indicating that MICA/B and HLA-E expression on senescent cells are regulated by different signaling pathways.

Together, these observations point out to an extrinsic mechanism of regulation of HLA-E expression through the stress-induced p38MAPK, rather than a DNA-damage driven response as observed for MICA/B.

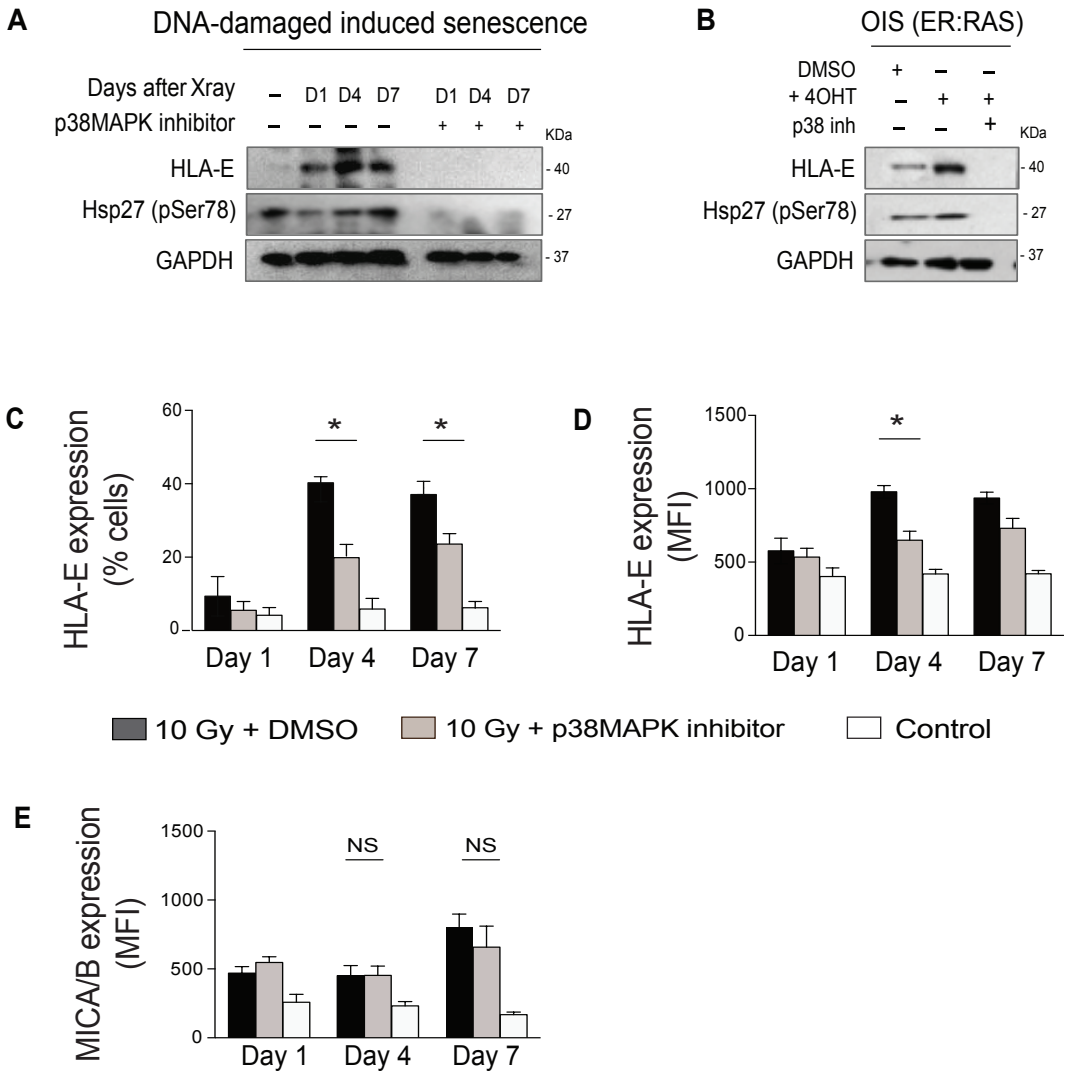

Figure 2.11 – HLA-E expression on senescent fibroblasts is regulated by p38MAPK signaling.

A) Primary human fibroblasts were treated with BIRB796 (0.5μM) 12 hours before irradiation and continuously after that, over 7 days. Expression of HLA-E was monitored by western blot at the indicated time points after irradiation and compared to DMSO-treated controls. Undetectable levels of phosphorylated Hsp27 confirm an effective inhibition of p38MAPK by BIRB796. **B)** IMR90 ER:RAS cells were treated with DMSO (first lane), 4OHT (second lane) or 4OHT + BIRB796 (third lane) and subjected to western blot for the analysis of HLA-E expression and pHsp27. GAPDH served as loading control. **C-E)** Cumulative data (n=4) of HLA-E and MICA/B expression analysed by flow cytometry on irradiated fibroblasts treated BIRB796 (0.5μM) or SB203580 (10μM), compared to DMSO-treated and non-irradiated controls. Comparison between treated and vehicle-treated groups performed with the Mann-Whitney *U* test (*$p < 0.05$).

2.3.5 HLA-E expression is induced by SASP-associated proinflammatory cytokines

The proposed role of p38MAPK as a major regulator of the senescence-associated secretory phenotype (SASP) (Freund et al., 2011) prompted us to investigate whether SASP factors could induce HLA-E expression on neighbouring cells, in a non-cell-autonomous manner (paracrine effect). To this end, early-passage fibroblasts were exposed to conditioned medium (CM) from senescent or non-senescent cells and HLA-E expression was monitored by flow cytometry, 48 hours after exposure. When indicated, a p38MAPK inhibitor (BIRB796) was added for 12 hours before collection of CM to block the secretion of SASP factors by senescent cells (Freund et al., 2011). It was found that HLA-E expression could indeed be induced 48 hours after exposure to CM from senescent cells ($p < 0.05$, **Figure 2.12**). Inhibition of p38MAPK activity during CM collection from senescent cells partially prevented the up-regulation of HLA-E on normal cells, although not significantly ($p > 0.05$, **Figure 2.12).**

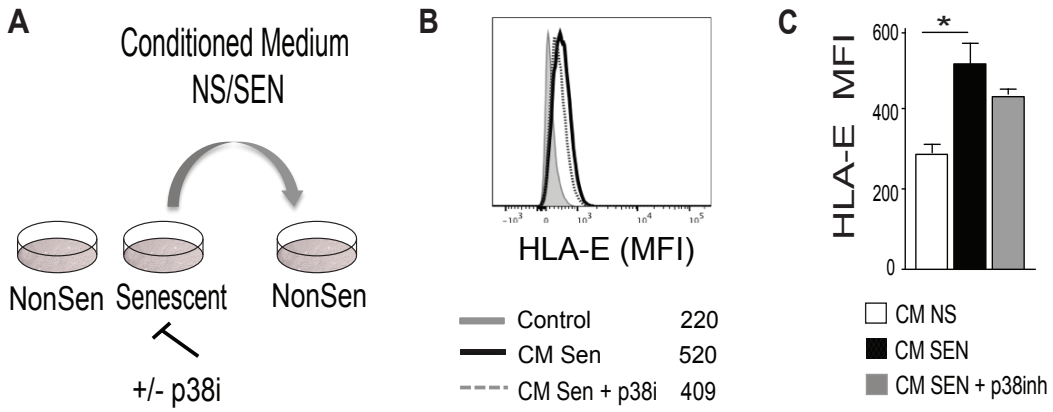

Figure 2.12 - Treatment of normal cells with conditioned medium from senescent cells induces an up-regulation of HLA-E expression, partially prevented by p38MAPK inhibition.

A) Normal fibroblasts were exposed to conditioned medium (CM) from senescent or non-senescent cells for 48 hours and the expression of HLA-E was analysed by flow cytometry. In one condition, a p38 inhibitor (p38i) was added for 12 hours before collection of CM from senescent cells. **B)** Representative FACS histograms of HLA-E expression after exposure to CM from senescent and non-senescent cells. Numbers indicate Mean Fluorescence Intensity (MFI). **C)** Summary data (n=3) acquired as in A and B.

Using cytometric bead arrays (CBA) we investigated the secretion of SASP-factors by irradiated primary human fibroblasts and identified IL-6, IL-8 and MCP-1 as the most highly secreted factors, compared to normal early-passage fibroblasts (**Figure 2.13A).** Exposure of normal early passage fibroblasts to treatment with some of these factors, revealed a significant effect of IL-6, individually or a combination of all factors, in inducing a significant up-regulation of HLA-E expression, not evident when cells were treated with IL-8 or IL-1β individually (**Figure 2.13B).**

IL6 has been previously implicated in many of the SASP-induced effects on surrounding cells (Coppe et al., 2010a). Moreover, increased levels of IL6 have been found in the serum of elderly patients, corroborating a role of this cytokine in "inflammaging" (Franceschi and Campisi, 2014). Collectively, these findings support a model in which the low-level chronic inflammation that underlies the SASP and aging contributes to the modulation of the expression of HLA-E on senescent cells.

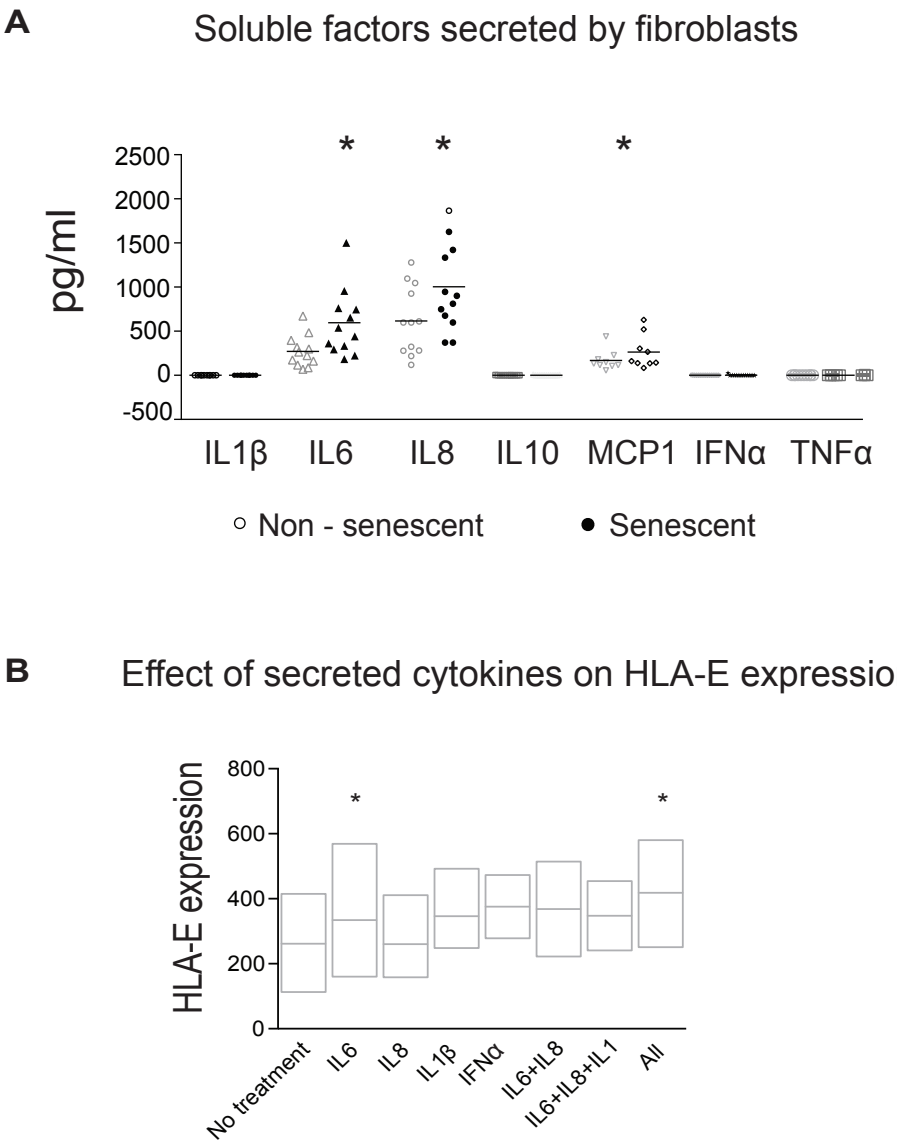

Figure 2.13 – Effect of soluble factors secreted by primary human fibroblasts on HLA-E expression.

Supernatant of irradiated senescent primary human fibroblasts or early-passage normal fibroblasts were collected and analysed by cytokine bead arrays. **A)** Summary data (n=12) of the concentration of the indicated cytokines (in pg/mL) demonstrating a significant increase in the secretion of IL-6, IL-8 and MCP1 by senescent cells relative to normal cells. The lower limit of detection was 1.5 pg/ml. **B)** Normal fibroblasts were exposed to IL-6 (20 ng/mL), IL-8 (20 ng/mL), IL-1β (20 ng/mL) or IFNα (500U/mL) or a combination of these, for 48 hours and analysed for the expression of HLA-E by flow cytometry. Summary data of 3 different experiments. Comparison between groups performed with Kruskal-Wallis test (*$p < 0.05$).

2.3.6 Expression of HLA-E on senescent cells in the p16-3MR reporter mouse model

To confirm the previous findings *in vivo*, the p16-3MR reporter mouse model was used to investigate whether senescent cells express the mouse homolog of HLA-E, the ligand Qa-1. This transgenic progeroid mouse model allows the identification of $p16^{INK4a}$ positive senescent cells via the concomitant expression of luciferase, which can be detected by luminescence. Most importantly, this model allows the selective deletion of senescent cells via the administration of ganciclovir, which selectively targets cells expressing $p16^{INK4a}$ and the herpes simplex virus 1 (HSV-1) thymidine kinase (HSV-TK) (Demaria et al., 2014). To induce senescence mice were treated with the chemotherapeutic drug bleomycin, an agent that causes pulmonary fibrosis in mice and has been shown to induce cellular senescence in lung epithelial cells (Aoshiba et al., 2003, Schafer et al., 2017).

Mice were treated with PBS or bleomycin for 14 days, with or without ganciclovir to eliminate senescent cells and whole lungs were harvested for analysis of Qa-1, $p16^{INK4a}$ (as a marker of senescence) and collagen expression (as a marker of fibrosis) by quantitative polymerase chain reaction (qRT-PCR). An increase in Qa-1 and $p16^{INK4a}$ mRNA levels were found in bleomycin-treated mice compared to PBS-treated controls, which were decreased after elimination of senescent cells with ganciclovir (**Figure 2.14 C,D**). Collagen mRNA levels followed the same trend as Qa-1 and $p16^{INK4a}$, suggesting that fibrosis is associated with the development of senescence and alleviated when senescent cells are cleared (**Figure 2.14D**).

Although further investigation would have been necessary to directly prove that Qa-1 is expressed by p16-positive senescent cells, these findings support the hypothesis that HLA-E /Qa-1 expression is associated with senescence, both *in vitro* an *in vivo*.

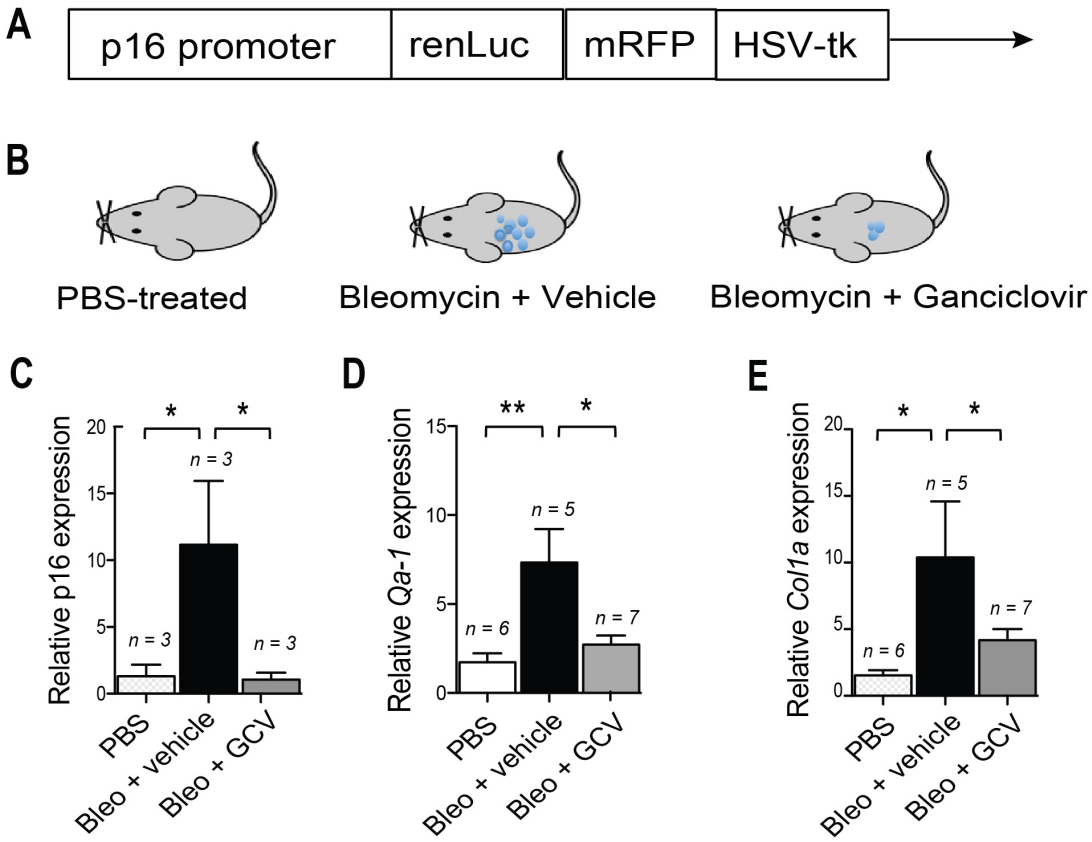

Figure 2.14 – Expression of Qa-1 (the mouse homolog of HLA-E) in the p16-3MR model of senescence.

A) Schematic of the p16-3MR (trimodality reporter) fusion protein, which contains functional domains of a synthetic Renilla luciferase (LUC), monomeric red fluorescent protein (mRFP), and truncated herpes simplex virus 1 (HSV-1) thymidine kinase (HSV-TK) associated with the p16 promoter. **B)** p16-3MR mice were treated with bleomycin (intra-tracheal injection of 1.9 UI/Kg), ganciclovir (GCV,25 mg/kg; daily i.p. injections) or PBS; **C-E)** qRT-PCR was used to quantify mRNA levels encoding $p16^{INK4a}$ (C), Qa-1 (D) and collagen1a (E) in lung tissue isolated from mice treated with PBS (white bar), bleomycin + vehicle control (black bars) and bleomycin + GCV (grey bars), (n=3 in each group). mRNA levels for the indicated genes were normalized to tubulin and presented as the relative fold difference to PBS-treated mice, set as 1. Statistical analysis performed with Kruskal-Wallis test with Dunn´s multiple comparison test.

2.4 DISCUSSION

In the present chapter, after establishing a valid model of premature senescence in primary human fibroblasts derived form the skin of healthy volunteers, an extensive analysis of the expression of MHC molecules on senescent cells relative to the non-senescent counterparts was performed, using flow cytometry. It was found that senescence is associated with a significant change in the surface expression of MHC molecules, with a significant increase in the expression of non-classical MHC molecules that have restricted expression on normal cells. Alongside the previously described up-regulation of MICA/B, we identified HLA-E as a novel immune ligand that is significantly up-regulated on senescent cells.

The up-regulation of MICA/B and other NKG2D ligands after DNA damage and the induction of senescence has been previously reported and implicated in the recognition by NK cells (Gasser et al., 2005, Krizhanovsky et al., 2008, Soriani et al., 2009, Iannello et al., 2013, Sagiv, 2016). By contrast, to our knowledge, the up-regulation of HLA-E in the context of senescence has never been described although microarray analysis of the transcriptome of human replicative senescent fibroblasts, included HLA-E in the list of senescence-regulated genes more than twofold up-regulated, as compared to young growing cells (Lackner et al., 2014).

Whilst the expression of this immune ligand is not specific to senescence, as it has also been reported in cancer and viral-infected cells (Tomasec et al., 2000, Kochan et al., 2013), the restricted expression of HLA-E on normal cells suggests that a pathological response may be driving the expression of HLA-E.

Mechanistically, it was demonstrated that the expression of HLA-E is regulated by p38MAPK signaling, independently of the DNA damage response that regulates the expression of activating immune ligands such as MICA/B. This finding supports a

theory where senescence may have two opposite sides – one beneficial and dependent on the activation of the DNA-damage response and the other detrimental, regulated by p38MAPK. Indeed, the activation of the DDR, which is responsible for cell growth arrest, leads to the expression of activating immune ligands such as MICA/B to ensure an eventual clearance of abnormal cells. In contrast, the activation of p38MAPK associated with the secretion of proinflammatory cytokines, leads to the expression of HLA-E which, (as it will be explored in the second chapter) may prevent an effective elimination of senescent cells.

Previous studies have reported that pro-inflammatory cytokines such as IL-1β, TNFα and IFNγ can induce the expression of HLA-E on endothelial cells (Coupel et al., 2007). We identified IL-6 as an important soluble factor secreted by senescent primary human fibroblast that induces HLA-E expression in normal neighbouring cells, in a paracrine fashion. IL-6 has been previously implicated in many of the SASP-induced effects on surrounding cells, including epithelial-to-mesenchymal transition (Laberge et al., 2012, Krtolica et al., 2001), tumour progression (Barnes et al., 2011, Kumari et al., 2016) as well as fibrinogenesis and endothelial cell activation (Coppe et al., 2010a, Barnes et al., 2011). Moreover, increased levels of IL-6 and IL-8 have been found in the serum of elderly patients, contributing to the systemic low-level chronic inflammation that has been linked to many age-related diseases (Franceschi and Campisi, 2014). The modulation of HLA-E expression by IL-6 thus suggests that sustained inflammation may contribute to the persistence of senescent cells in tissues (through a mechanism of immune evasion that we will explore in the following chapter), further contributing to the pathogenesis of age-related diseases. This mechanism may be common to other inflammatory settings, explaining why HLA-E expression is not only found in senescence.

We cannot exclude that other transcriptional and post-translational mechanisms may be involved in the regulation of HLA-E expression. In fact, HLA-E expression is also

regulated at a post-translational level through the *de novo* generation of peptides that will be presented by HLA-E and stabilize its expression at the cell surface (Braud et al., 1998b, Bland et al., 2003). Previous studies have demonstrated that the stability of HLA-E molecules at the cell surface depends on the availability of highly conserved 9mer peptides commonly generated after trimming of the leader sequence of classical MHC class I molecules (Lee et al., 1998, Llano et al., 1998, Petrie et al., 2008). However, in recent years it has been demonstrated that HLA-E can present a much more diverse repertoire of peptides, derived not only from viral particles, such as during CMV, EBV and HCV infection, but also from intracellular self-proteins, such as heat-shock proteins (Michaelsson et al., 2002, van Hall et al., 2010). This deserves further investigation to explore whether HLA-E can present self-peptides derived from intracellular signalling pathways activated in senescent cells and use these as reporters of cellular stress.

The activation of p38 MAPK has been implicated in other forms of senescence, not only premature senescence induced by irradiation or oncogene activation, but also in replicative senescence and in premature aging syndromes, such as Werner Syndrome and Ataxia Telangiectasia (Iwasa et al., 2003, Davis and Kipling, 2009). Prof.Akbar´s group has also demonstrated how p38 MAPK activation is involved in the regulation of some of the most important features of senescence in T cells (Di Mitri et al., 2011, Henson et al., 2014, Lanna et al., 2014). It would be interesting to explore whether these forms of senescence are also associated with an up-regulation of HLA-E and MICA/B.

As for MICA/B, HLA-E expression is frequently up-regulated in a wide variety of cancers and during viral infections. In some types of solid tumours, such as melanoma, surface expression of HLA-E was reported as an independent risk factor for adverse patient outcome and prognosis. Interactions with the immune system are crucial to the effects of HLA-E expression under physiological and pathological

conditions. HLA-E is recognized by both activating (NKG2C) and inhibitory (NKG2A) receptors, which are commonly found on NK cells. In the following chapter, the interplay between senescent cells and the immune system mediated via the interaction between MICA/B and HLA-E with the respective specific receptors expressed on immune cells will be investigated.

In conclusion of this first part of the study, it is proposed that an altered MHC expression profile is a feature of senescence that may be involved in the communication with the immune system and regulation of the immune surveillance of senescent cells.

2.5 MATERIALS AND METHODS – CHAPTER ONE

2.5.1 Skin explant culture for derivation of primary human fibroblasts

Primary human fibroblasts were derived from skin obtained from 4mm punch biopsies taken in sun-protected areas of the forearm of previously consented volunteers in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Royal Free Hospital and University College London. Donors did not have any co-morbidity nor were on any immunosuppressive drugs that could influence the results. Explanted skin was anchored onto the bottom of a six-well culture plate (VWR International) in Dulbecco modified Eagle's medium (DMEM; Invitrogen, UK), supplemented with 20% fetal calf serum (FCS), 2 mM glutamine and 100 IU/ml penicillin/streptomycin (all from Sigma Aldrich, UK) and placed in a humidified incubator at 37°C and 5% CO2, as previously described (Vangipuram et al., 2013). In this skin explant culture, fibroblasts appear 7-14 days after the first outgrowth of keratinocytes and are eventually selected over keratinocytes by the use of DMEM supplemented with 20% FBS. Media was changed every 2-3 days after attachment and outgrowth of cells. Once the monolayers had reached confluence the fibroblasts were passaged and cultured in 25-cm^2 tissue culture flasks.

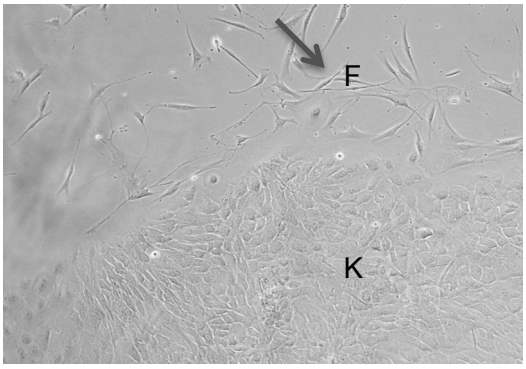

Figure 2.15 – Microscopic appearance of a skin-explant culture.
Images obtained at day 7 showing fibroblasts (F) outgrowing from the keratinocyte layer (K).

2.5.2 Cell culture

Primary human fibroblasts were cultured in DMEM supplemented with L-glutamine (2 mM), 100 U/ml of streptomycin–penicillin and 10% FCS, at 37°C in 5% CO_2 humidified incubator. Cells were passaged into new flasks whenever they had reached 80% confluence and cell count and viability was assessed using the trypan blue method.

Where indicated, population doublings (PD) were calculated using the following equation: PD log (number of cells counted after expansion)-log (number of cells seeded)/log2.

2.5.3 Induction of senescence

Cells were irradiated with a total dose of 10 Gy (X-ray) at rates of 5 Gy/min, using the following settings (AGO-HS X-ray machine): 215 Kv per 12 mA, using an aluminium filter at a distance of 30 cm. Cells were allowed to recover in culture and harvested after trypsinization at different time points for the analysis of the expression of senescence-associated markers and changes in MHC expression, as indicated in each section. Early-passage fibroblasts (between passages 3 and 9) were used as controls.

A dose of 10Gy of X-ray was established based on preliminary experiments, in which doses between 2Gy and 20Gy were tested to determine the lowest effective dose in inducing senescence without increasing the levels of apoptosis, measured by the detection of Annexin V using flow cytometry.

2.5.4 Oncogene-induced senescence

IMR90 ER:RAS and IMR90 ER:STOP cells obtained from Dr. Juan Carlos Acosta (MRC Institute of Genetics & Molecular Medicine, Edinburgh, UK) were used to model oncogene-induced senescence as previously described (Acosta et al., 2013).

Briefly, IMR90 human diploid fibroblasts transduced with a vector expressing *H-RAS*^{G12V} or a control vector (STOP) associated with the tamoxifen-induced ER fusion protein were treated with 4-hydroxytamoxifen (4OHT) (Sigma Aldrich, St Louis, Mo, USA) at 200 nM to activate *H-RAS*^{G12V} oncogene. After 7 days of treatment, cells were harvested and analysed for the expression of senescence markers and for the analysis of MHC expression.

2.5.5 Treatment with inhibitors

ATM signaling was inhibited using the specific ATM inhibitor KU55933 (EMD Millipore) at a final concentration of 10μM, as previously described (Hickson et al., 2004). Alternatively, the ATM/ATR inhibitor CGK733 (Calbiochem), at 10μM final concentration, was used as previously described (Alao and Sunnerhagen, 2009).

Signaling through p38 MAPK was inhibited using the small-molecule BIRB796 (Selleck Chemicals), at a final concentration of 0.5μM in the culture media or using the small molecule SB203580 (Calbiochem), at 10μM final concentration, as previously described (Di Mitri et al., 2011, Freund et al., 2011).

Where indicated, cells at 80% confluence were pre-treated with specific inhibitors, 12 hours before irradiation and continuously over a period of 7 days, with daily replenishment with fresh media and inhibitors. A solution of 0.1% DMSO in DMEM was used as vehicle-control.

2.5.6 Treatment with Conditioned Medium and Cytokines

Conditioned media (CM) was collected after incubation of senescent versus non-senescent cells with serum-free DMEM for 24 h, as previously described (Freund et al., 2011). After that CM were collected, clarified by centrifugation and filtration and diluted 1:1 with 10%FBS-DMEM. Normal fibroblasts (between passages 3-9) were incubated with the diluted CM for 48 hours until analysis of HLA-E expression by flow cytometry. When indicated, a p38MAPK inhibitor (BIRB796, 0.5μM) was added along the culture with CM to block the secretion of SASP factors by senescent cells and observe the effects on HLA-E expression.

For cytokine treatment, recombinant human cytokines IL-6 (20 ng/mL), IL-8 (20 ng/mL), IL-1β (20 ng/mL) or IFNα (500U/mL, all from Peprotech,UK) were diluted in complete DMEM at the indicated final concentrations.

2.5.7 Cytometric Bead Array

To measure soluble factors secreted by senescent fibroblasts compared to non-senescent controls, Cytometric Bead Arrays (CBA, BD Biosciences) were performed, by Dr. Emma Chambers. Briefly, conditioned media from senescent and normal early-passage fibroblasts were collected as previously described. 50μl of each sample was incubated with 20μl of cytokine-specific beads in a microtitre plate, for 3hours at room temperature (protected from light). After that, plates were washed twice and 20μl of detection antibody was added for a further 2 hours, at room temperature. After a final wash, samples were resuspended in FACS flow buffer and analysed in a BD Fortessa flow cytometer (BD Biosciences).

2.5.8 Flow cytometry

For fibroblast staining, cells were washed in PBS and harvested after trypsin treatment, centrifuged at 1200 rpm for 10 minutes and resuspended in ice-cold 1xPBS. Flow cytometric analysis of the surface expression of MHC molecules was performed after a 30-minute incubation at 4°C with the following antibodies: Live/Dead Fixable Blue Dead Cell Stain (Invitrogen, UK), HLA-ABC PE (clone W6/32, eBioscience, UK), HLA-E APC (clone 3D12, eBioscience, UK) and MICA/B Alexa-fluor 488 (clone 159207, R&D Systems, UK).

Proliferation of irradiated versus non-irradiated fibroblasts was assessed by staining for the nuclear antigen Ki67 using the PE or FITC-labelled mouse Anti-Human Ki-67 Set (BD Biosciences, UK), using the Foxp3 Staining Buffer Set (Miltenyi Biotec, UK), according to the manufacturer's instructions. The intracellular staining for p16 expression was performed using the same protocol as for Ki67, using the anti-CDKN2A/p16INK4a antibody conjugated to Alexa-fluor 647 (Abcam, UK).

Apoptosis was assessed using the FITC Annexin V Apoptosis detection kit (BD Biosciences, UK).

Samples were acquired on a BD LSR II or LSR Fortessa flow cytometer (BD Biosciences, UK) after fixation with 2% formaldehyde (Sigma-Aldrich, UK). Data were analysed using FlowJo software (Tree Star, Ashland, OR).

2.5.9 Phosphoflow cytometry

For the detection of phosphorylated γ-H2AX using flow cytometry, cells were fixed for 10 min at 37 °C with warm Cytofix Buffer (BD Biosciences). Cells were washed and permeabilized for 30 minutes at 4 °C with 100µl of ice-cold Perm Buffer III (BD Biosciences). Next, cells were washed twice in Stain Buffer (BD Biosciences) and

incubated for 30 minutes at room temperature with Alexa Fluor 488-conjugated antibody to γH2AX phosphorylated at Ser139 (clone 2F3; BioLegend). Cells were finally washed in Stain Buffer and immediately analysed by flow cytometry.

2.5.10 Senescence-associated β-galactosidase staining

Cells were fixed and stained using the Senescence-Associated β-Galactosidase Staining Kit (Cell Signaling, #9860), following the manufacturer´s instructions. After staining, cells were incubated for 12-16 hours at 37°C until visualization by phase-contrast microscopy.

2.5.11 Immunofluorescence

Cells were cultured in sterile 8-well chamber-slides (Nunc), fixed in 4% paraformaldehyde and permeabilized in PBS containing 0.5% Triton X-100 for 10 minutes. After blocking with protein block serum-free reagent (Dako) for 30 minutes, cells were incubated with primary antibodies overnight at 4°C, followed by a 1 hour incubation at room temperature with Alexa Fluor® 594 or Alexa Fluor® 488-conjugated secondary antibody (Invitrogen). After a final washing step, slides were mounted with Vectashield containing DAPI (Vector laboratory) and visualized on a Zeiss Axiovert 200M fluorescence microscope.

Primary antibodies used for immunofluorescence: rabbit polyclonal anti-histone γH2A.X Phospho (pS139) (Cell Signaling #9718), rabbit monoclonal to CDKN2A/p16INK4a (Abcam, ab108349) and mouse monoclonal to HLA-E purified (clone 3D12, eBioscience).

2.5.12 Western blot analysis

Cells were harvested after trypsinization, washed in PBS and lysed with Radio-Immunoprecipitation Assay (RIPA) buffer (Sigma-Aldrich, UK), supplemented with

protease and phosphatase inhibitors (GE Healthcare, Amersham, UK) during 30 minutes on ice. Concentrations of protein lysates were determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Fisher Scientific, UK). 10 or 20 µg of total protein were diluted in SDS sample buffer (NuPage, NP0007, Life Tecnologies) plus Sample Reducing Agent (NuPAge, NP0004) and boiled for 5' at 95°C to allow protein denaturation. Cell lysates were separated by protein electrophoresis at 120V for 2 hours using 10% Bis-Tris pre-cast gels (NuPage) and MOPS-SDS 1X running buffer (0,05 M MOPS; 0,05 M Tris; 0,1% (p/v) SDS; 1,025 mM EDTA). Proteins were wet-transferred overnight at 4°C onto Hybond-P PVDF membranes (GE Healthcare, Amersham, UK) with freshly-made transfer buffer (25 mM Tris; 200 mM glycine; 20% (v/v) methanol). After blocking for 1h at room temperature with 5% non-fat dry milk in PBS-Tween 0.1%, membranes were incubated with primary antibodies overnight at 4ºC. Membranes were then washed and incubated with HRP-conjugated secondary antibodies (GE Healthcare, Amersham, UK, 1:4000) for 1 hour at room temperature. After a final washing step, chemiluminescent reaction was detected using the ECL detection kit (GE Healthcare, Amersham, UK). Prior to re-probing with different antibodies, membranes were stripped at 37°C in agitation using Restore™ Western Blot Stripping Buffer (Thermo Scientific, 46430). The protein bands were quantified using Image J software (Version 1.44o). The integrated density of each band was measured using the gel analysis function of ImageJ, normalized to GAPDH.

Primary antibodies used for western blotting were: rabbit polyclonal anti-histone γH2A.X Phospho (pS139), anti-Hsp27 Phospho (pS78), anti-p38 MAPK Phospho (pThr180/Tyr182), anti-p53 and anti – GAPDH (all from Cell Signaling Technology). For HLA-E expression we used the mouse monoclonal anti-HLA-E (MEM-E/02, Santa Cruz Biotechnology), as previously described (Menier et al., 2003). All primary antibodies were used at a dilution of 1:1000.

2.5.13 The p16-3MR progeroid mouse model

All animal experiments with the p16-3MR mice were performed in Prof. Judith Campisi´s laboratory (Buck Institute for Research on Aging, Novato, California, USA), under protocols approved by the Buck Institute´s Comittee for Animal care and handling. This transgenic mouse model was developed by members of the Campisi lab (Demaria et al., 2014) to enable the detection and live tracking of $p16^{INK4a}$ positive senescent cells in vivo by luminescence via the concomitant expression of luciferase. This model also enables the selective deletion of senescent cells by treatment with ganciclovir, which has high affinity for the herpes simplex virus 1 (HSV-1) thymidine kinase (HSV-TK) and low affinity to cellular thymidine kinase, thus reducing possible toxic effects.

To induce senescence in these mice, bleomycin was administered via intra-tracheal instillation (1.9U/Kg, daily) during 14-21 days. Ganciclovir (25 mg/kg; daily) was administered via intra-peritoneal injection 5 days before the animals were sacrificed. Lung, liver and brain tissues were excised and analyzed by luminescence using a Xenogen imager or cryopreserved for future analysis.

2.5.14 RNA Isolation, RT-PCR, and Real-Time Quantitative PCR

Total RNA from mouse lung tissues was isolated using Directzol RNA miniprep (Zymo Research), following the manufacture's protocol. RNA was amplified and reverse-transcribed using TaqMan Universal PCR Master Mix (Applied Biosystems) as follows: 95°C for 10 min and 40 cycles of 95°C for 15 sec, 70°C for 5 sec, and 60°C for 1min. cDNA was analysed by real-time quantitative PCR using the Roche Universal Probe Library system (Indianapolis, IN, USA). mRNA levels were normalized to averages of actin and tubulin, unless noted otherwise. All reactions were performed in triplicate and included the following: 1 μL of cDNA; 5 μM of each primer; 2x SYBR Green PCR Master Mix (Applied Biosystems); and water added to a

final volume of 25 *μ*L. The relative amount of mRNA was determined using the comparative threshold (Ct) method by normalizing target cDNA Ct values to that of GAPDH. Fold increase ratios were calculated relative to the control (PBS) for each group using the formula $2e - \Delta\Delta Ct$.

Primer sequences and probes used:

- Mouse actin F 5'- CTAAGGCCAACCGTGAAAAG-3**'**, R 5' ACCAGAGGCATACAGGGACA-3**'**, UPL Probe #64;
- Mouse tubulin F 5'-CTGGAACCCACGGTCATC-3**'**; R 5'-GTGGCCACGAGCATAGTTATT-3**'**,UPL Probe #88;
- Mouse Qa-1 F 5´- AGCCCCTCACCCTGAGAT-3**'**, R 5´-ACCACAGCTCCAAGGATGAT-3**'**, UPL Probe #94;
- Mouse p16INK4a: F 5**'**-AATCTCCGCGAGGAAAGC-3**'**;R 5**'**-GTCTGCAGCGGACTCCAT-**3', #**91;

2.5.15 Statistical analysis

Statistical analysis was performed using GraphPad Prism Software, version 6.00 (GraphPad Software). Distribution of data was assessed by D'Agostino-Pearson or the Shapiro-Wilk test and depending on the normality of the data, analysis was performed using parametric or non-parametric tests: Student *t* test or the Mann-Whitney *U* test for comparison between two groups; If more than two groups were compared simultaneously, one-way ANOVA (for parametric data) or the Friedman test (for non-parametric data, paired samples) or Kruskal Wallis test (non-parametric data, non-paired) were applied, followed by post-hoc tests for multiple comparison adjustments. Probability values less than 0.05 were considered significant. Data are presented as mean ± standard error of the mean (SEM), unless otherwise stated.

Tests were used to determine data distribution and depending on the normality of the data, comparisons were performed using the Student *t* test, the non-parametric Mann–Whitney U test (for two groups), the Wilcoxon signed rank test (for > 2 paired groups), Kruskal–Wallis (for > 2 nonpaired groups) or Friedman (for > 2 paired groups) one-way ANOVA tests, as appropriate. Linear regression analysis was performed to generate lines of best fit and correlations between variables were analysed using Pearson´s or Spearman's rank correlation coefficient (r).

3 Immune surveillance of senescent cells: why do senescent cells accumulate with age?

3.1 ABSTRACT

Recent evidence demonstrate that senescent cells may be cleared by the immune system however fail to explain how senescent cells accumulate with age and at sites of age-related diseases. In this chapter, the immune surveillance of senescent cells was studied using an autologous *in vitro* co-culture system with primary human fibroblasts and immune cells from the same individuals. A possible mechanism of immune evasion of senescent cells was identified, dependent on the expression of HLA-E by senescent fibroblasts and interaction with the inhibitory receptor NKG2A, present in NK and a subset of CD8+ T cells. Importantly, *in vitro* blockade of the interaction between HLA-E and NKG2A results in a more effective immune response towards senescent cells, mediated through NKG2D recognition of MICA/B. With the increasing recognition that the accumulation of senescent cells negatively affects lifespan and is associated with many age-related diseases, these findings demonstrate the potential benefit of strategies aiming to improve the immune system´s ability to clear senescent cells from tissues to reduce the burden of co-morbidities with age.

KEY POINTS:

- Both NK and CD8+ T cells can selectively target senescent cells, via a NKG2D-dependent mechanism;
- Expression of HLA-E by senescent cells leads to an immune escape mechanism, through the interaction with NKG2A on NK and CD8+ T cells;
- Blockade of HLA-E/NKG2A interaction effectively boosts immune responses against senescent cells *in vitro*.

3.2 INTRODUCTION

In recent years, both the innate and adaptive immune systems have been implicated in the elimination of senescent cells (Xue et al., 2007, Krizhanovsky et al., 2008, Rakhra et al., 2010, Kang et al., 2011, Lujambio et al., 2013). The elimination of senescent cells has gained considerable interest not only for cancer therapy but also for preventing age-related diseases (Hoenicke and Zender, 2012, Naylor et al., 2013, Lujambio, 2016). Despite recent evidence for the existence of immune responses against senescent cells (Sagiv and Krizhanovsky, 2013), the immune surveillance of these cells is not totally efficient otherwise senescent cells would not accumulate with age and at sites of age-related pathologies (Erusalimsky and Kurz, 2005, Herbig et al., 2006, Boccardi et al., 2015). Human melanocytic nevi, which are enriched in cells with senescence markers (Michaloglou et al., 2005, Gray-Schopfer et al., 2006, Mooi and Peeper, 2006), represent an intriguing example of how senescent cells can persist for decades in the organism, protected from an effective immune response.

In the present chapter, we will investigate possible mechanisms of immune evasion regulated by the expression of immunomodulatory ligands such as HLA-E on senescent cells. To assess the relevance of the expression of this molecule *in vivo*, human melanocytic nevi samples will be analysed for the expression of HLA-E. The immune surveillance of senescent cells will be studied using an autologous system with skin-explanted primary human fibroblasts and immune cells derived from the same individuals. It is shown that overexpression of HLA-E and interaction with the inhibitory NKG2A receptor, present not only in NK cells but also on a subset of CD8+ T cells, protects senescent cells from an effective immune clearance. Importantly, *in vitro* blockade of the interaction between HLA-E and NKG2A results in a more effective lysis of senescent cells by the immune system, pointing towards an attractive strategy to improve the elimination of senescent cells.

3.3 RESULTS

3.3.1 MICA/B and HLA-E are recognized by receptors present on NK cells and CD8+ T cells

To elucidate the role of HLA-E and MICA/B expression in regulating the immune surveillance of senescent cells, we first interrogated which cells could specifically recognize these immune ligands.

HLA-E and MICA/B are ligands for immune receptors belonging to the Natural Killer Group 2 (NKG2) family, which are important regulators of NK cell function (Lopez-Botet et al., 2000, Petrie et al., 2008). MICA/B and ULBP antigens are ligands of NKG2D, one of the most potent stimulatory NK cell receptors (Raulet, 2003, Lanier, 2015). HLA-E binds both NKG2A and NKG2C receptors, which interestingly have opposite effects on NK cell function (Braud et al., 1998a, Llano et al., 1998, Wada et al., 2004). NKG2A contains an ITIM-motif and functions as an inhibitory receptor that has a major role in the education of NK cells, preventing NK cell-reactivity against normal cells that express HLA-E bound to peptides derived form the signal sequence of class I molecules (Petrie et al., 2008, Cheent et al., 2013). In contrast, NKG2C through its association with DAP-12, an adaptor molecule containing an ITAM motif, acts as an activating receptor, leading to degranulation and cytokine secretion of NK cells (Lanier et al., 1998a, Pegram et al., 2011). Although commonly attributed to NK cells, an increasing body of evidence indicates that other immune cells express NKG2 receptors (Tarazona et al., 2000, Uhrberg et al., 2001, Derre et al., 2002).

We first analysed the distribution of the receptors for HLA-E and MICA/B across distinct immune cells types, using flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers (*n*=50; median age 52; range, 25-83) and stained with monoclonal antibodies for NKG2D, NKG2A and NKG2C in

combination with antibodies for the phenotypic markers CD3, CD4 and CD8 and analysed by multicolor flow cytometry. The frequency of cells expressing NK receptors was calculated for each of the following compartments – CD3-negative, CD3+, CD8+, CD4+ - using the gate strategy shown in **Figure 3.1**.

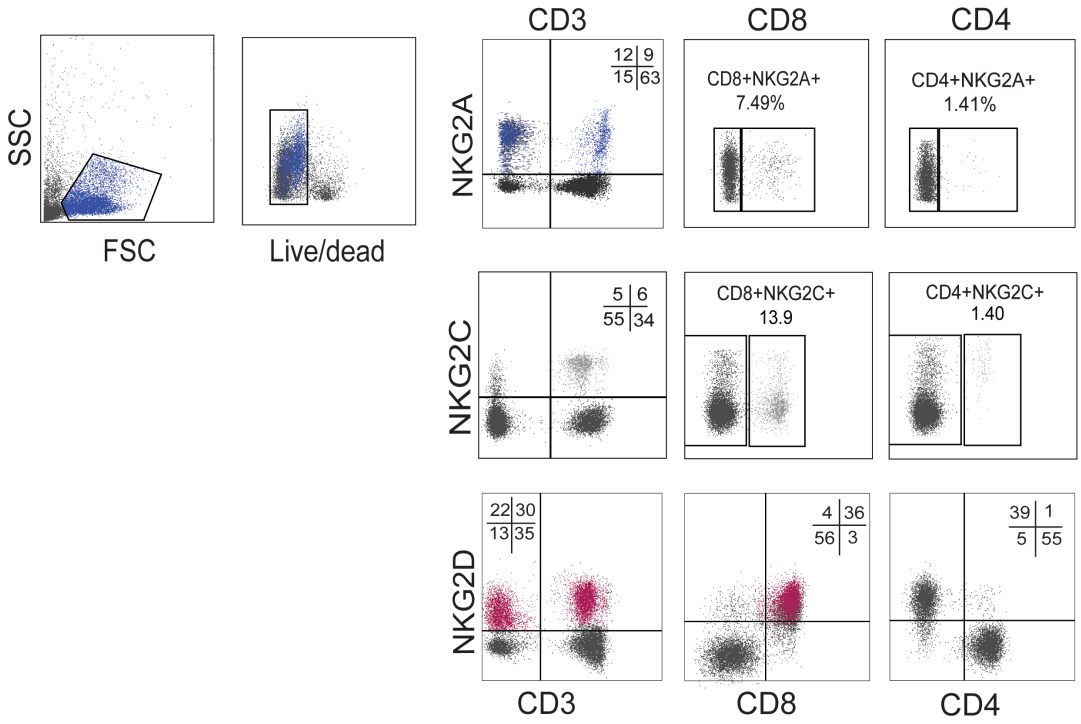

Figure 3.1 - Expression of the NKG2 family of receptors in human peripheral blood mononuclear cells.

PBMCs were isolated from peripheral blood of healthy volunteers (*n*= 50) and stained with specific antibodies for NKG2A, NKG2C and NKG2D combined with the phenotypic markers CD3, CD4, CD8 and analysed by multicolor flow cytometry. The figure shows the gating strategy used in a representative healthy donor to determine the frequency of NKG2A+ (top panel), NKG2C+ (middle panel) and NKG2D+ cells (bottom panel) in each compartment – CD3-negative, CD3-positive, CD4-positive, CD8-positive. Numbers indicate percentages of gated cells in each compartment.

Confirming previous reports, NKG2D+ cells were identified in both CD3-negative (which includes NK cells) and CD3+ (representing T cells) compartments. We then gated in the CD3-positive compartment to assess the distribution of NKG2D+ cells among CD8+ and CD4+ T cells. In line with previous studies (Bauer et al., 1999, Lanier, 2015), we confirmed that NKG2D was constitutively expressed on the majority of CD8+ T cells (mean 78.1 ± 9.3%) whereas only a very small percentage of CD4+ T cells expressed this receptor (mean 1.39 ± 1.72%, **Figure 3.2**).

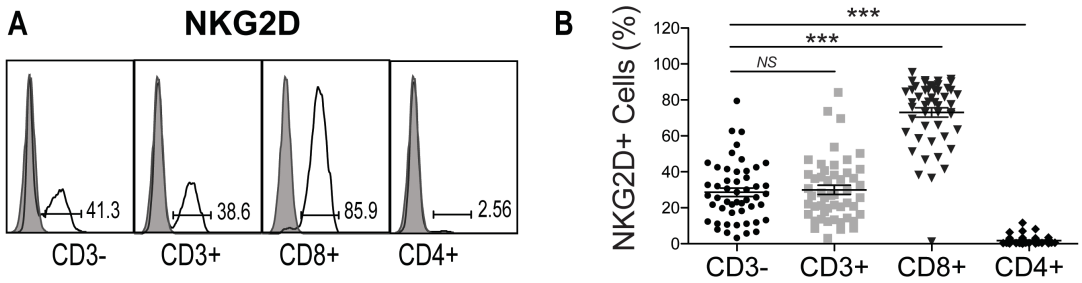

Figure 3.2 – NKG2D is highly expressed on CD8+ T cells and NK cells.

A) Representative FACS histograms of the expression of NKG2D on the indicated subsets of human lymphocytes. Shaded histograms represent isotype controls. Numbers indicate percentages of cells. **B)** Summary data (*n*= 50) presented as percentages of NKG2D+ cells in each compartment, determined as in A). Comparison between groups performed with the non-parametric Friedman test with Dunn´s correction for multiple comparisons (**p< 0.05*, ***p<0.01*, ****p< 0.001)*.

Regarding the receptors for HLA-E (**Figure 3.3**), we observed that the expression of both the inhibitory NKG2A and activating NKG2C receptors was not only found on the CD3-negative compartment, but also a significant proportion of CD8+ T cells also expressed these receptors (NKG2A+ CD8+ cells 3.18± 3.67%; NKG2C+ CD8+ cells 3.78 ± 9.13%). The high standard error of mean of NKG2C expression on CD8+ T cells indicates a high variability among donors, which is consistent with previous

reports indicating that the expression of this receptor is influenced by the gene copy number (Guma et al., 2004, Muntasell et al., 2016).

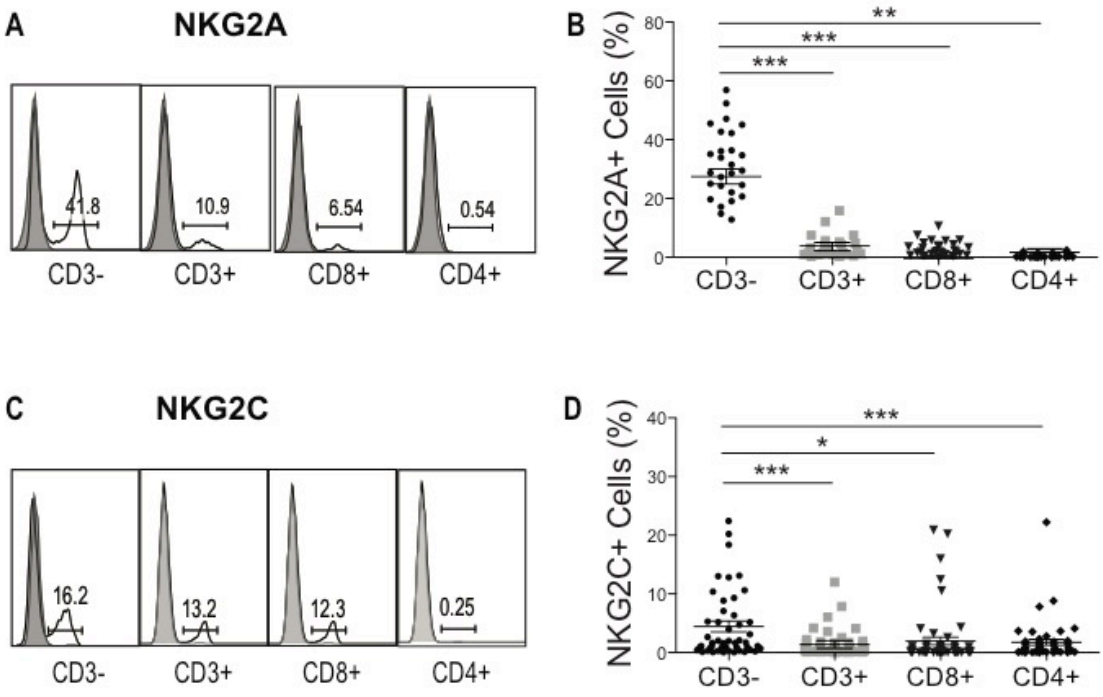

Figure 3.3 – HLA-E specific receptors are expressed on NK cells and a proportion of human CD8+ T cells.

Representative FACS histograms (**A and C**) and summary data (**B and D**) of NKG2A (top) and NKG2C (bottom) expression on the indicated subsets of human lymphocytes. Numbers in A and C indicate percentages of cells in each compartment. Statistical analysis performed with Friedman test with Dunn´s correction for multiple comparisons (*$p< 0.05$, **$p<0.01$, ***$p< 0.001$).

In conclusion, with this data it was confirmed that not only NK cells but also CD8+ T cells expressed significant levels of NKG2 receptors and therefore may be able to recognize MICA/B and HLA-E expressing cells.

3.3.2 Expression of NKG2 receptors increases in highly differentiated T cells

Previous studies have reported an increase in expression of NK cell receptors in T cells with a late differentiated phenotype (Vallejo et al., 2011). Therefore, the expression profile of NKG2A/NKG2C and NKG2D during different stages of differentiation was investigated.

T cell differentiation is associated with a profound change in the expression of surface receptors and the loss of costimulatory receptors is commonly used as an indicative of advanced differentiation (Effros, 1997, Weng et al., 2009, Mahnke et al., 2013). CD8+ T cells were therefore divided according to the expression of CD27 and CD28 receptors and the distribution of the NKG2 family of receptors was assessed within each subset. This classification has been previously used to distinguish CD8+ T cells according to their stage of differentiation (van Lier et al., 2003, Appay et al., 2008, Henson et al., 2009) and allows the distinction of three populations (**Figure 3.4A**): an early differentiated population (CD28+27+), an intermediate population (CD28-27+) and a population with a late differentiation phenotype (CD27-28- cells).

In keeping with previous observations, the majority of CD8+ T cells expressing NKG2A and NKG2C receptors exhibited a highly differentiated phenotype (**Figure 3.4C-D**), defined by the absence of co-stimulatory receptors CD28 and CD27.

Regarding NKG2D, it was found that this receptor was highly expressed across all subsets of CD8+ T cells, nevertheless a significant increase in the proportion of CD8+ T cells expressing NKG2D was observed in late differentiated subset as compared to less differentiated cells (**Figure 3.4E**, $p < 0.01$).

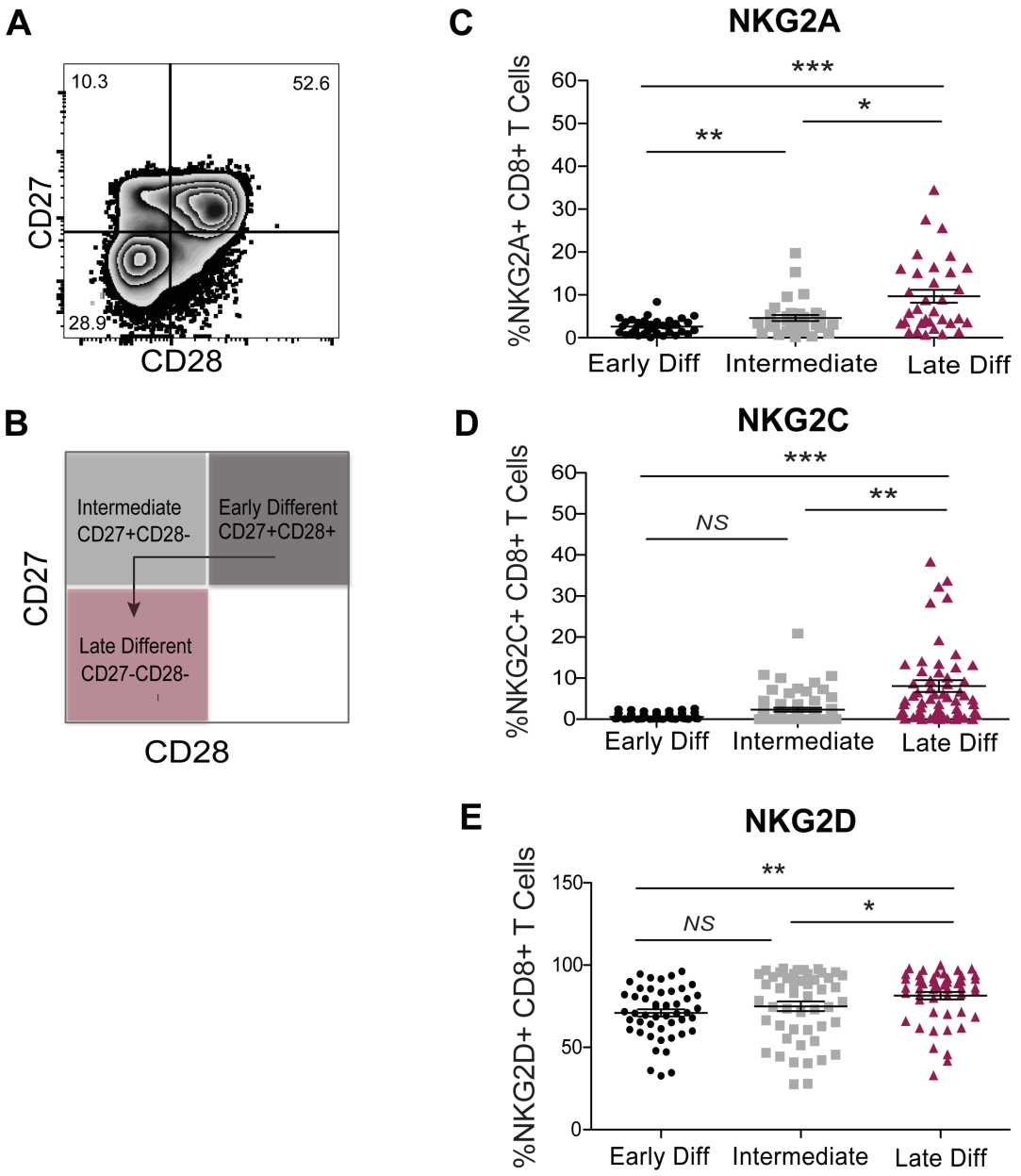

Figure 3.4 - Expression of NKG2 receptors increases in highly differentiated CD8+ T cells.

Representative FACS plot (**A**) and diagram (**B**) of the subsets defined by CD28/CD27 expression as: early (CD27+28+), intermediate (CD27+-28-) and late (CD27-28-) differentiated cells. **C-E**) Summary data of the distribution of NKG2A+ (*n*= 30), NKG2C+ (*n*= 50) and NKG2D+ (*n*= 50) cells within the subsets of CD8+ T cells defined as in A. Comparison between groups done with Friedman test with Dunn´s correction for multiple comparisons (*$p< 0.05$, **$p<0.01$, ***$p< 0.001$, ****$p< 0.0001$).

We next investigated whether the expression of NKG2 receptors is altered with age. The proportion of NKG2A-positive CD8+ T cells significantly increased with age (r = 0.4691, p = 0.0136), whereas no significant changes in the frequency of NKG2C and NKG2D expressing cells were observed with age (**Figure 3.5**).

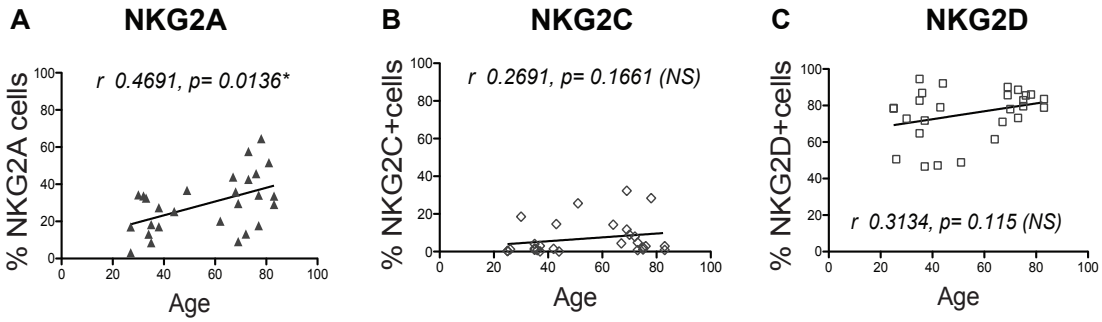

Figure 3.5 – Correlation between age and the frequency of CD8+ T cells expressing NKG2A (A), NKG2C (B) and NKG2D (C).

Correlation assessed by Spearman test showing a significant increase of NKG2A-expressing cells with age.

In summary, this data demonstrates that the expression of receptors for HLA-E and MICA/B is significantly increased in CD8+ T cells exhibiting a terminal differentiated phenotype (CD27-28- T cells). Interestingly, although the expression of NKG2 receptors on CD4+ T cells was much lower than on CD8+ T cells, the proportion of CD4+ T cells expressing NKG2 receptors was also predominantly found in the CD27-CD28- late differentiated subset, with very low frequencies in the other subsets (**Figure 3.6**).

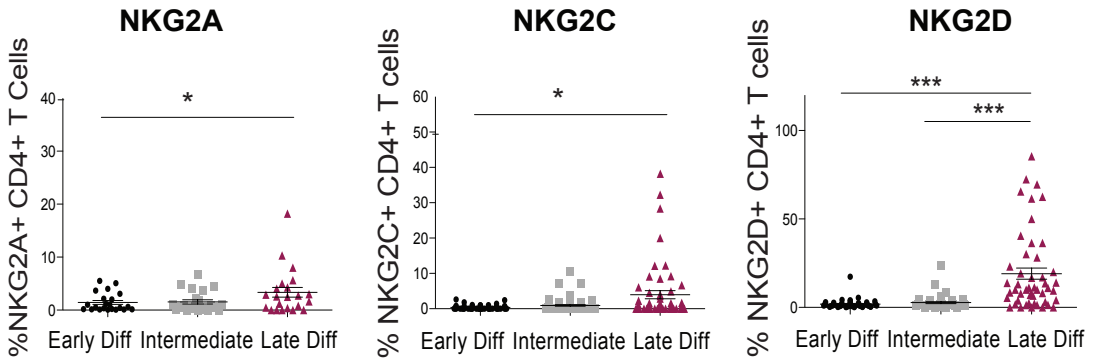

Figure 3.6 – Expression of NKG2 receptors within the CD4+ T cell compartment is mainly restricted to cells with a late differentiated phenotype.

Summary data of the distribution of NKG2A+ (*n*= 30), NKG2C+ (*n*= 50) and NKG2D+ cells (*n*= 50) among the subsets defined by CD27/CD28 within the CD4+ T cell compartment. Statistical analysis with Friedman test (*$p< 0.05$, **$p<0.01$, ***$p< 0.001$).

Both CD8+ and CD4+ CD28-27- terminally differentiated cells have been extensively characterized by Prof. Akbar´s group and others as exhibiting multiple features of immunesenescence (Plunkett et al., 2007, Henson et al., 2009, Weng et al., 2009, Akbar and Henson, 2011, Lanna et al., 2014). From this data it may be hypothesized that an increased expression of atypical ligands observed in senescent fibroblasts goes hand in hand with an increase in expression of their cognate receptors in the immune system, suggesting that the immune system is able to adapt to the aged-microenvironment and to an increased expression of senescence-induced ligands on fibroblasts.

3.3.3 Both NK and CTLs selectively target senescent cells, through an NKG2D-dependent mechanism

NK cells have been previously implicated in the surveillance and elimination of senescent cells, through the interaction between NKG2D and its ligands expressed on senescent cells (Iannello et al., 2013, Sagiv, 2016). The observation that CD8+ T lymphocytes also express specific receptors recognizing the senescence-induced ligands MICA/B and HLA-E prompted us to study whether these cells were also implicated in the immune surveillance of senescent cells.

To study the immune surveillance of senescent cells an autologous co-culture system was developed using primary human fibroblasts derived from the skin of healthy volunteers and NK and CD8+ T cells isolated from peripheral blood of the same donors (**Figure 3.7 and Materials and Methods).** After an effective induction of premature senescence using ionizing radiation (as demonstrated in chapter two), senescent irradiated and normal proliferating fibroblasts were used as targets in i*n vitro* cytotoxic assays with autologous IL2-activated NK and CD8+ T cells.

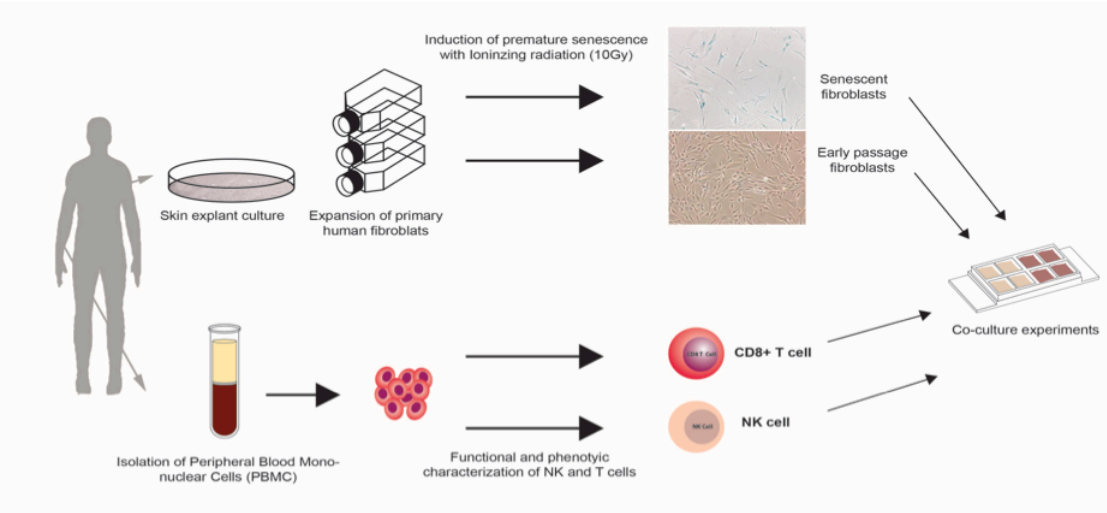

Figure 3.7 – Schematic model of the autologous co-culture system developed to study the interactions between the immune system and senescent cells.

Different flow cytometry-based methods, extensively described in the literature as good alternatives to chromium-release assays (Zaritskaya et al., 2010), were employed to determine cytotoxicity levels: active caspase 3 expression as a measure of induced apoptosis of target cells (Jerome et al., 2003) and CD107a expression as a marker of degranulation of effector cells (Aktas et al., 2009).

We initially tested different effector to target (E:T) ratios and measured the expression of active caspase 3 on target cells after a 6-hour incubation period with purified NK and CD8+ T cells (**Figure 3.8A)**. Consistent with previous reports (Xue et al., 2007, Krizhanovsky et al., 2008), NK cells preferentially targeted senescent cells as shown by the significantly higher levels of active caspase 3 in senescent cells cultured with NK cells, compared to non-senescent cells (p < 0.01, **Figure 3.8B**). Interestingly, CD8+ T cells could also induce significant lysis of senescent fibroblasts, suggesting that CD8+ T cells may be involved in the surveillance of senescent cells (p < 0.01, **Figure 3.8B).**

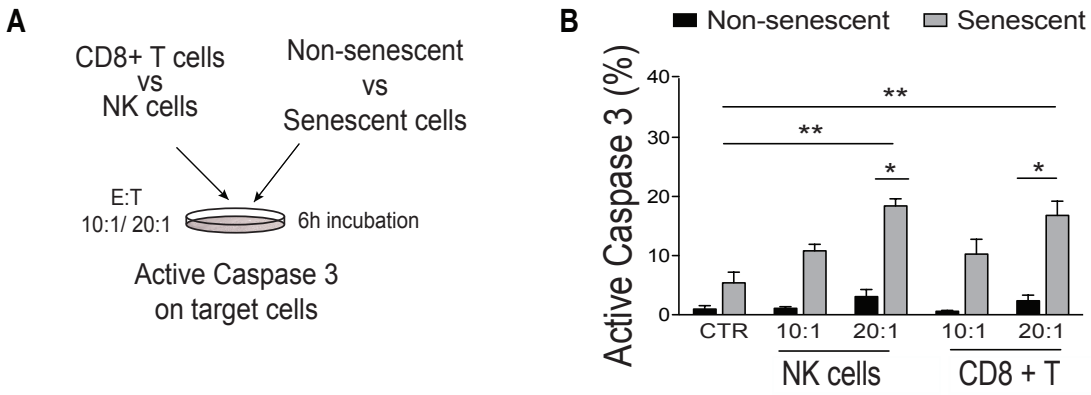

Figure 3.8 - Both NK and CD8+ T cells selectively target senescent cells.

A) Experimental design of active caspase 3 assay performed with purified human NK and CD8+ T cells in culture with autologous senescent and normal fibroblasts. **B)** Summary data of five independent experiments performed as in A), using different effector to target (E:T) ratios and presented as the percentage of active caspase 3 expression on senescent (grey bars) and non-senescent fibroblasts (black bars), after a 6h-incubation with NK and CD8+ T cells. Controls (CTR) indicate spontaneous expression of caspase 3 on fibroblasts cultured without effector cells. Statistical analysis performed with 2-way Anova test (*p< *0.05*, **p<0.01).*

Of note, the basal expression of active caspase 3 (labelled as CTR, **Figure 3.8B**) was higher on senescent fibroblasts compared to non-senescent fibroblasts, most likely due to the effects of radiation, making fibroblasts more prone to apoptosis. In every experiment a control for the spontaneous expression of caspase 3 as well as a positive control (to assess maximum lysis) was used. A similar method was used in the CD107a degranulation assay to assess specific cytotoxicity using a negative control with unstimulated effector cells and a positive control with PMA+ ionomycin stimulated effector cells (**Materials and Methods**).

The results obtained with the active caspase 3 assay were further confirmed with the CD107a degranulation assay using a fixed E:T ratio (20:1, representing the optimal ratio determined in the previous assays), indicating that both NK and CD8+ T cells can target and induce cytolysis of senescent fibroblasts *in vitro* ($p < 0.001$, **Figure 3.9A/B).**

As NKG2D had been previously implicated in NK-cell mediated killing of senescent cells we next investigated whether NKG2D was also involved in CD8+ T cell responses against senescent cells. Pre-incubation of effector cells with specific NKG2D blocking antibodies lead to a significant decrease in cytotoxicity towards senescent fibroblasts, in both NK and CD8+ T cells (**Figure 3.9B**). Similar results were obtained after transfection of CD8+ T lymphocytes with a small interfering (siRNA) to silence NKG2D expression (**Figure 3.9C and 3.9D**), indicating that both NK and CD8+ T cells target senescent cells, via an NKG2D-dependent mechanism. The reduction of cytotoxicity observed upon NKG2D blockade or silencing was only partial as compared to controls, thus suggesting that additional activating receptors may be implicated in the killing of senescent cells, as previously reported (Soriani et al., 2009). The fact that we did not observe a significant decrease in the expression of MHC class I molecules at the surface of irradiated fibroblasts (Figure 2.6A), argues against the involvement of MHC class I-specific receptors of the KIR family.

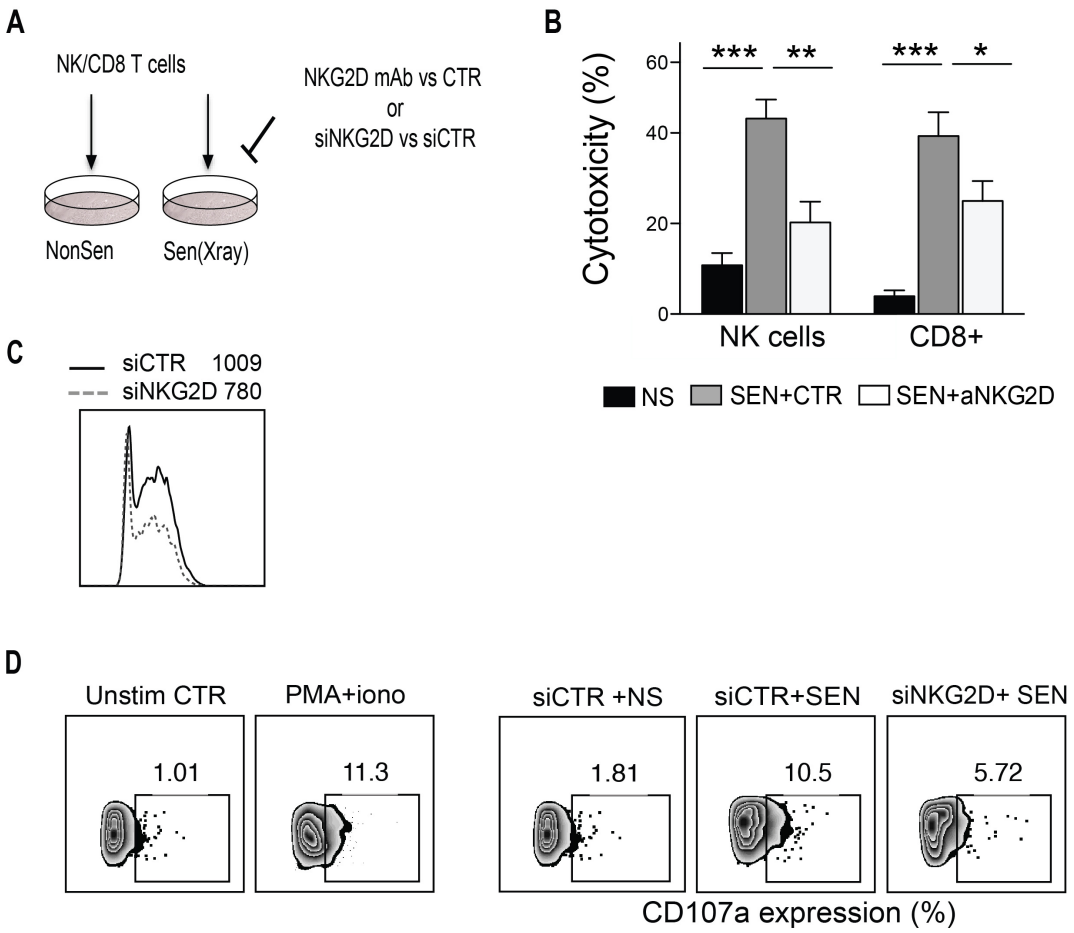

Figure 3.9 – Killing of senescent cells by NK and CD8+ T cells is partially regulated by NKG2D.

A) Experimental design of CD107a degranulation assays performed after antibody blockade of NKG2D or after manipulation of NKG2D expression in human CD8+ T cells with RNA interference. **B)** Summary data of 5 independent experiments performed as in A), using a fixed E:T ratio (20:1) and measuring CD107a expression on effector cells after incubation with mAb to NKG2D (1D11) or an isotype control. Results are presented as the percentage of specific cytotoxicity calculated using the formula (sample degranulation − spontaneous degranulation) / (maximum degranulation − spontaneous degranulation) × 100. Groups were compared using Kruskal Wallis test. **C)** Transfection of human CD8+ T cells with siRNA for NKG2D effectively reduced surface expression of NKG2D as compared to siRNA control. Numbers indicate MFI of NKG2D. **D)** CD107a expression in CD8+ T cells transfected with siNKG2D after co-culture with senescent vs non-senescent cells is effectively reduced as compared to cells transfected with siRNA control. Numbers indicate percentages of CD107a+ cells. CD8+ T cells cultured without targets (unstim CTR) and CD8+ T cells stimulated with PMA+ ionomycin were included as negative and positive controls, respectively. Representative of two different experiments *(*$p< 0,05$, **$p<0.01$*, and ***$p< 0.001$)*.

3.3.4 Blockade of NKG2A/HLA-E interaction enhances the immune-mediated clearance of senescent cells

Effector functions of NK and CD8+ T cells are tightly regulated by the balance between activating and inhibitory signals (Lanier, 2005). Therefore it was reasoned that positive signals delivered by NKG2D ligation may be blocked if the inhibitory receptor NKG2A is engaged with its ligand HLA-E expressed on senescent cells.

To investigate this, *in vitro* assays were performed to assess degranulation of NK and CD8+ T cells after pre-incubation with specific blocking antibodies to NKG2A (Z199) or NKG2D (1D11), compared to isotype controls. As previously shown, blockade of NKG2D significantly suppressed both NK and CD8+ T cell responses against senescent cells ($p < 0.01$, **Figure 3.9B and 3.10B**). By contrast, blockade of NKG2A receptor engagement significantly boosted the cytotoxic activity of NK cells against senescent cells ($p < 0.05$) and enhanced CD8+ T cell activity, although not significantly ($p > 0.05$, **Figure 3.10B**).

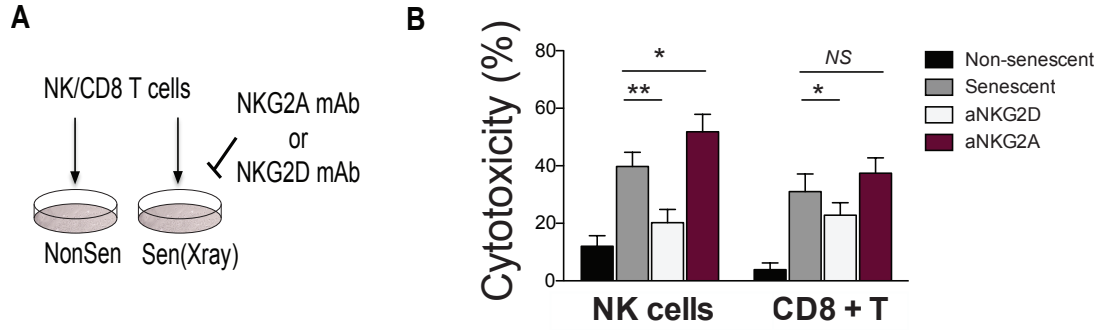

Figure 3.10 - Blockade of NKG2A receptor enhances immune-mediated lysis of senescent cells.

A) Experimental design of CD107a degranulation assays performed after pre-incubation of effector cells with blocking antibodies to NKG2A (Z199) and NKG2D (1D11) or the respective isotype controls, using a fixed E:T ratio of 20:1. **B)** Summary data (n=4) presented as the percentage of specific lysis mediated by CD8+ and NK cells. Statistical analysis done with Kruskal-Wallis test with Bonferroni correction. (*$p< 0,05$, **$p<0.01$).

To directly investigate the role of HLA-E in inhibiting immune responses against senescent cells, we used RNA interference to inhibit HLA-E expression. After 36 hour post-transfection, a significant decrease in HLA-E expression was observed in senescent fibroblasts transfected with with siRNA for HLA-E compared to siRNA control (**Figure 3.11A)**. This resulted in a significant increase in degranulation (**Figure 3.11B)** and cytokine secretion (**Figure 3.11C)** by autologous NK and CTL, as compared to cells transfected with a scramble siRNA control.

Overall, these findings suggest that HLA-E expression on senescent cells decreases their susceptibility to immune-mediated lysis, by protecting them from elimination by NK and CD8+ T cells expressing the inhibitory receptor NKG2A.

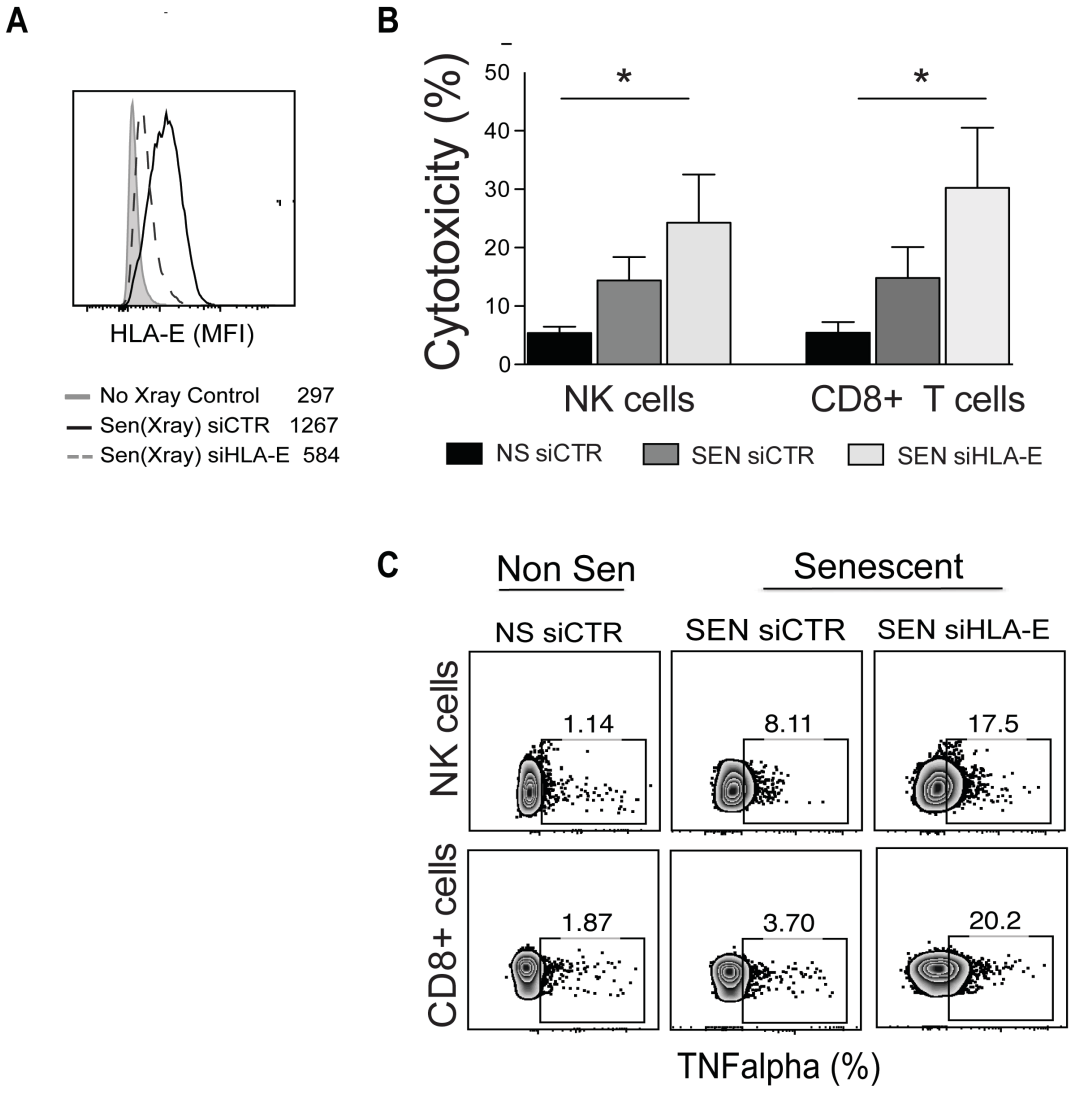

Figure 3.11 – Depletion of HLA-E expression significantly boosts NK and CD8+ T cell responses to senescent cells.

Normal and senescent fibroblasts were transfected with siRNA to HLA-E (sc-62470, Santa Cruz) or a control siRNA (sc-37007, Santa Cruz) before co-culture with autologous NK and CD8+ T cells. **A)** HLA-E expression on fibroblasts was confirmed 36h after transfection using flow cytometry. Values indicate the Mean Fluorescence Intensity (MFI) of HLA-E. **B)** Cumulative data of CD107a expression in NK (n=4) and CD8+ T cells (n=3), presented as the percentage of cytotoxicity (calculated as in Figure 2.8). Statistical analysis done with Kruskal Wallis test (*$p< 0,05$). **D)** Representative dot plots of TNF-alpha secretion by NK (top) and CD8+T cells (bottom) after 6-hour incubation with normal and senescent fibroblasts transfected with siHLA-E or siRNA control. Data is representative of 3 independent experiments.

3.3.5 Expression of HLA-E is found in cells from human melanocytic nevi

Studies have shown that human melanocytic nevi are highly enriched with cells with markers of senescence and thus represent an example of oncogene-induced senescence *in vivo*. In fact, despite the presence of oncogenic mutations, most frequently in *BRAF* and *RAS* (Pollock et al., 2003, Bauer et al., 2007), the progression of human melanocytic nevi to melanomas is not frequent (Bevona et al., 2003) and this is thought to be due to the induction of senescence, keeping these cells growth-arrested for decades (Michaloglou et al., 2005, Gray-Schopfer et al., 2006, Mooi and Peeper, 2006). At the same time, human melanocytic nevi represent an example of how senescent cells can reside in tissues for decades without being effectively eliminated by the immune system.

To assess the biological relevance of HLA-E expression on senescent cells *in vivo*, we investigated whether cells from human melanocytic nevi expressed HLA-E. To this end, formalin-fixed, paraffin-embedded (FFPE) tissue arrays of congenital and acquired human melanocytic nevi were stained for HLA-E and CD8 using immunohistochemistry (**Figure 3.12).** To confirm that cells expressing HLA-E were senescent cells, sections were also stained for Ki67 as an indirect marker of proliferation demonstrating that cells expressing HLA-E were rarely positive for Ki67 confirming that they are in cell cycle arrest (**Figure 3.12, bottom panel).** In each tissue array, normal skin and melanoma samples were used as controls.

Samples were analysed using an Image Analysis Software which identified each nuclei (in blue) and counted each HLA-E positive cell (in brown) and CD8+ cell (in red). The software was also able to calculate the distance between each red and brown dot, allowing us to determine the co-localization of HLA-E and CD8+ cells (for more details and complete data set, please refer to **Materials and Methods**).

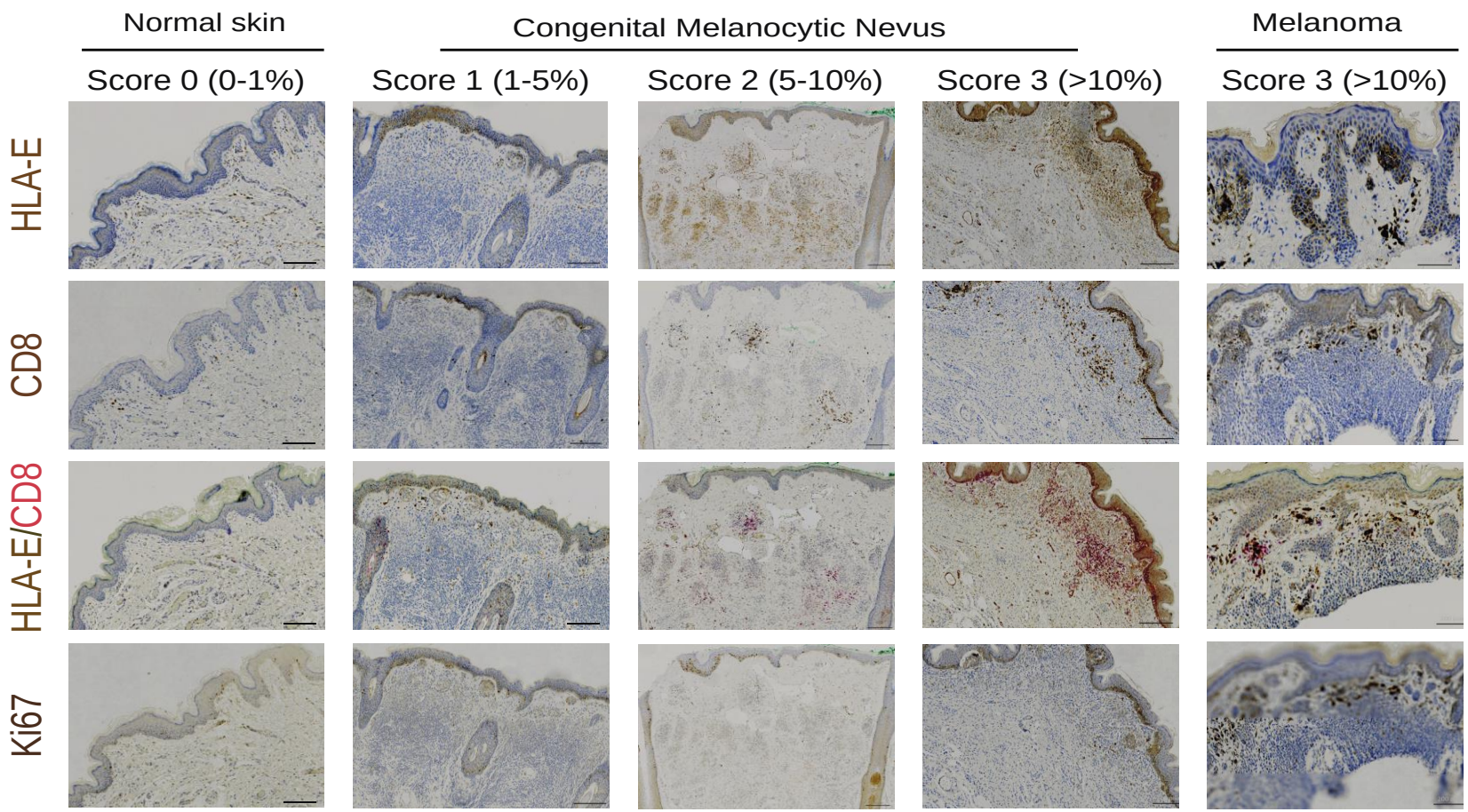

Figure 3.12 - Immunohistochemical staining of human melanocytic nevi.

Formalin-fixed paraffin-embedded tissue arrays of human melanocytic nevi were analysed by immunohistochemistry using HLA-E (MEM-E/02), CD8 and Ki67 specific antibodies. When double-staining for HLA-E and CD8, different chromogens reveal HLA-E (brown) and CD8 (red). Images are representative staining patterns of normal skin, congenital melanocytic nevi and melanoma showing the different scores of intensity for HLA-E, based on the percentages of positive cells for HLA-E (indicated in brackets). Scale bar = 50 μm.

A score for the intensity of HLA-E expression was defined based on the percentage of HLA-E positive cells (number of brown cells/number of blue nuclei), as negative (score 0: between 0-1%), mild (score 1: 1-5%), moderate (score 2: 5-10%) and intense (score 3: >10%). Among 24 melanocytic lesions studied, HLA-E expression was detectable in most (23/24) human melanocytic nevi (mean 12.87%, range 0.08%-38.6%). In 10/24 (41.7%) lesions we observed a strong positivity for HLA-E (score 3), with frequencies of HLA-E positive cells up to 30% (**Table 3.1**).

	HLA-E Intensity Score	
	n	**%**
Score 0 (0-1%)	1	4.2
Score 1 (1-5%)	7	29.2
Score 2 (5-10%)	6	35.0
Score 3 (>10%)	10	41.7
Total	24	100

Table 3.1- IHC staining scores of HLA-E expression and CD8 infiltration in human melanocytic nevi (n=24).

To assess CD8+ T cell infiltration, the percentage of CD8+ T cells (number of red/number of blue nuclei) was determined and varied between 0.05% and 16.5% (mean 5.29 ± 4.01%) amongst samples (**Table 3.4 in Materials and Methods**). Interestingly, CD8+ cells were frequently detected in the vicinity of HLA-E+ cells as shown in the representative images in **Figure 3.13.** To assess more accurately the distribution of CD8+ T cells in relation to HLA-E+ cells, the image analysis software was used to calculate the distance between each red and brown dot (for more details please refer to **Materials and Methods**). A high degree of co-localization between CD8+ cells and HLA-E+ cells (defined as >75% of red bordering brown/total red) was found in 7/24 (29%) of samples. Accordingly, a positive correlation was observed between the frequency of HLA-E+ cells in melanocytic lesions and the percentage of CD8+ cell infiltration (**Figure 3.13E**).

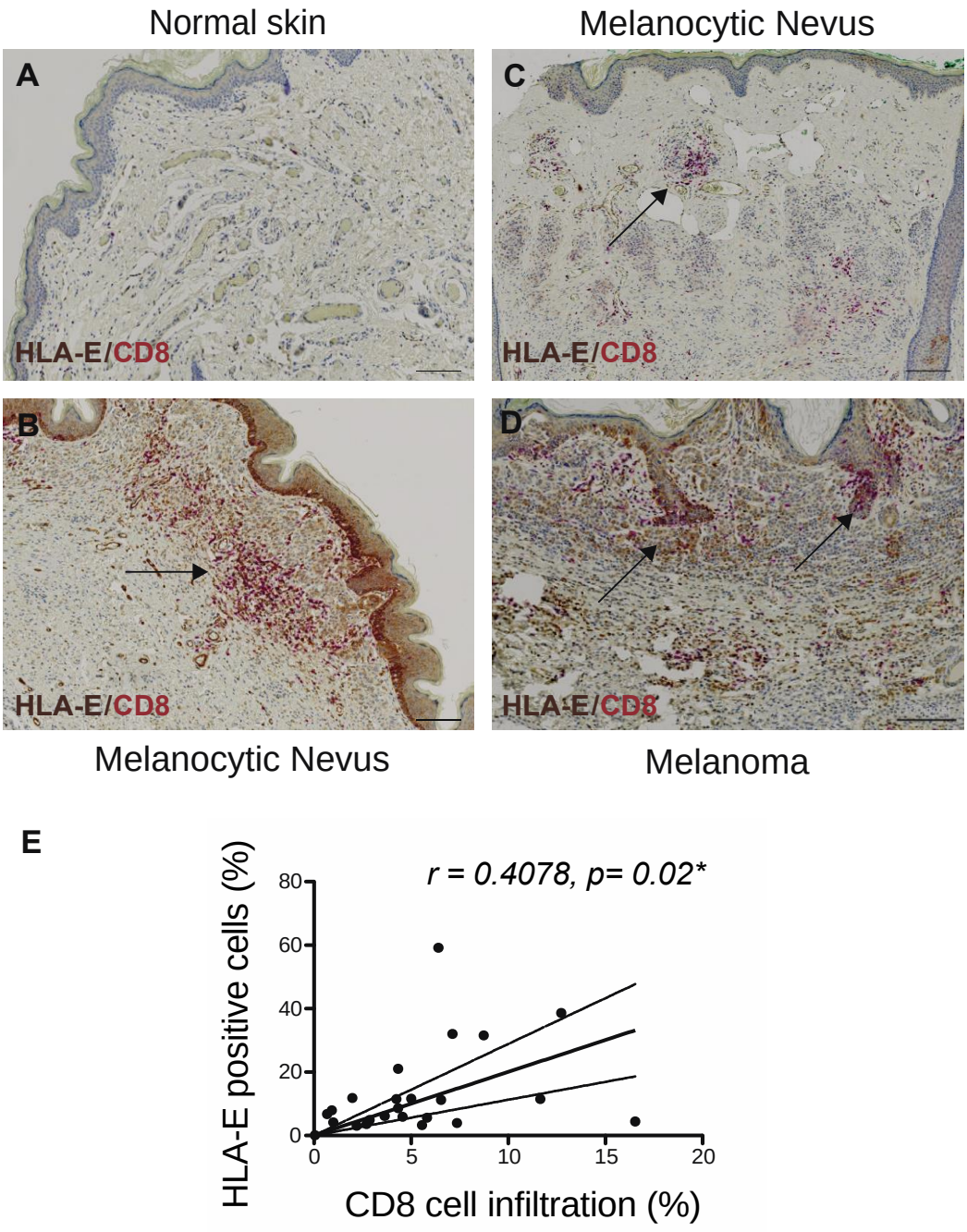

Figure 3.13 – Immune infiltrates of CD8+ cells frequently colocalize with areas with strong HLA-E expression.

Formalin-fixed paraffin-embedded sections of normal skin **(A)**, melanocytic nevi **(B-C)** and melanoma **(D)** were analysed by immunohostochemistry after co-staining for HLA-E (brown) and CD8 (red). Arrows indicate areas with CD8+ infiltrates surrounding HLA-E positive cells, rarely seen in normal skin and more intense in melanoma samples relative to benign nevi. **E)** Correlation assessed by Spearman test between the frequency of HLA-E+ cells and CD8+ infiltrates in human melanocytic nevi (n=24).

As NK cells can also stain positively for CD8, we used the CD3 marker to distinguish between NK and T cells and confirmed that CD3+ CD8+ T lymphocytes constitute an important component of the immune cell infiltrate (**Figure 3.14 A-D),** arguing in favour of a role of CD8+ T cells in the surveillance of senescent cells.

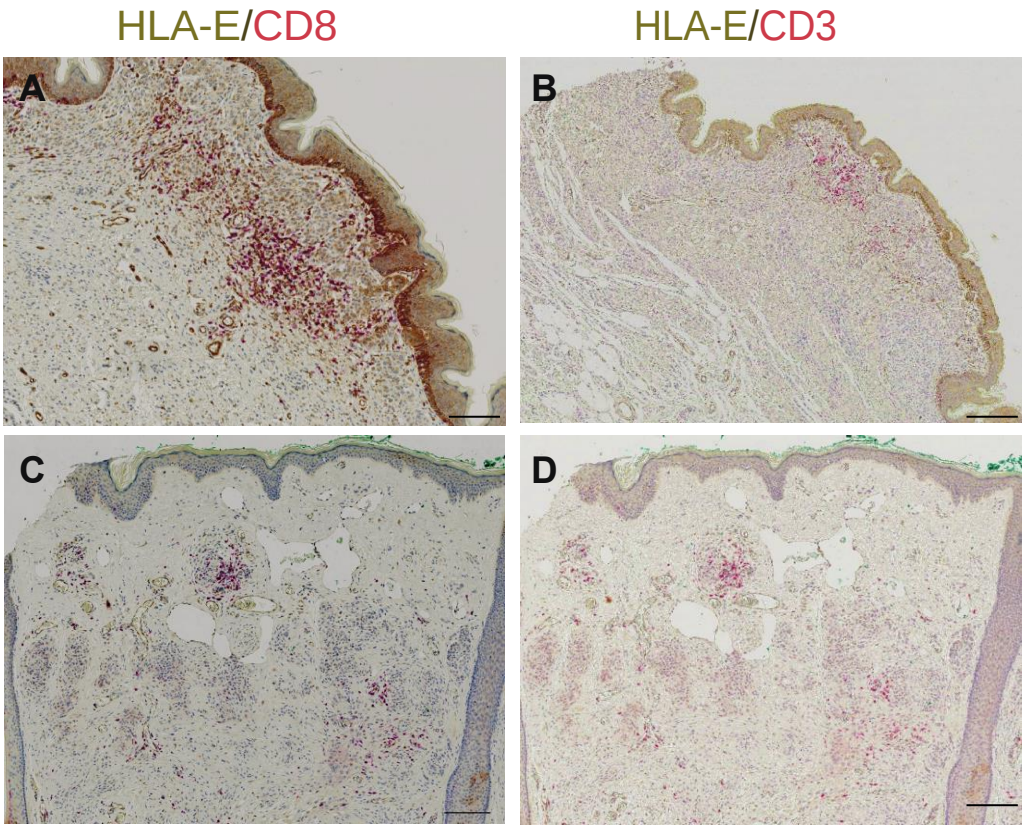

Figure 3.14 – Immune infiltrates surrounding HLA-E positive melanocytes include CD3+ CD8 T cells.

Consecutive sections of human melanocytic nevi lesions were double-stained with specific antibodies to HLA-E and CD8 **(A and C)** and HLA-E and CD3 (**B and D**), showing that part of the CD8+ infiltrates are also positive for CD3, thus corresponding to CD8+ T cells.

Collectively these findings indicate that HLA-E expression is common *in vivo* among cells of human melanocytic nevi and may explain why these cells persist for decades in tissues, protected from an effective immune-mediated clearance.

3.4 DISCUSSION

In the previous chapter, HLA-E and MICA/B were identified as significantly up-regulated ligands on senescent cells, compared to non-senescent counterparts. Both ligands are recognized by NK cell receptors belonging to the NKG2 family, which are not only found on NK cells but also on CD8+ T cells. In the present work, we extend observations from other groups indicating that senescent cells can be targeted by the immune system, however demonstrating for the first time that not only NK but also CD8+ T cells play an important role in the immune surveillance of senescent cells.

Functionally, it is demonstrated that both NK and CD8+ T lymphocytes can recognize and selectively target senescent cells, through an NKG2D-dependent mechanism. The concept that T cells can break the traditional rules and recognize cells using the receptors that are traditionally attributed to NK cells is not new and has been previously shown in tumour-infiltrating lymphocytes able to recognize NKG2D ligands expressed on tumour cells (Maccalli et al., 2007, Vivier et al., 2002, Nausch and Cerwenka, 2008). The findings described here suggest that CD8+ T cells may be equally important in the immune response to senescent cells, using receptors that are normally found on NK cells to recognize senescence-induced ligands. Consistent with this, an increased expression of the cognate receptors for MICA/B and HLA-E was detected in highly differentiated CD8+ T cells, which are significantly expanded with age. This suggests that the immune system is able to adapt to the age-dependent accumulation of senescent cells, however for some reason fails to eliminate these cells efficiently.

In this study, we propose a mechanism that may explain why senescent cells evade the immune system and accumulate with age. We propose that the expression of HLA-E on senescent cells may compromise an effective immune surveillance by NK and CD8+ T cells expressing the inhibitory receptor NKG2A.

Overall, our findings support a model for the immune surveillance of senescent cells in which a balance between the expression of activating NKG2D ligands and inhibitory HLA-E will determine the outcome of the immune response to senescent cells, mediated by both NK and CTLs (**Figure 3.15**). Blockade of the interaction between HLA-E and NKG2A may tip the balance towards the activation of immune cells and therefore enhance the immune-mediated lysis of senescent cells.

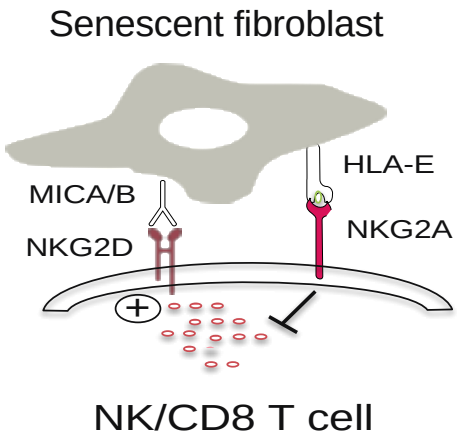

Figure 3.15 - Model for the interactions between senescent cells and the immune system.

NKG2A is an inhibitory C-type lectin receptor that recognizes HLA-E and is commonly found on NK cells but also expressed by some cytotoxic CD8+ T cells (Braud et al., 1998a, Borrego et al., 2006). Here we demonstrate that this receptor is predominantly expressed by CD8+ T cells with a terminally differentiated effector phenotype and is significantly increased with age. Despite expressing high levels of the activating receptor NKG2D, the simultaneous presence of the inhibitory receptor NKG2A in late differentiated CD8+ T cells may compromise their function and therefore explain why the immune system is less effective in eliminating senescent cells with age. Even if the activating counterpart of NKG2A, the receptor NKG2C is co-expressed on the same cells, studies indicate that inhibitory receptor signaling is

dominant and overrides activation signals (Saez-Borderias et al., 2009, Beziat et al., 2011). These results thus bring new insights to our understanding of how immunesenescence may affect the capacity of the immune system to fight senescent cells.

Importantly, evidence is provided that *in vitro* blockade of the interaction between HLA-E and the inhibitory receptor NKG2A results in a more effective clearance of senescent cells mediated by NKG2D, thus confirming a role of NKG2A in supressing immune responses to senescent cells. Ligation of NKG2A with the cognate ligand HLA-E induces a signaling cascade downstream of the immunoreceptor tyrosine-based inhibition motif (ITIM) that suppresses effector cytotoxic activity (Borrego et al., 2005). This is thought to represent an important immune checkpoint, preventing NK cell-reactivity against normal cells that express HLA-E bound to peptides derived form the signal sequence of class I molecules (Petrie et al., 2008, Cheent et al., 2013). Interestingly, accumulating evidence now indicates that HLA-E is able to present a diverse repertoire of peptides that includes epitopes derived from stress-induced heat shock proteins, not only to NK but also to CD8+ T cells (Michaelsson et al., 2002, van Hall et al., 2010).

In recent years, the manipulation of inhibitory signaling pathways in immune cells has gained great attention and represents an attractive approach to boost the host natural immune responses to abnormal cells. Immune checkpoint inhibitors, such as PD-1/PD-L1 and CTLA-4 inihibitors, are currently approved as fist-line therapies for advanced cancers, such as melanoma and lug cancer (Pardoll, 2012, Li et al., 2016). Similarly, humanized monoclonal antibodies targeting NKG2A (Monalizumab, formerly IPH2201) are currently in phase I/II development both in monotherapy and as adjuvants for gynecologic (NCT02331875) and head and neck (NCT02459301) cancers. Due to a restricted expression of HLA-E on healthy tissues, interventions

that specifically block the interaction of this ligand with its inhibitory receptor may be promising therapeutic approaches to selectively remove senescent cells from tissues.

Increasing evidence indicates that the accumulation of senescent cells negatively influences lifespan and removal of senescent cells may be beneficial to prevent age-related diseases and cancer (Baker et al., 2011, Baker et al., 2016, Lujambio, 2016). The persistence of senescent cells in tissues is thought to contribute to the maintenance of a low-level chronic inflammatory state that underlies many age-related diseases (Campisi and d'Adda di Fagagna, 2007, van Deursen, 2014). This has prompted the quest to develop drugs (termed senolytic drugs) that selectively target senescent cells to alleviate the progression of age-related diseases and reverse some aspects of aging (Chang et al., 2016, Zhu et al., 2016).

On the other hand, an increasing number of studies have reported the presence od senescence markers in tumour cells after treatment with chemotherapeutic drugs or after ionizing radiation (Efimova et al., 2010, Zhu et al., 2013, Kalathur et al., 2015) and the use of therapies to induce cellular senescence in tumours has recently emerged as a promising approach for treating cancer (Nardella et al., 2011, Acosta and Gil, 2012, Toso et al., 2015). However, the success of such senescence-based therapies depends on the effective clearance of senescent tumor cells from the organism (Toso et al., 2014, Soriani et al., 2009). Collectively, these facts reinforce the importance of strategies aiming to boost the natural capacity of the immune system to selectively remove senescent cells and ensure the success of senescence-based anticancer therapies.

The observation that cells from human melanocytic nevi express HLA-E supports the relevance of this mechanism *in vivo.* Human melanocytic nevi are benign lesions associated with oncogenic mutations, most frequently in *BRAF* and *RAS* (Pollock et al., 2003, Bauer et al., 2007). The identification of cells displaying senescence

markers in these benign lesions whilst absent in the malignant counterparts, is a compelling evidence that cellular senescence is indeed an important tumour suppressive mechanism *in vivo* (Michaloglou et al., 2005, Gray-Schopfer et al., 2006, Mooi and Peeper, 2006). However, even if nevi are stable for years, they pose a risk for the development of melanoma (Bennett, 2016), further highlighting the advantages of eliminating senescent cells. In addition to representing a good example of oncogene-induced senescence *in vivo*, human nevi are also an intriguing example of how senescent cells may persist for decades in tissues without inducing an effective immune response. Our observations these cells express high levels of HLA-E may thus provide a plausible explanation to why human melanocytic nevi escape the immune system.

In conclusion, this work provides important insights on the immune surveillance of senescent cells and identifies a novel mechanism of immune evasion that may contribute for the persistence of senescent cells in tissues, such as cells of human melanocytic nevi. This may open new promising therapeutic approaches based on the use immune modulators such as anti-NKG2A antibodies to improve the clearance of senescent cells and reduce the burden of co-morbidities and cancer with age. Further studies would be necessary to confirm the effectiveness of anti-NKG2A treatments in eliminating senescent cells in animal models.

3.5 MATERIALS AND METHODS

3.5.1 Blood and skin sample collection

Paired blood and skin samples were obtained from healthy donors in accordance with the Declaration of Helsinki and approved by the Ethical Committee of the Royal Free Hospital, London (Research Ethics number: 11/0473).

Primary human fibroblasts were derived from skin explant cultures, as described in Chapter One. Cells were grown in culture with DMEM supplemented with 10% FCS, 2 mM glutamine and 100 IU/ml penicillin/streptomycin (all from Sigma-Aldrich, UK), at 37°C in a humidified 5% CO2 incubator. Sub-confluent cultures were X-irradiated with a total dose of 10Gy and allowed to recover in culture for 7-14 days, during which time they developed the characteristic features of senescence.

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood using Ficoll–Hypaque density gradient centrifugation (Amersham Biosciences, UK) from heparinized blood of healthy volunteers between the ages of 25 and 83 years (*n*= 50; median age 52, range 25-83) and resuspended in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamineand 100 IU/ml penicillin/streptomycin (all from Sigma-Aldrich, UK), at 37°C in a humidified 5% CO^2 incubator.

3.5.2 NK and CD8+ T Cell purification

NK and CD8+ T cells were purified from freshly isolated PBMCs by negative selection using magnetic activated cell sorting (MACS, Miltenyi Biotec, UK) according to the manufacturer's instructions. The purity of sorted cells (typically ≥ 95%) was assessed by flow cytometry using the following panel of phenotypic markers: CD3 PE CF594, CD8 APC, CD16 FITC and CD56 Brilliant Violet 421 (all from BD

Biosciences, UK). After sorting cells were resuspended in complete RPMI medium with IL2 (100U/mL) until incubation with target cells.

3.5.3 Autologous Co-culture system

Primary human fibroblasts were derived from skin explant cultures, irradiated and allowed to recover in culture until developing a senescent phenotype, as described in the first chapter. Senescent fibroblasts (between day 14 and 21 post-irradiation) were seeded onto flat-bottom 48-well plates ($20x10^4$ cells per well) and grown to confluence at 37 °C and 5% CO_2. Early-passage fibroblasts (between passages 3 and 9) were platted under the same conditions and used as non-senescent controls. When confluent, the monolayers were washed with Hanks' solution (Life Technologies, Inc.) and effector cells were added at the desired effector:target ratio, typically 20:1 and 10:1. IL-2- activated NK and CD8+ T cells freshly purified from the peripheral blood of the same donors were used as effector cells in cytotoxic assays.

3.5.4 Cytotoxic assays: Active Caspase 3 Assay

Senescent and non-senescent fibroblasts were incubated with autologous NK and CD8+ T cells, at increasing effector to target ratios (10:1; 20:1). After a 6-hour incubation period, cells were harvested after trypsinization, washed, fixed and permeabilized with BD Cytofix/Cytoperm (BD 554722) at 4°C for 30 min followed by washing with BD Perm/Wash Buffer and staining for 30 minutes at 4°C with Anti-Active Caspase 3 Antibody PE (BD 550821, all from BD Biosciences, UK). Quantification of active caspase 3 expression in fibroblasts incubated with medium only without effector cells was used as a negative control to assess spontaneous lysis. Fibroblasts treated with Camptothecin (8 µg/mL; Sigma-Aldrich) were used as positive controls.

3.5.5 Cytotoxic assays - CD107a degranulation assay and cytokine secretion

CD107a (lysosomal-associated membrane protein-1, LAMP-1) expression was used as a marker of degranulation of $CD8^+$ T and NK cells, as previously described (Aktas et al., 2009). Briefly, MACS- purified NK and CD8+ T cell subsets were incubated at 37°C for 6 h with senescent or non-senescent fibroblasts, at a fixed effector to target ratio of 20:1, in the presence of APC-conjugated CD107a antibody (BD Biosciences). Brefeldin A (1 µg/ml; Sigma-Aldrich) and Monensin (1 µg/ml; Sigma-Aldrich) were added in the final 5h-incubation period. Effector cells incubated alone in the presence of phorbol-12-myristate-13-acetate (PMA, 50 ng/ml, Sigma-Aldrich) with Ionomycin, (250 ng/ml, Sigma-Aldrich) and were used as positive control whereas medium alone served as unstimulated (US) control. After incubation, cells were harvested, washed and stained for surface markers for 30 min on ice, followed by fixation/permeabilization and intracellular detection of cytokines (TNF-alpha and IFNγ) and analysed by flow cytometry on a LSR II flow cytometer (BD Biosciences). Analysis was performed with FlowJo software (TreeStar, Ashland, OR).

Data are presented as percent specific cytotoxicity calculated using the formula (sample lysis/degranulation − spontaneous lysis/degranulation) / (maximum lysis/degranulation − spontaneous lysis/degranulation) × 100, a method that is widely used to assess cytotoxicity more accurately.

3.5.6 Antibody Blockade experiments

When indicated, freshly purified NK and CD8+ T cells were pre-incubated with 1 µg/mL of anti-NKG2D (1D11, Ebioscience), 1 µg/mL of anti-NKG2A (Z199, Bekcman Coulter) or the respective isotype controls. After washing in PBS, effector cells were incubated with senescent and non-senescent fibroblasts, at a fixed effector to target

ratio of 20:1 for 6 hours in the incubator, in the presence of an anti-CD107a antibody. Brefeldin A (1 µg/ml; Sigma-Aldrich) and Monensin (1 µg/ml; Sigma-Aldrich) were added in the final 5h-incubation period. CD107a expression as a marker of degranulation of effector cells and cytokine secretion (IFNγ and TNFα) were evaluated after 6hours incubation period and percent cytotoxicity was calculated as described previously.

3.5.7 Transfection of cells with small interfering RNAs

Primary human CD8+ T cells were transfected with small interfering RNA (siRNA) by electroporation using the Amaxa Human T Cell Nucleofector Kit and Nucleofector technology (Lonza), according to the manufacturer's instructions. Briefly, 2-5x10^6 cells were resuspended in Nucleofector solution with 200 nmol of siRNA for NKG2D (sc-42948) or a scrambled control siRNA (sc-37007; Santa Cruz). Cells were transferred into 24-well cell culture plates containing pre-equilibrated complete medium (RPMI) and incubated at 37°C. Efficiency of siRNA transfection was confirmed by measuring the expression of NKG2D by flow cytometry, typically 36-48 hours after transfection (please refer to Figure 3.9C for a representative example).

For transfection of human fibroblasts with siRNA for HLA-E (sc-62470) or a scrambled control siRNA (sc-37007; Santa Cruz), we used the Amaxa Human Dermal Fibroblast Nuvleofector Kit (Lonza), as per manufacturer´s protocol. HLA-E siRNA (h) is a pool of 3 target-specific 19-25 nt siRNAs designed to inhibit HLA-E gene expression. Efficiency of siRNA transfection was confirmed by measuring the expression of HLA-E by flow cytometry, typically 36-48 hours after transfection (Figure 2.11B for a representative example).

3.5.8 Flow Cytometry

Multi-parametric flow cytometry was used for phenotypic analysis of PBMCs. For

analysis of surface expression of NK cell receptors, staining was performed at 4°C for 30 min in the presence of saturating concentrations of antibodies listed in **Table 3.2,** using a live/dead fixable Near-Infrared stain (Thermo Scientific, L10119).

For the detection of CD107a expression and intracellular cytokine staining, cells were fixed and permeabilized with the Fix & Perm® Kit (Invitrogen, Life Technologies, UK), before incubation with indicated antibodies or the respective isotype controls (**Table 3.2**). For the detection of Active Caspase 3 expression, cells were fixed and permeabilized with BD Cytofix/Cytoperm (BD 554722) at 4°C for 30 min followed by washing with BD Perm/Wash Buffer and staining with Anti-Active Caspase 3 Antibody PE (BD 550821), all included in the Active Caspase 3 PE kit (all from BD Biosciences, UK).

After staining, cells were washed in PBSA and resuspended in 2% paraformaldehyde until acquisition on a LSR II flow cytometer (BD Biosciences). Analysis was performed with FlowJo software (TreeStar, Ashland, OR).

Antibody	Conjugate	Clone	Isotype	Manufacturer	Catalog #	Dilution	Dilution
CD3	BUV 395	UCHT1	Mouse IgG1	BD	563546	1/100	1:100
CD4	PercP Cy 5.5	SK3	Mouse IgG1	Biolegend	344608	Jan-50	1:50
CD8	BV 421	RPA-T8	Mouse IgG1	Biolegend	301036	01:50	1:50
CD27	BV 786	L128	Mouse IgG1	BD	563327	01:50	1:50
CD28	BV 510	T44	Mouse IgG1	Biolegend	302936	01:50	1:50
CD45RA	BV 605	HI100	Mouse IgG2b	Biolegend	304134	01:50	1:50
NKG2A	AF 700	131411	Mouse IgG2a	R&D Systems	FAB 1059N	01:20	1:20
NKG2C	APC	134591	Mouse IgG1	R&D Systems	FAB138A	01:20	1:20
NKG2D	PE	149810	Mouse IgG1	R&D Systems	FAB139P	01:20	1:20
NKp30	PE	AF29	Mouse IgG1	Miltenyi Biotec	130-099-706	01:20	1:20
IFNgamma	PE-Cy7	B27	Mouse IgG1	BD	557643	01:20	1:20
TNFalpha	APC	MAb11	Mouse IgG1	BD	340534	01:10	1:10

Table 3.2 – List of antibodies used in Flow Cytometry.

3.5.9 Tissue arrays of Congenital Melanocytic Nevi

Tissue arrays of Congenital Melanocytic Nevi (CMN) were obtained from Dr. Veronica Kinsler in the Paediatric Dermatology Department at the Great Ormond Street Hospital, in agreement with the Research Ethics Committee and Research Development Office of Great Ormond Street Hospital and the University College London Institute of Child Health. Samples included in these tissue arrays had been obtained for clinical indications and informed consent was obtained for all patients involved.

Each array included 14-16 formalin-fixed paraffin-embedded tissue sections of CMN from different patients and sections of normal skin, acquired melanocytic nevus and malignant melanoma as controls (**Figure 3.16).**

Figure 3.16 – Representative images of tissue arrays of human congenital melanocytic nevi.

3.5.10 Immunohistochemical staining

Immunohistochemical staining of formalin-fixed paraffin-embedded tissue arrays of congenital melanocytic nevi was performed by Alex Virasami (with the supervision of Professor Neil Sebire) at the Department of Histopathology, Great Ormond Street Hospital for Children. Stainings were performed on the automated Leica BOND-MAX immunostainer (Leica Biosystems) following deparaffinization and heat-induced epitope retrieval (with Leica HIER solution ER2 at pH9). Staining was detected using Leica Bond polymers.

When indicated, dual staining of HLA-E and CD8/CD3 was performed in the same tissue section using a sequential staining technique with the Chromoplex Dual Staining Detection system (Leica Biosystems): after the first primary antibody incubation, sections were stained with a peroxidase-DAB detection reagent (brown staining). The second primary antibody was applied after the excess DAB was rinsed off, followed by detection with an alkaline phosphatase detection system labeled with a fast red chromogen (red staining).

All antibodies used were optimized for use on paraffin sections with appropriate positive and negative controls (**Table 3.3**).

Antibody	Clone	Isotype	Manufacturer	Catalog #
HLA-E	MEM-E/02	Mouse IgG1	Abcam	Ab2216
CD8	4B11	Mouse IgG2b	Leica	PA0183
CD3	LN10	Mouse IgG1	Leica	PA0553
Ki67	MM1	Mouse IgG1	Leica	PA0118

Table 3.3 – List of primary antibodies used for Immunohistochemistry.

3.5.11 Image Analysis

Quantification of HLA-E, CD8 and CD3-positive on CMN tissue sections was performed with image analysis software, in collaboration with Mathew Ellis (Division of Neuropathology at UCL Institute of Neurology).

Briefly, tissue array slides were scanned on a LEICA SCN400F digital slide scanner (LEICA Microsystems). Scanned images were then processed and analysed on the SlidePath Digital Image Hub (LEICA) with Definiens Tissue Studio 3.6 (Definiens AG Munich, Germany). After manual selection of the regions of interest (ROI) in each section, image analysis algorithms were defined as follows: the software determined the area of each ROI (in μm^2) and identified and counted the number of nuclei (# blue), the number of HLA-E positive cells (# brown), the number of CD8-positive cells (# red) and the number of CD8+ cells bordering HLA-E+ cells (# red bordering brown) per region of interest. All counts were expressed as cells/ μm^2. Using this information we then defined a function for HLA-E intensity ((# brown/# blue)*100), CD8 infiltration ((# red/# blue)*100) and co-localization of CD8 and HLA-E ((# red bordering brown/# red)*100). The scores for HLA-E intensity and CD8 infiltration were defined according to frequency of HLA-E+ and CD8+ cells as: negative (score 0: between 0-1%), mild (score 1: 1-5%), moderate (score 2: 5-10%) and intense (score 3: >10%). Due to image quality problems some sections with low quality of tissue morphology were excluded and a total of 24 sections of congenital human nevi were analysed. The complete data analysis is available in **Table 3.4**.

CMN label	Area (µm²)	# Nucleus(blue)	# Nucleus(Red)	Red bordering Brown	# Nucleus(Brown)	Area IHC_Brown	#Red/#Blue*100	#Brown/#Blue*100	Red bordering Brown/total red
MDI	2409184	11107	507	405	658	61662	4.56	5.92	79.88
MDI	5294618	16327	1427	254	5155	495109.5	8.74	31.57	17.80
MDI	4884588	13731	879	724	8125	450164.5	6.40	59.17	82.37
MDI	2536287	9366	185	52	1111	159056.25	1.98	11.86	28.11
MDI	668465	644	82	35	249	59336	12.73	38.66	42.68
MDI	3309243	18117	167	107	1439	107703	0.92	7.94	64.07
MDI	2789191	10397	520	176	1211	86756.5	5.00	11.65	33.85
MDI	1865116	6399	43	42	435	25136.5	0.67	6.80	97.67
MDI	469211	1617	90	21	53	2265.75	5.57	3.28	23.33
MDI	2847470	7633	544	200	2444	152364	7.13	32.02	36.76
MDI	902587	2885	105	58	177	12452.25	3.64	6.14	55.24
MDI	3403055	14296	606	312	1641	148881.75	4.24	11.48	51.49
MDI	937157	3794	164	78	328	15894.75	4.32	8.65	47.56
MDI	2498848	9442	409	145	1989	113468.25	4.33	21.07	35.45
SNDI	1914257	9160	599	195	1026	91804.25	6.54	11.20	32.55
SNDI	2338713	13905	1024	467	556	141636.75	7.36	4.00	45.61
SNDI	1472323	3790	627	147	169	7365.5	16.54	4.46	23.44
SNDI	941658	7332	4	4	6	4922.25	0.05	0.08	100.00
SNDI	1417008	9293	541	377	524	57882.75	5.82	5.64	69.69
SNDI	1252299	9202	263	220	453	42254.75	2.86	4.92	83.65
SNDI	1190083	10202	101	67	427	45777.5	0.99	4.19	66.34
SNDI	1263369	7964	175	148	248	51623	2.20	3.11	84.57
SNDI	1575296	11112	301	249	407	80523.75	2.71	3.66	82.72
SNDI	1259527	5114	596	288	589	63354.5	11.65	11.52	48.32
Mean	**2059981.38**	**8867.88**	**414.96**	**198.79**	**1225.83**	**103224.83**	**5.29**	**12.87**	**55.55**
STDEV	**1244673.12**	**4507.07**	**348.91**	**169.43**	**1834.12**	**123721.85**	**4.02**	**14.07**	**24.87**

Table 3.4 – Quantification of HLA-E and CD8 positive cells performed by digital image analysis of human melanocytic nevi stained using immunohistochemistry.

3.5.12 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 6.00. Tests were used to determine data distribution and depending on the normality of the data, comparisons were performed using the Student *t* test, the non-parametric Mann–Whitney U test (for two groups), the Wilcoxon signed rank test (for > 2 paired groups), Kruskal–Wallis (for > 2 nonpaired groups) or Friedman (for > 2 paired groups) one-way ANOVA tests, as appropriate. Linear regression analysis was performed to generate lines of best fit and correlations between variables were analysed using Pearson´s or Spearman's rank correlation coefficient (r). Values of $p < 0.05$ were considered significant for all tests. Data are presented as means ± standard error of the mean (SEM) unless otherwise stated.

4 Reprogramming of senescent CD8+ T cells into NK-like T cells during human aging

4.1 ABSTRACT

In the previous chapter, an increased expression of receptors belonging to the NKG2 family was found in CD8+ T cells with a highly differentiated phenotype. Following on these observations, an extended analysis of the expression of NK cell receptors on human peripheral blood lymphocytes was performed. It was found that highly differentiated CD8+ T cells not only express phenotypic markers of NK cells but also acquire NK-like effector functions, mediated independently of the TCR. Interestingly, highly differentiated CD8+ T cells exhibit decreased TCR responsiveness while showing an enhanced sensitivity to NKG2D ligation, suggesting that as T cells differentiate, they become less dependent on TCR-driven signals and more responsive to innate-like stimulation. In the present chapter, the molecular mechanisms underlying the acquisition of NK-like functions by highly differentiated human CD8+ T cells were investigated. It is demonstrated that the *de novo* expression of the adaptor DAP12 by senescent CD8+ T cells is determinant for the acquisition of innate-like functions, mediated by NKG2D, independently of the TCR. Furthermore, the expression of NK receptors and signaling adaptors are coordinately induced as cells lose key components of the TCR machinery. This is evident at the transcriptional level, supporting the hypothesis of a general reprogramming of CD8+ T cells to NK-like T cells as a compensatory mechanism to the contraction of the TCR repertoire during human aging.

KEY POINTS

- Highly differentiated CD8+ T cells exhibit phenotypic, functional and transcriptomic changes resembling NK cells;
- The acquisition of NK cell receptors and signaling molecules occurs in coordination with the suppression of conventional TCR signaling;
- The acquisition of innate-like functions by CD8+ T cells depends on the association of NKG2D with DAP12, not present in less differentiated cells.

4.2 INTRODUCTION

Aging is associated with a general decline in immune function, contributing to a higher risk of infection, cancer and autoimmune diseases in the elderly. Such faulty immune responses are the result of a profound remodeling of the immune system that occurs with age, generally termed as immunosenescence (Vallejo, 2007). While the number of naïve T cells emerging from the thymus progressively decreases with age as a result of thymic involution (Lynch et al., 2009), the memory T cell pool expands and exhibits significant changes in the phenotype and function of differentiated T cells (Linton and Dorshkind, 2004). Chronic immune activation due to persistent viral infections, such as cytomegalovirus (CMV) and Epstein-Barr Virus (EBV) are recognized as one of the main drivers contributing to the accumulation of antigen-specific T cells with characteristics of replicative senescence (Ouyang et al., 2003, Pawelec and Gouttefangeas, 2006, Brunner et al., 2011). In combination with the depletion of the naïve T cell pool, the accumulation of terminally differentiated T cells with age skews the immune repertoire and has been implicated in the impaired immune responses to new antigens and vaccination in the elderly (Akbar et al., 2004, Blackman and Woodland, 2011, Arnold et al., 2011, McElhaney et al., 2012).

Functionally, terminally differentiated or senescent T cells are often regarded as a defective population, exhibiting limited proliferative potential and self-renewal capacity associated with low telomerase activity (Libri et al., 2011, Di Mitri et al., 2011, Henson et al., 2014, Lanna et al., 2014). Such functional defects are inexorably linked with the age-related decline in immune responses (Akbar et al., 2004, Weng et al., 2009, Akbar et al., 2016). Paradoxically however, accumulating evidence indicates that terminally differentiated cells maintain potent effector functions and are able to respond robustly to stimulation (Libri et al., 2011, Lachmann et al., 2012, Henson et al., 2014). The molecular mechanisms underlying these

divergent observations remain elusive. Recent studies indicate that senescent T cells have impaired TCR signalling activity (Larbi et al., 2004, Li et al., 2012a, Lanna et al., 2014, Goronzy et al., 2012), predicting the existence of TCR-independent pathways that may regulate effector functions in these cells.

Interestingly, as T cells differentiate they express receptors that are normally attributed to natural killer (NK) cells, especially in the CD8+ T cell compartment (Strauss-Albee et al., 2014). We have shown in the previous chapter that the expression of NKG2 family of receptors is highest in CD8+ T cells that exhibit a terminally differentiated phenotype. Other studies have demonstrated an increased expression of NK-lineage receptors in conditions associated with chronic immune activation and during aging (Tarazona et al., 2000, Vivier and Anfossi, 2004, Abedin et al., 2005, Vallejo et al., 2011). However, the biological significance of the acquisition of NK receptors by T cells during aging remains poorly understood. Here we set out to explore the molecular pathways regulating effector functions of senescent CD8+ T cells and revealed an important role for NK receptors, in particular NKG2D, in mediating innate-like functions, independently of the TCR.

NKG2D is a C-type lectin activating receptor, which recognizes MHC-related self-ligands induced by various forms of cellular stress and is the prototypical example of a NKR that is highly expressed on CD8+ T cells (Bauer et al., 1999, Raulet, 2003) although the exact role of this receptor in CD8+ T cell function is still controversial. NKG2D has no intrinsic signalling capacity and an association with transmembrane adaptor proteins is required to activate signalling cascades and to maintain a stable expression of this receptor at the cell surface (Lanier, 2015). Comparative studies of NKG2D signaling have revealed that NKG2D is a versatile receptor that may induce direct activation or co-stimulation depending on the availability and association with different adaptors (Snyder et al., 2004). In human CD8+ T cells, NKG2D is thought to exclusively associate with the adaptor DAP10, which contains an YxxM-motif

similarly to CD28, thus resulting in co-stimulatory signals, mediated by the phosphatidylinositol-3-kinase (PI3K) signalling pathway (Wu et al., 1999, Groh et al., 2001, Upshaw et al., 2006, Lanier, 2015). In certain circumstances, however, NKG2D has been shown to provide direct stimulatory signals to human αβ and γδCD8+ T cells, independently of the TCR, provided that cells are stimulated with cytokines such as IL-15 or high doses of IL-2 (Meresse et al., 2004, Verneris et al., 2004, Chu et al., 2013). The molecular determinants of this functional switching are still poorly understood. An alternative pathway for NKG2D signaling has been described in activated NK cells in mice, where the expression of a short splice isoform of NKG2D and pairing with the ITAM-bearing adaptor DAP12 (also known as KARAP or TYROBP), leads to the recruitment of Zap70 (zeta-chain-associated protein kinase 70) and Syk (spleen tyrosine kinase), resulting in direct activation of NK cells, cytokine production and cytotoxicity (Diefenbach et al., 2002, Gilfillan et al., 2002).

In the present chapter we demonstrate that terminally differentiated CD8+ T cells in humans express NK-cell receptors and acquire signaling molecules traditionally linked to the NK-cell receptor machinery, in particular the adaptor DAP12 which is able to pair with NKG2D and recruit Zap70/Syk protein kinases, allowing senescent CD8+ T cells to become permissive to innate signals and respond directly to NKG2D, independently of the TCR. Hence, despite the attenuation of TCR responsiveness, senescent CD8+ T cells display an increased sensitivity to NKG2D ligation and are maintained in a poised effector state that endows them with the capacity to respond rapidly to stimulation. We further demonstrate that such innate-like characteristics are imprinted at the transcriptional level supporting the hypothesis of a general reprogramming of highly differentiated CD8+ T cell into innate-like T cells during human aging (Pereira and Akbar, 2016).

4.3 RESULTS

4.3.1 Impaired TCR signalling is a distinctive feature of human senescent CD8+ T cells

Multiple phenotypic and functional features have been proposed to define highly differentiated or senescent T cells. Loss of costimulatory receptors, CD28 and CD27, is one of the most consistent markers T cell differentiation (Effros, 1997, van Lier et al., 2003, Weng et al., 2009, Mahnke et al., 2013). The differential expression of these markers enables the distinction of three subsets within the CD8+ T cell compartment (**Figure 4.1A**): an undifferentiated (CD28+CD27+), an intermediate (CD28-CD27+) and a highly differentiated subset (CD28-CD27-) negative for both markers (Appay et al., 2002, Henson et al., 2009). Alternatively, T cell subsets may be stratified according to the expression of CD45RA and CD27 (**Figure 4.1B)** as: naïve (T_{naive} CD27+CD45RA+), central memory (T_{CM}; $CD45RA^{-}CD27^{+}$), effector memory (T_{EM}; $CD45RA^{-}CD27^{-}$) and effector memory T cells that re-express CD45RA (T_{EMRA}; $CD45RA^{+}CD27^{-}$)(Appay et al., 2008). Re-expression of CD45RA identifies a subpopulation of effector memory T cells with features of terminal differentiation, denominated as T effector memory re-expressing CD45RA or T_{EMRA} (Henson et al., 2012). As CD8+ T cells tend to sequentially downregulate CD28 followed by CD27 (Rufer et al., 2003), the population defined by the loss of CD27 and re-expression of CD45RA (T_{EMRA}), is in its vast majority negative for both CD28 and CD27 and therefore included in the subset of late differentiated CD28–27– CD8+ T cells (**Figure 4.1E)** (Romero et al., 2007). Chronological aging is typically associated with a contraction of the naïve T cell pool and expansion of the CD28-CD27-CD45RA+ terminally differentiated subset (**Fig. 4.1C,D)** (Akbar and Fletcher, 2005, Koch et al., 2008, Arnold et al., 2011, Akbar and Henson, 2011)**.**

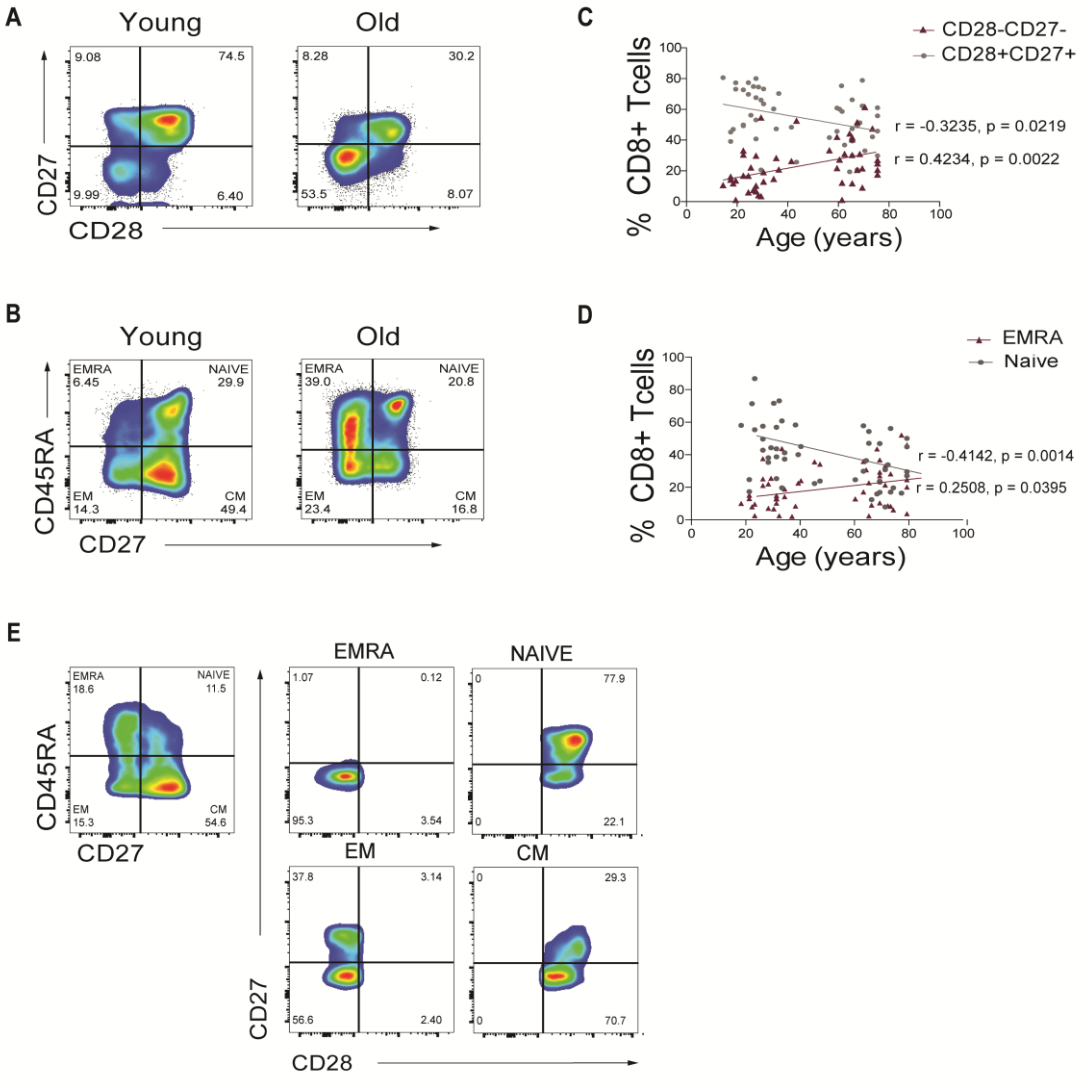

Figure 4.1 – Aging is associated with the accumulation of T cells with a terminally differentiated phenotype, characterized by the loss of CD27 and CD28 costimulatory receptors and re-expression of CD45RA.

Representative FACs plots showing the subsets stratified by the expression of CD27/CD28 (**A)** and CD27/CD45RA **(B)** in CD8+ T cells derived from a young (≤ 35 yo) and old (≥65 yo) healthy donor. Numbers in quadrants represent percentages of cells in each subset. **C)** Correlation between the frequencies of early (CD28+27+) and late (CD28-27-) differentiated CD8+ T cells and age, gated as in A, in PBMCs derived from healthy donors (n=50, median age of 52, age range 25-83) and determined by multicolour flow cytometry. **D)** Correlation between the frequencies of naive (CD27+45RA+) and EMRA (CD27-45RA+) CD8+ T cells and age, gated s in B in the same donors ((n=50). Lines of best fit were generated by linear regression and the correlation (*r* value) and significance were assessed by Spearman rank. **E)** CD8+ T subsets defined by CD27/CD45RA expression were gated according to the expression of CD27/CD28 receptors, demonstrating that EMRA CD8+ T cells are in their vast majority included in the CD28-27- subset.

CD27-CD28-CD45RA+ T cells have been shown to exhibit features of senescence that include accumulation of DNA damage markers, short telomeres, low proliferation and loss in the capacity to activate the enzyme telomerase (Plunkett et al., 2007, Di Mitri et al., 2011, Henson et al., 2014, Lanna et al., 2014). However, despite the loss of proliferative capacity, terminally differentiated cells are polyfunctional, reflecting their ability to simultaneously carry out multiple functions, including secretion of IFN-γ and TNF-α and cytotoxicity (Libri et al., 2011, Lachmann et al., 2012, Henson et al., 2014), and this is an important observation that distinguishes senescent from exhausted T cells (Di Mitri et al., 2011).

To investigate the mechanisms responsible for reduced proliferative responses to TCR stimulation in highly differentiated CD8+ T cells (Henson et al., 2009), the first signaling events downstream of the TCR were analysed in freshly isolated human PBMCs using phospho-flow cytometry. After crosslinking the TCR with a monoclonal antibody specific to CD3 (OKT3, 10 ug/mL), it was consistently observed that phosphorylation of TCR-proximal components such as CD3ζ (Tyr142) and Zap70 (Tyr319) was significantly impaired in highly differentiated CD27-CD28- CD8+ T cells compared to the less differentiated cells ($p< 0.001$, **Figure 4.2A,B**).

Recent observations from our group indicate that senescent CD4+ T cells lose key components of the TCR machinery (Lanna et al., 2014). Therefore, it was next investigated whether the expression of the TCR-proximal machinery was altered between the different subsets of CD8+ T cells. As shown in the immunoblots in **Figure 4.2C**, a significant downregulation of Lck expression, Lat and PLCγ was observed as cells progressed towards terminal differentiation explaining the overt impairment in phosphorylation after TCR stimulation. Interestingly, the expression of Zap70 was conserved across all subsets of CD8+ T cells ($p< 0.05$, **Figure 4.2C,D)**, whereas senescent CD4+ T cells lack this component of the TCR (Lanna et al., 2014).

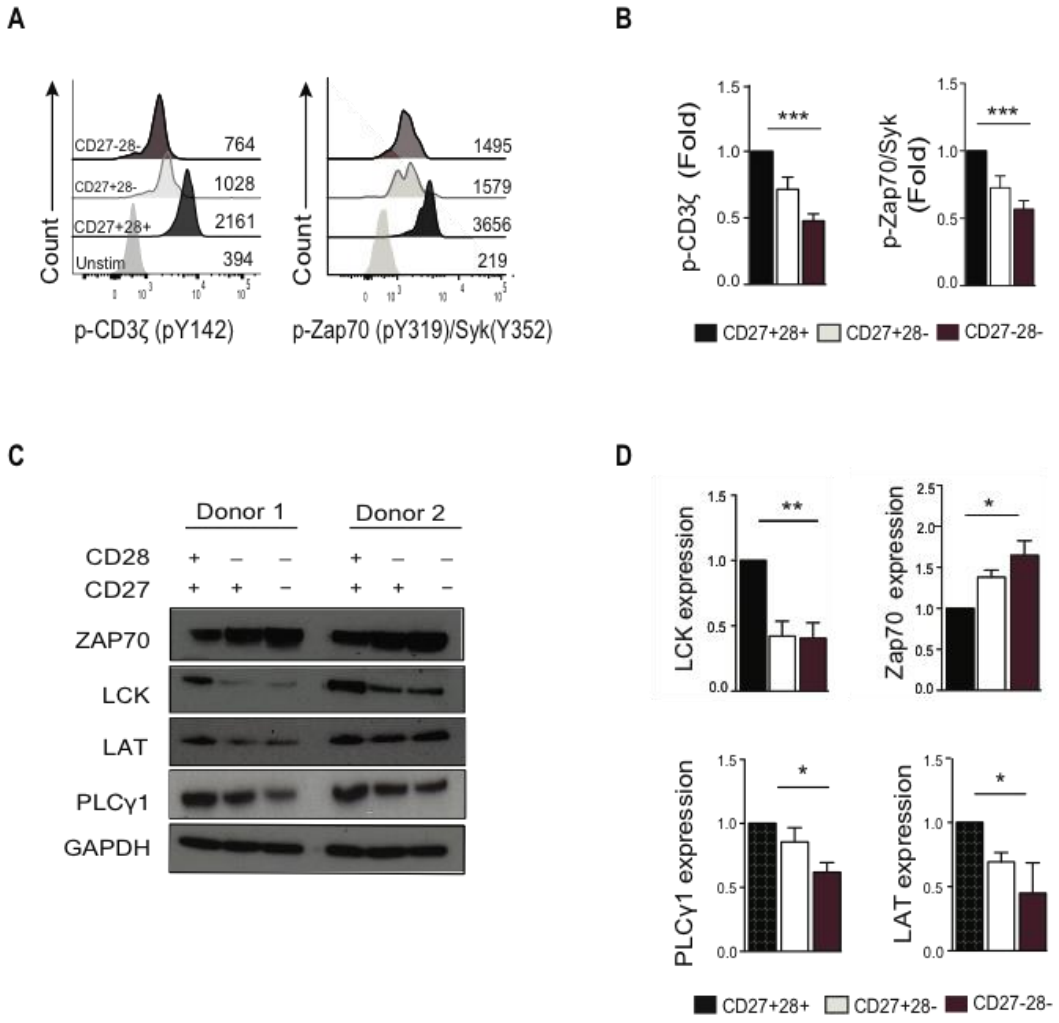

Figure 4.2 - Highly differentiated CD8+ T cells exhibit impaired TCR proximal signaling.

Freshly isolated human CD8+ T cells were analysed for **(A)** phosphorylation of CD3ζ (left) and Zap70/Syk (right), determined 10 minutes after TCR crosslinking with anti-CD3 (OKT3, 10 µg/mL) in CD8+ CD27/CD28 subsets, assessed by phosphoflow cytometry. Numbers represent the mean fluorescence intensity (MFI) for each subset. Light grey histograms represent unstimulated controls. **B)** Summary results of CD3ζ and Zap70/Syk phosphorylation in CD8+ subsets, presented as the MFI relative to that of CD27+CD28+ T cells, set as 1 (*n*=4*).* **C)** Immunoblot analysis of the expression of proximal TCR components on human $CD8^+$ CD27/CD28 subsets from two different donors purified directly *ex-vivo* using magnetic beads. GAPDH serves as loading control. **D)** Quantification of LCK, Zap70, PLCGγ and LAT expression normalized to the loading control (GAPDH) and presented relative to the basal expression in naïve T cells (CD27+CD28+) set as 1 (*n*=4*)*. Analysis of variance between groups determined with Friedman Test with Dunn´s correction for multiple comparisons (**p< 0.05*, ***p<0.01*, ****p< 0.001, ****p< 0.0001)*.

According to the canonical model for TCR signaling (**Figure 4.3)**, activation of Lck is one of the first events after TCR ligation, leading to the recruitment and rapid phosphorylation of CD3ζ chains, Zap70 and the assembly of Lat signalossome, which is central for the integration of TCR-proximal signals to downstream effector pathways (Chakraborty and Weiss, 2014, Malissen et al., 2014).

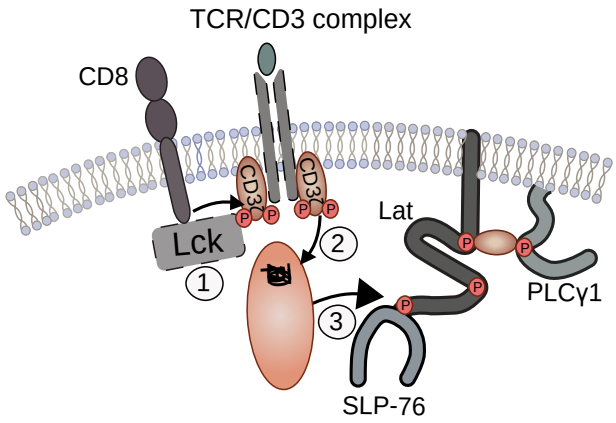

Figure 4.3 – Schematic model of T Cell Receptor (TCR) proximal signaling.
ITAM, immunoreceptor tyrosine-based activation motif; Zap70, zeta-chain protein kinase 70; LCK, lymphocyte-specific tyrosine kinase; LAT, Linker for Activation of T cells: PLCγ, Phospholipase Cγ; SLP-76, Src homology 2-domain containing leukocyte protein of 76 kDa.

The observation that highly differentiated CD8+ T cells are severely depleted of proximal components of the TCR signalossome may explain the impaired phosphorylation of CD3ζ and Zap70 through the canonical TCR signaling pathway. However the conserved expression of total Zap70 suggests that this Syk-family tyrosine kinase may be crucial to activate alternative signaling pathways in senescent CD8+ T cells. In line with previous studies suggesting that T cell signalling pathways are impaired with age (Larbi et al., 2004, Li et al., 2012a, Lanna et al., 2014), this data indicates that impairment in classical TCR proximal signaling is an important characteristic of senescent CD8+ T cells.

4.3.2 Human highly differentiated T cells express high levels of cytolytic effectors

Despite TCR hyporesponsiveness and lack of proliferative capacity (Henson et al., 2009, Henson et al., 2014), highly differentiated T cells are armed effector cells, as demonstrated by the constitutive high expression of cytolytic molecules, granzyme B and perforin, compared to less differentiated subsets ((*p< 0.0001,* **Figure 4.4**). Interestingly, a significant *ex-vivo* expression of cytolytic mediators on CD4+ T cells was also found, mainly restricted to the highly differentiated subsets, predicting the acquisition of cytotoxic functions by these cells.

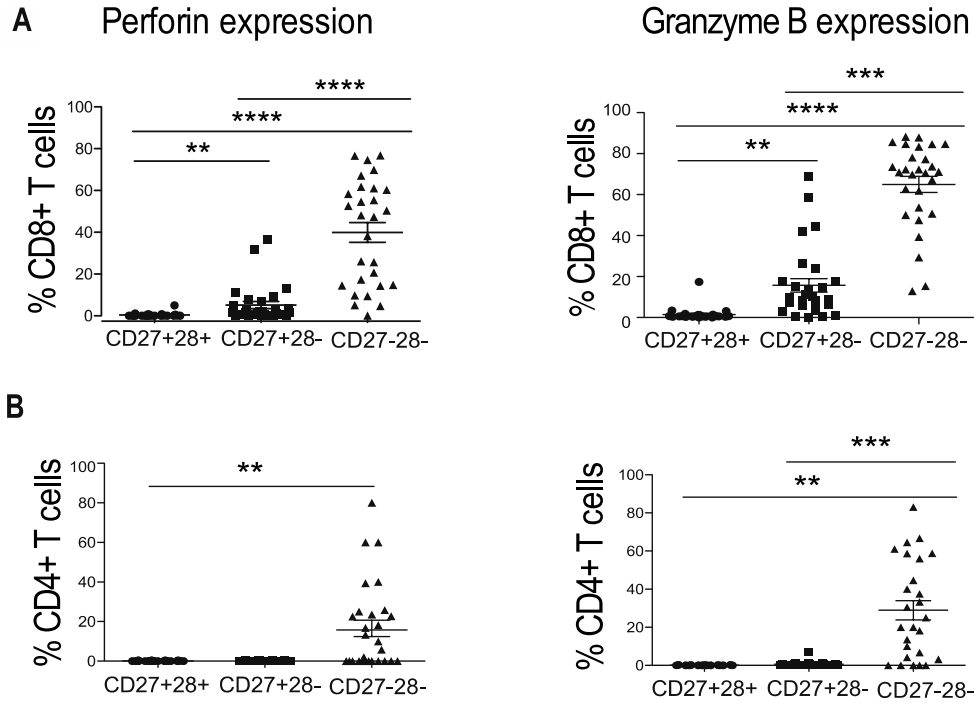

Figure 4.4 – Terminally differentiated cells show the highest expression of cytolytic mediators compared to less differentiated cells.

Ex-vivo flow cytomery analysis of the expression of effector mediators, perforin (left panel) and granzyme B (right) across T cell subsets, defined by CD27/CD28, in both CD8+ **(A**) and CD4+ T cells (**B**) (*n*=25). Analysis of variance between groups determined with Friedman Test with Dunn´s correction for multiple comparisons (**p< 0.05*, ***p<0.01*, ****p< 0.001, ****p< 0.0001)*.

4.3.3 Highly differentiated CD8+ T cells acquire NK cell phenotypic markers

A growing body of evidence indicates that, as T cells differentiate, they acquire the expression of receptors that are normally attributed to NK cells (Tarazona et al., 2000, Vallejo et al., 2011, Strauss-Albee et al., 2014). Using flow cytometry, we performed an extensive analysis of the surface expression of NK cell receptors on human peripheral blood mononuclear cells (PBMCs) isolated from healthy donors across different ages (*n*= 22; median age 52; range, 25-83). Cells were stained with specific antibodies for each NK cell receptor, in combination with antibodies for TCRαβ and the phenotypic markers CD3, CD4, CD8, CD27, CD28 and CD45RA and analysed by multicolor flow cytometry. The frequency of cells expressing NK cell receptors was calculated for each compartment – CD3-negative, CD3-positive, CD8-positive, CD4-positive - using the gating strategy shown in **Figure 4.5A.** Within CD8+ and CD4+ T cell compartments, the frequency of cells expressing NK cell receptors was calculated for each subset defined by CD27/CD28 expression (**Figure 4.5B)**, as well as in the subsets defined by CD7/CD45RA expression (**Figure 4.5C)**.

In line with previous studies, the expression of the majority of NK cell receptors studied was not unique to NK cells and the highest expression on non-NK cells was observed in the peripheral αβCD3+CD8+ T cell compartment (**Figure 4.5A)**. Amongst CD8+ T cells, the expression of NK cell receptors was found to be highest in cells with a terminally differentiated phenotype, characterized by the loss of CD28 and CD27 and re-expression of CD45RA. Importantly, it was confirmed that CD3+ cells expressing NK cell receptors did not express the semi-invariant TCR Vα24/Vβ11 chains, thus excluding that they represent an expansion of classical NKT cells, which only represented a minor fraction (0.1–1% amongst donors) of T cells in peripheral blood (**Figure 4.5A**).

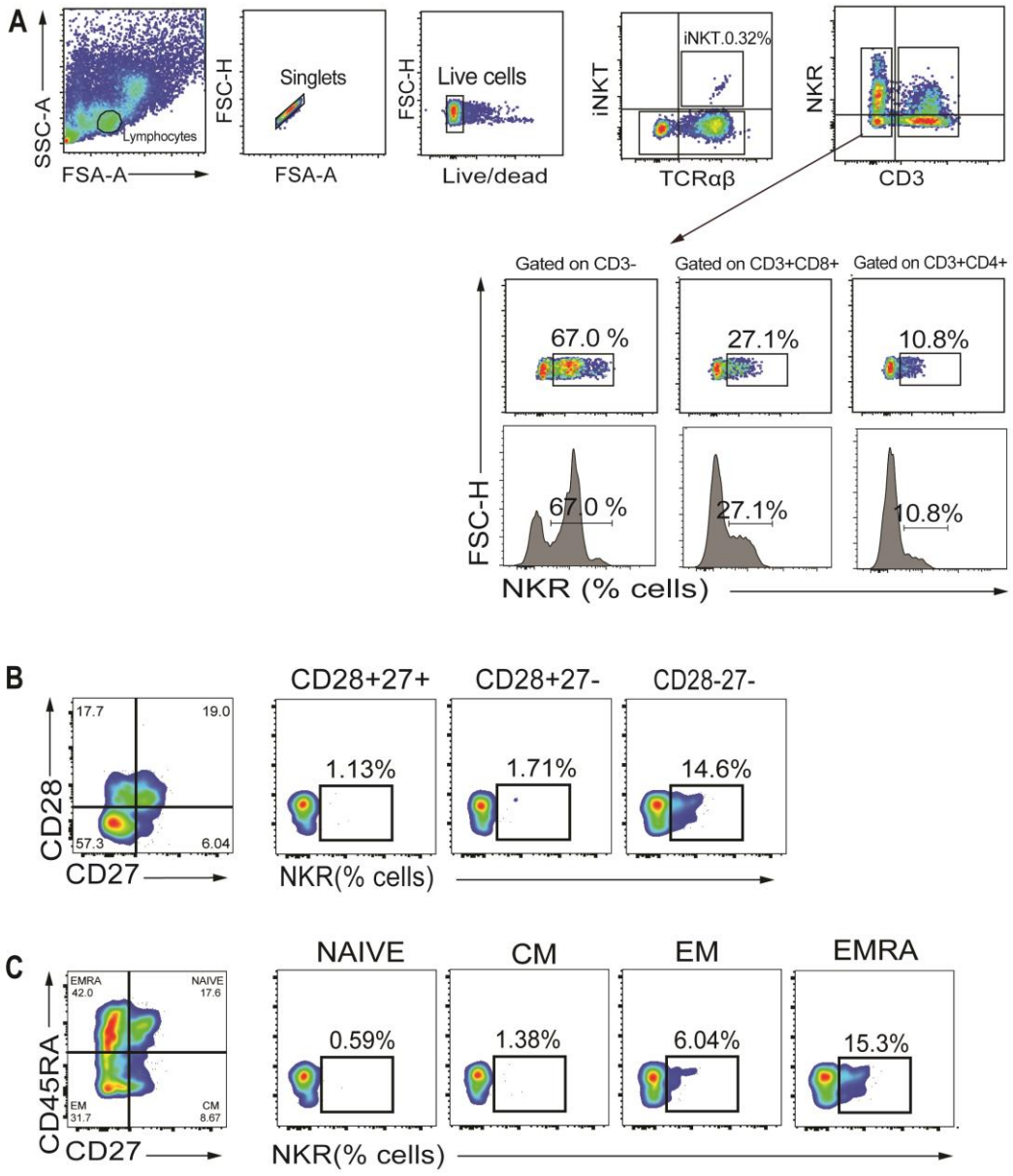

Figure 4.5- T cells expressing NK cell receptors are mostly αβCD8+ T cells with a terminally differentiated phenotype.

A. Gating strategy used to determine the frequency of cells expressing NK cell receptors (NKR), within the different cell subtypes in PBMCs stained by multicolour flow cytometry. Plots are from a representative staining for CD56 expression. **B.** The frequency of cells expressing NK receptors was determined amongst the subsets of CD3+ CD8+ T cells, defined by CD27/CD28 expression (in B) and CD27/CD45RA (**C**). Naïve (CD27+ 45RA+), CM, central memory (CD27+ 45RA-), EM, effector memory (CD27- 45RA-) and EMRA, effector memory re-expressing CD45RA (CD27- 45RA+). Numbers represent percentages of cells within each compartment.

The repertoire of NK receptors expressed on highly differentiated αβCD8+ T cells is highly diverse and includes both activating (NKG2D, NKG2C, NKp44, KIR2DS) and inhibitory receptors (NKG2A, KIR2DL/KIR3DL and CD244) as well as other canonical NK cell (CD16 and CD56) and maturation (KLRG1, CD57) markers (**Figure 4.6**).

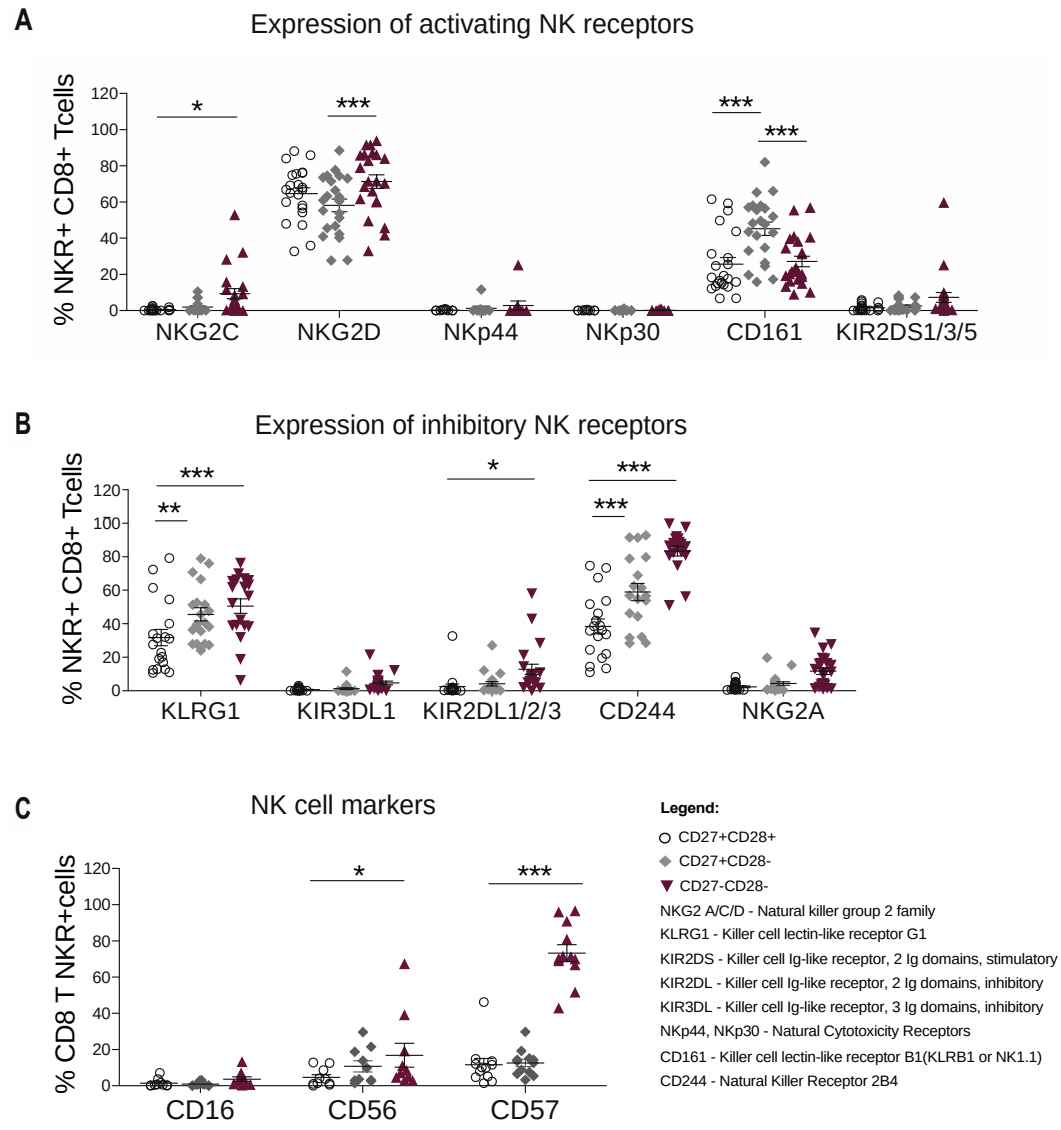

Figure 4.6– Expression of NKR on CD8+ T cell subsets defined by CD27/CD28. Expression of activating (**A**), inhibitory (**B**) and canonical NK markers (**C**) on human CD8+ T subsets, defined by CD27/CD28 expression, from 22 healthy donors (median age = 52, range 25-83 yo) analyzed by flow cytometry. (NKG2A data does not correspond to the same 22 donors, but to the data showed in Chapter 2, Fig.3.4C). Analysis of variance across subsets calculated with Friedman test with Dunn´s correction for multiple comparisons (**p< 0.05*, ***p<0.01*, ****p< 0.001)*.

A similar distribution of NKR was observed in CD8+ T cells subsets defined by the expression of CD27 and CD45RA (**Figure 4.7**).

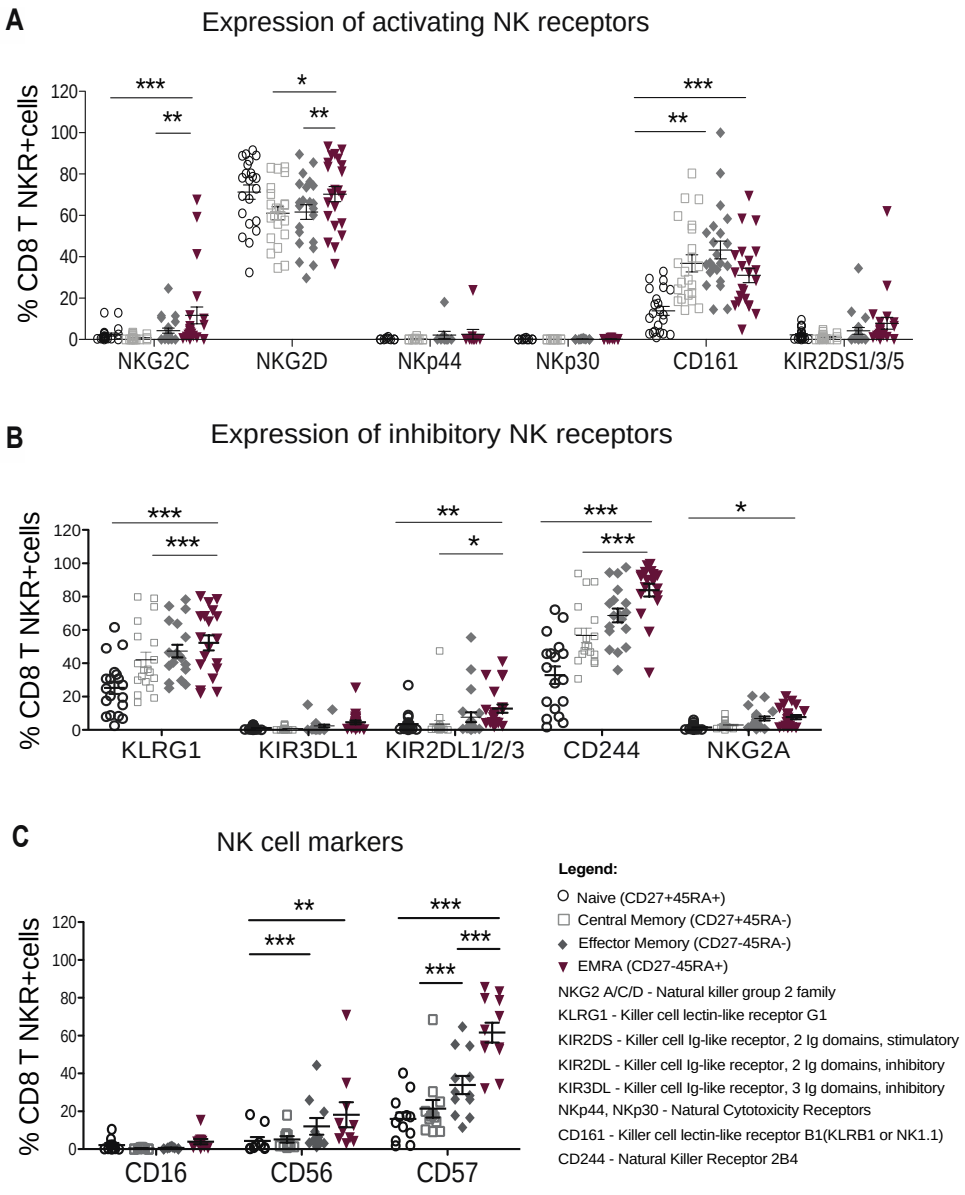

Figure 4.7 - Distribution of NKR on CD8+ T cell subsets defined by CD27/CD45RA.

The expression of activating (**A**), inhibitory (**B**) and other NK cell markers (**C**) was analysed on human CD8+ T subsets now defined by CD27/CD45RA expression, from the same 22 donors, analysed by flow cytometry (NKG2A data does not correspond to the same 22 donors, but to the data showed in Chapter 2, Fig.3.4C). Analysis of variance between subsets calculated with Friedman test with Dunn´s correction for multiple comparisons (*$p< 0.05$, **$p<0.01$, ***$p< 0.001$, ****$p< 0.0001$).

Although the expansion of T cells expressing NK receptors is most noticeable in the CD8+ T cell compartment, the expression of NK cell markers was also found on human CD4+ T cells, predominantly in the highly differentiated subsets (**Figure 4.8),** although the magnitude of expression is higher in CD8+ compared to CD4+ T cells.

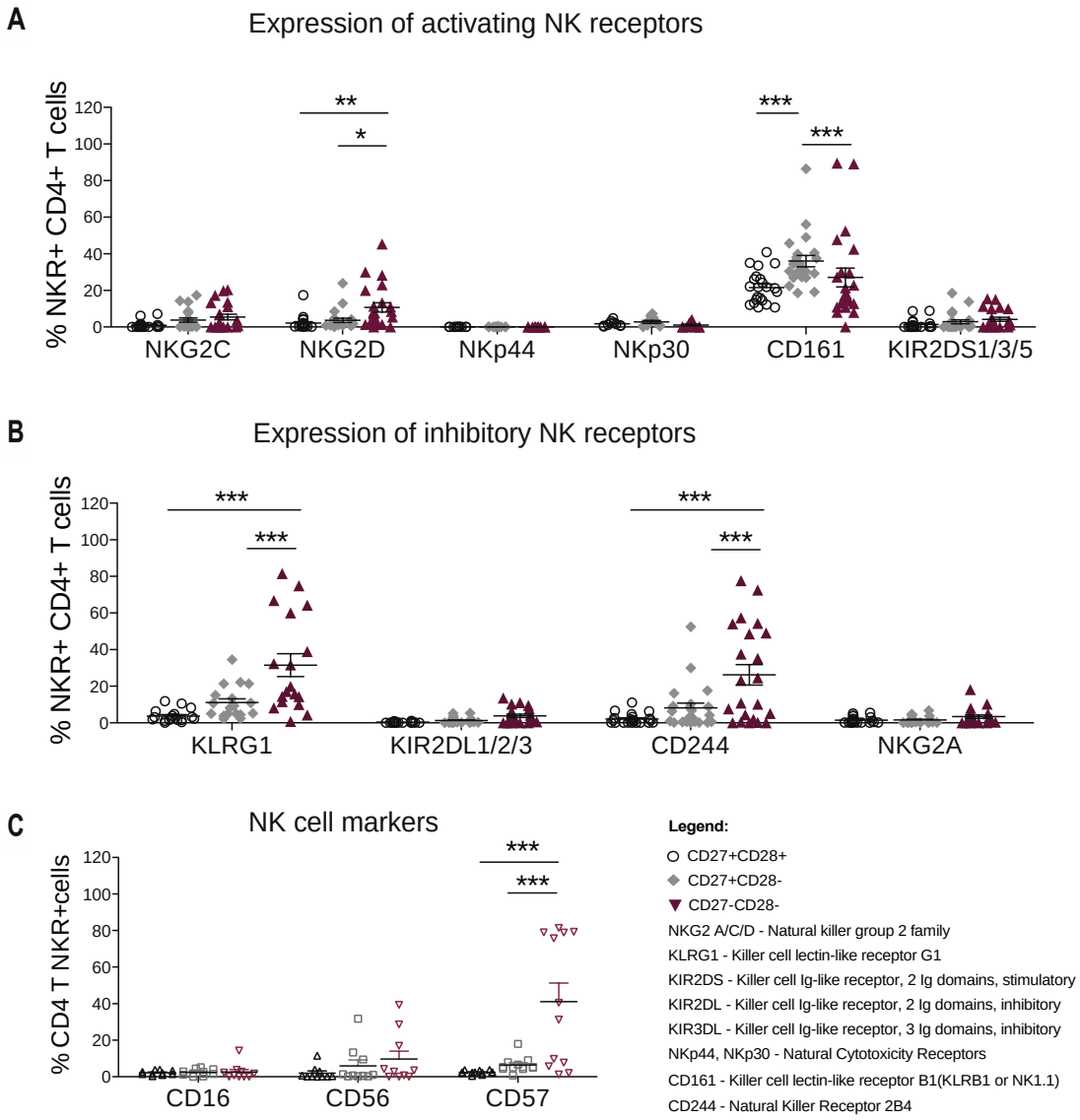

Figure 4.8 – Expression of NKR on CD4+ T cell subsets defined by CD27/CD28. Flow cytometry analysis of the expression of activating (**A**), inhibitory **(B**) and canonical NK cell markers (**C)** on human CD4+ T subsets, defined by CD27/CD28 expression (*n*= 22). Analysis of variance between subsets calculated with Friedman test with Dunn´s correction for multiple comparisons (*$p< 0.05$, **$p<0.01$, ***$p< 0.001$, ****$p< 0.0001$).

For some receptors, there was considerable variability among donors in the expression of NK cell receptors. This is most likely due to genetic polymorphism among donors, as it is known that for some receptors, such as NKG2C or KIRs, surface expression is highly dependent on the genotype (Horowitz et al., 2013).

Together with NKG2D, natural cytotoxicity receptors (NCR), such as NKp44 and NKp30, are the main mediators of direct cytotoxicity in NK cells whereas CD16 (FCγ receptor III) is essential for antibody-dependent cellular cytotoxicity (ADCC) (Long et al., 2013). The acquisition of such activating receptors by CD8+ T cells suggests a possible role in mediating cytolytic responses by highly differentiated CD8+ T cells.

Killer cell Ig-like receptors (KIRs) are mainly involved in inhibiting immune responses (inhibitory KIRs) via interaction with MHC class I molecules. Some KIRs, however, have been found to deliver stimulatory signals (activating KIRs, KIR2DS family) via the association with the ITAM-bearing adaptor DAP12 (Lanier et al., 1998b). Similarly, NKG2C has been shown to couple with DAP12, providing direct activating signals to both NK and CD8+ T cells, whereas the inhibitory isoform NKG2A negatively regulates NK and T cell function.

CD161 (the product of KLRB1 gene) is a C-type lectin NK receptor that has been identified in subsets of CD4+ and CD8+ αβ T cells with a memory phenotype and has been proposed as a marker of IL17-producing cells (Maggi et al., 2010). Consistent with previous reports, we found that the majority of CD161-expressing cells in the peripheral CD8+ T cell compartment had a memory rather than a terminally differentiated (**Figure 3.7**). Nevertheless, it is known from the literature that the majority of CD8+ CD161^{high} T cells are represented by mucosal-associated invariant T (MAIT) cells, an innate-like T cell subset that represents ≈ 5% of the T cell pool, enriched at mucosal sites such as the liver and gut (Dusseaux et al., 2011).

The SLAM family receptor 2B4 (CD244) has been shown to provide co-inhibitory signals to T cells, in particular exhausted CD8+ T cells in synergy with PD-1 (Blackburn et al., 2009, Raziorrouh et al., 2010). Similarly, KLRG1 has been shown to deliver inhibitory signals, supressing proliferation and effector functions in both CD8+ T and NK cells (Henson et al., 2009, Muller-Durovic et al., 2016).

KLRG1 and CD57 are considered markers of terminal differentiation or senescence in both NK and CD8+ T cells (Brenchley et al., 2003, Voehringer et al., 2002, Henson et al., 2009). The coincidental increase in these maturation markers and other NK cell markers corroborates that the expression of NK cell receptors is co-ordinately induced as cells differentiate and the immune system matures.

Collectively, these findings support and extend previous studies regarding the expression of NK cell markers on CD8+ T cells and underscore that these receptors are preferentially expressed on cells with a terminally differentiated phenotype. The acquisition of such a repertoire of inhibitory and activating receptors by terminally differentiated T cells is reminiscent of an NK-like regulation of effector functions, where the functional outcome is determined by the integration of inhibitory and activating signals.

4.3.4 Determinants of the expression of NKR on highly differentiated CD8+ T cells

Although the expression of NK lineage receptors by differentiated αβCD8+ T cells is increasingly well documented, the determinants controlling the acquisition of such receptors by human αβCD8+ T cells remain elusive.

To investigate whether the expression of NK receptors was altered with age, the frequency of CD8+ T cells expressing NK cell receptors was correlated with the age of donors. There was a positive correlation between the frequencies of CD8+ T cells expressing both activating and inhibitory and age (**Figure 4.9A),** significant for CD16 and CD244.

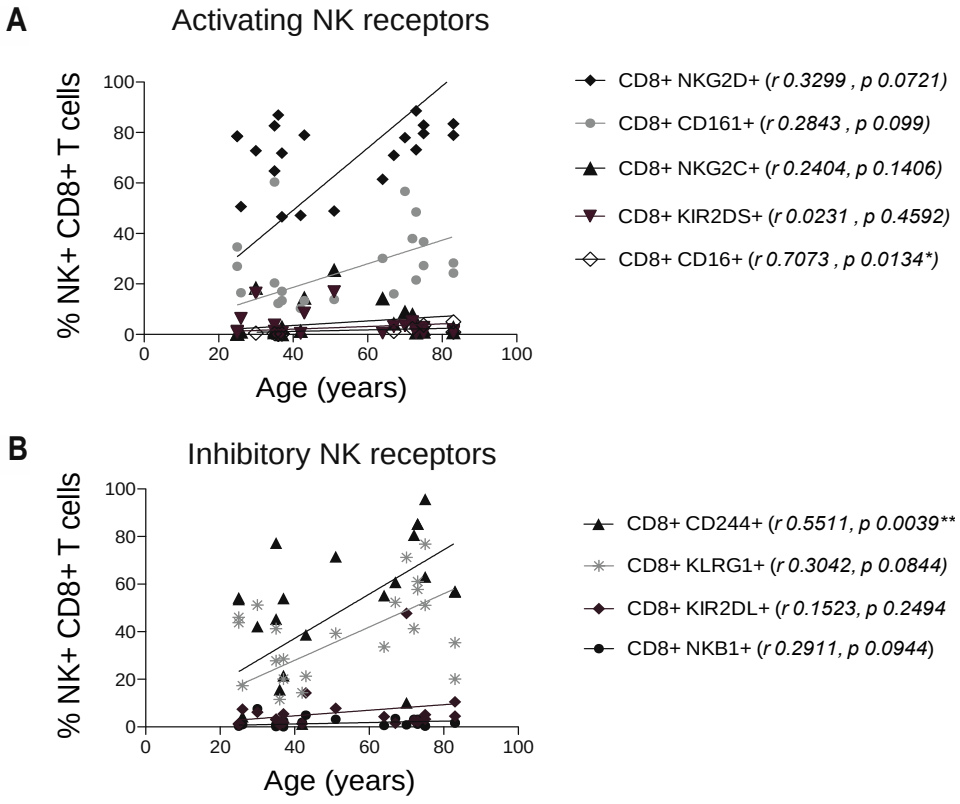

Figure 4.9 - Frequency of CD8+T cells expressing NK cell receptors increases with age.

Correlation between the frequency of activating **(A**) and inhibitory (**B**) NK cell receptors on CD8+ T cells with age of donors (*n*=22). Correlation was analysed using Spearman's rank correlation coefficient (r) (*$p< 0,05$, **$p<0.01$).

Similarly, as expected the frequency of CD8+ T cells expressing NK cell receptors correlated with the expression of KLRG1, indicating that individuals with a mature immune system are most likely to express NKR on CD8+ T cells (**Figure 4.10**).

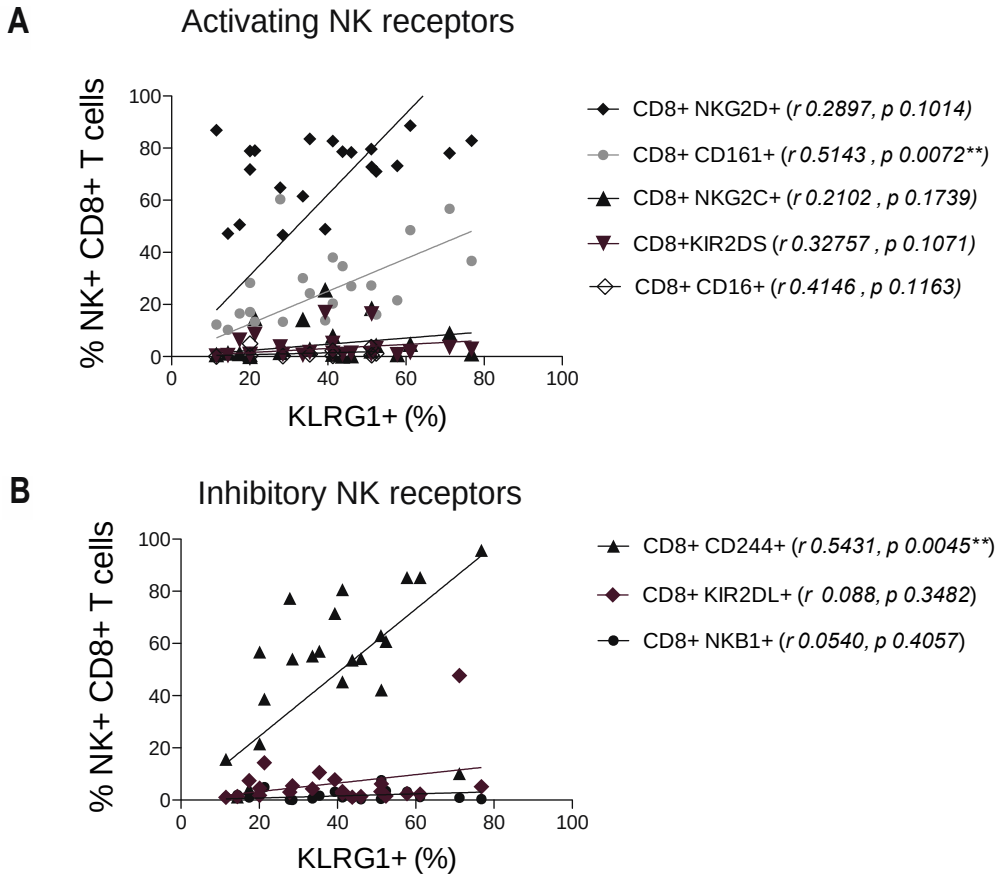

Figure 4.10 – Expression of NK receptors on CD8+ T cells correlates with T cell differentiation.

Correlation between the frequency of activating (**A**) and inhibitory **(B)** NK receptors and the expression of KLRG1 on CD8+ T cells (*n*=22), as a marker of T cell maturation/differentiation. Correlation was analysed using Spearman's rank correlation coefficient (r) (*$p< 0,05$, **$p<0.01$).

Chronic viral infections, in particular CMV infection, were shown to have a profound impact on CD8+ T cell phenotype, driving T cell differentiation and maturation (van Lier et al., 2003, Fletcher et al., 2005, Pawelec and Gouttefangeas, 2006, Brunner et al., 2011). To investigate the contribution of latent viral infections in shaping the NKR repertoire on T cells, we first stratified donors according to their serostatus for CMV and EBV infection and compared the frequency of CD8+ T cells expressing NKR, in both groups. In line with previous reports (Guma et al., 2004), it was found that CMV infection was associated with a significant expansion of NKG2C+ CD8+ T cells (**Figure 4.11**, $p < 0.01$), however the expression of all the other receptors analysed was comparable between CMV positive and negative individuals.

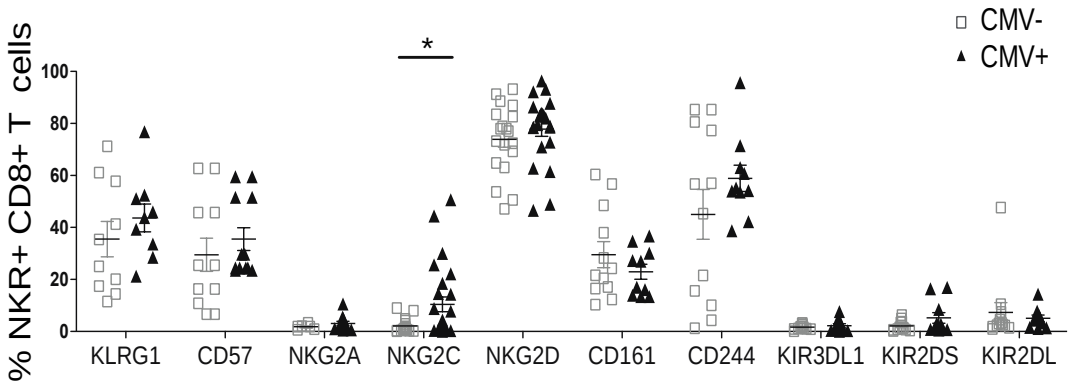

Figure 4.11 – Frequency of NKR+ CD8+ T cells amongst CMV-seropositive and seronegative individuals.

Comparative analysis of the expression of NKR on total CD8+ T cells between CMV+ (n = 10) and CMV-negative (n = 12) donors, using multicolour flow cytometry. Comparison between groups was done using the non-parametric Mann-Whitney U test (*$p< 0,05$).

We next examined the expression of NK cell receptors on virus-specific CD8+ T cells obtained from a pool of CMV and EBV-positive donors after staining with HLA-specific dextrameric complexes loaded with CMV and EBV-derived peptides. To avoid analysing T cell responses to only one viral epitope, two dextramers loaded with different CMV-specific and EBV-specific peptides were used (**Materials and Methods**). Remarkably, the frequency of NKR-expressing cells among dextramer-positive CD8+ T cells was generally lower than in global CD8+ T cells (**Figure 4.12).** This was evident for the majority of the NK receptors analysed in both CMV- and EBV-specific CD8+ T cells (reaching significant differences in CD57, NKG2C and CD161 expression, $p < 0.05$, **Figure 4.12**), with the exception of NKG2D, which showed a trend towards an increased expression in virus-specific CD8+ T cells.

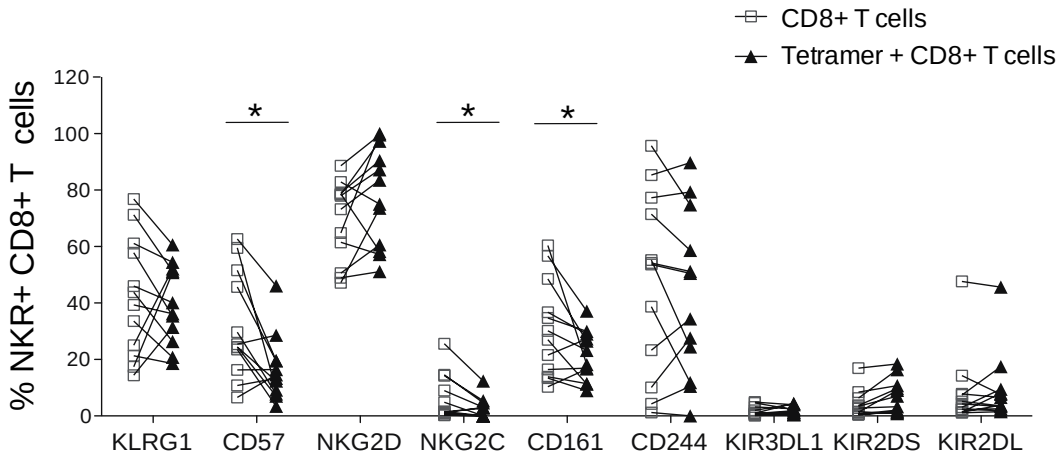

Figure 4.12 – NKR+ CD8+ T cells do not represent an expansion of virus-specific CD8+ T cells.

Flow cytometry analysis of the expression of NKR on virus-specific CD8+ T cells obtained from CMV- or EBV-positive donors after dextramer staining (*n*=12). Data is shown as percentages of NKR+ cells within dextramer-positive CD8+ T cells, compared to global CD8+ T cells. Comparison between dextramer-positive and global CD8+ T cells was done by Wilcoxon signed rank test for paired samples (*$p< 0,05$, **$p<0.01$, ***$p< 0.001$).

Studies indicate that the differentiation phenotype of virus-specific CD8+ T cells varies significantly in different virus infections (Appay et al., 2002, van Lier et al., 2003) and it is possible that such heterogeneity could contribute to the discrepancy observed in the expression of NKR on virus-specific T cells. In line with this, EBV-specific T cells preferentially showed an intermediate differentiated phenotype, distributed amongst the central and effector memory subsets, whereas CMV-specific T cells consisted primarily of highly differentiated CD8+ T cells, confirming previous studies (Appay et al., 2002). However, a stratified analysis according to viral specificities showed no significant differences in the pattern of expression of NKR on virus-specific CD8+ T cells relative to total CD8+ T cells (**Figure 4.13).**

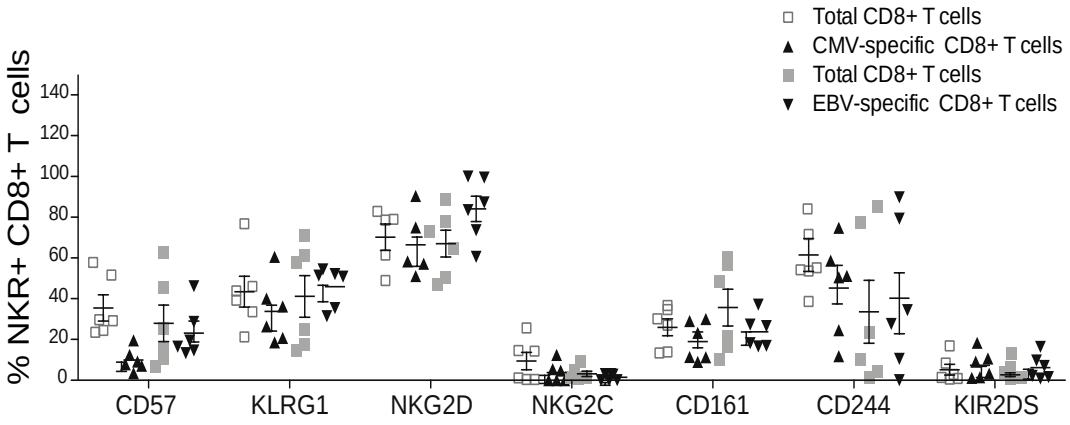

Figure 4.13 – Dextramer-positive CD8+ T cells with distinct viral specificities generally express lower levels of NKR compared with global CD8+ T cells.

Flow cytometry analysis of the expression of NKR between CMV- specific (*n*=6) and EBV-specific CD8+ T cells (*n*=6) compared with global CD8+ T cells from the same donors. Statistical significance tested with the Wilcoxon signed rank test for paired samples.

Although the number of donors tested is low and does not allow us to draw definitive conclusions, this data suggests that CD8+ T cells expressing NK receptors do not represent an expansion of virus-specific CD8+ T cells, pointing to other possible factors as drivers of NKR expression on T cells.

Homeostatic cytokines, in particular IL-15 have been implicated in antigen-independent differentiation of human CD8+ T cells (Alves et al., 2003, Geginat et al., 2003) and can induce the loss of CD28 (Chiu et al., 2006) as well as re-expression of CD45RA on memory CD8+ T cells (Dunne et al., 2005, Griffiths et al., 2013). Further, IL-12 and IL-15 were shown to induce bystander-activation of CD8+ T cells independently of TCR specificity. (Chu et al., 2013, Soudja et al., 2012, Liu et al., 2002). Therefore, the role of these cytokines in inducing the expression of NK receptors on CD8+ T cells was investigated. For this, MACS-purified CD8+ CD28+27+ T cells were cultured in the presence of IL15, IL12 or plate-bound anti-CD3+IL-2 (to reproduce the effects of TCR stimulation) and the expression of NKR was monitored by flow cytometry. Interestingly, it was found that cytokines had a stronger effect on the induction of NKR expression compared to TCR stimulation (**Figure 4.14**), suggesting a possible role in driving the expansion of NK-like T cells.

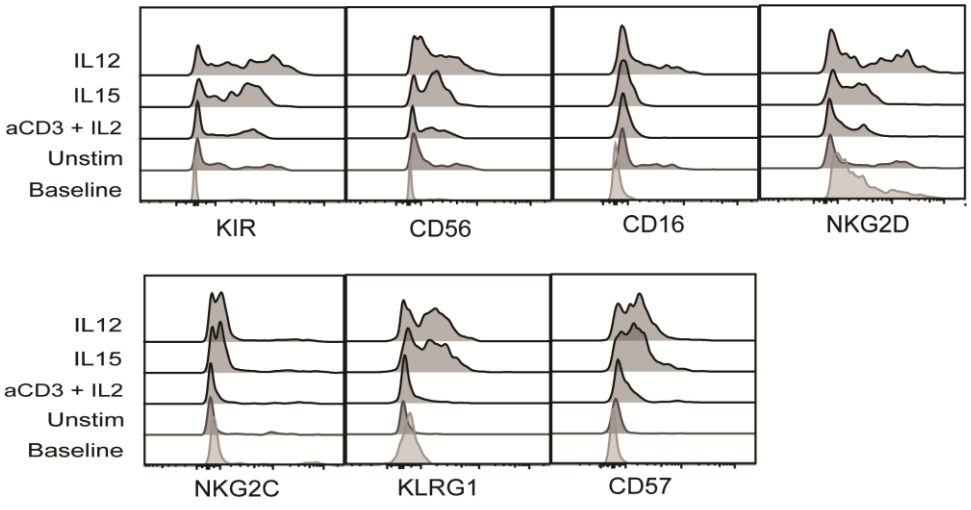

Figure 4.14 – Expression of NKR on CD8+ T cells is induced by inflammatory cytokines.

Freshly isolated human naive CD8+ T cells (CD28+27+) were cultured for 7 days in the presence of plate-bound anti-CD3 (OKT3, 0.5 µg/mL) and IL-2 (100 U/mL), IL-15 (20 ng/mL) or IL-12 (20 ng/mL). A) Histograms of the expression of the indicated NKR analysed after 7 days by multiparameter flow cytometry, compared to the baseline expression (Day 0) and the unstimulated control (unstim). Representative example of three different donors with similar results.

4.3.5 Highly differentiated CD8+ T cells are more cytotoxic and can kill independently of the TCR-MHC interaction

In a first approach to explore the functional significance of the acquisition of NKR by terminally differentiated CD8+ T cells, the expression of cytolytic mediators, perforin and granzyme B, was compared between cells expressing NK receptors and the negative counterparts. As observed in **Figure 4.15** with the examples of NKG2D and NKG2A, the expression of granzyme B was significantly higher in cells expressing both NKG2D and NKG2A than in cells negative for these receptors, supporting a role of NK cell receptors in the regulation of T cell cytotoxic functions. The difference was more striking in the CD4+ T cell compartment, where the expression of cytotoxic granules is uncommon. However, the finding that CD4+ T cells may become cytotoxic is not new and has been previously described for highly differentiated CD4+ T cells (Libri et al., 2011).

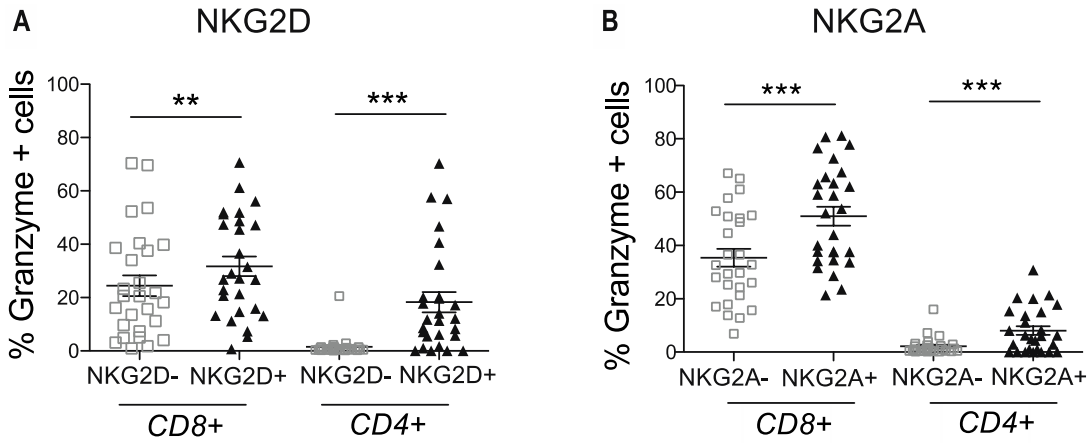

Figure 4.15 – Expression of cytolytic mediators is higher on T cells expressing NKR compared to the negative counterpart.

Distribution of granzyme B expression on CD8+ T and CD4+ T stratified according to NKG2D (**A**) and NKG2A (**B**) expression, analyzed *ex-vivo* from PBMCs by flow cytometry (*n*=28). Similar results were obtained for perforin expression. Comparison between groups calculated with Wilcoxon matched pairs test. (**p< 0.05*, ***p<0.01*, ****p< 0.001, ****p< 0.0001)*.

We next investigated whether CD8+ T cells expressing NK receptors were able to mediate immune responses, in an innate fashion, independently of the TCR-MHC recognition. To this end, the classical NK cell target K562 cell line was used as target cells in *in vitro* cytotoxic assays with purified CD8+ T cells, using NK cells as positive controls. As a MHC-class I deficient cell line, K562 cells cannot present peptides to CD8+ T cells via the classical MHC/TCR antigen-presenting pathway. Two complementary flow cytometry-based methods of assessing cytotoxicity were used, as previously described: the active caspase 3 assay and the CD107a degranulation assay. As observed in the representative histograms for the active caspase 3 assay (**Figure 4.16)**, the cytotoxic activity of CD8+ T cells was lower compared to NK cells, however significant levels of up to 40% were achieved using an E:T ratio of 20:1 or more, suggesting that CD8+ T cells are also capable of killing K562 tumour cells.

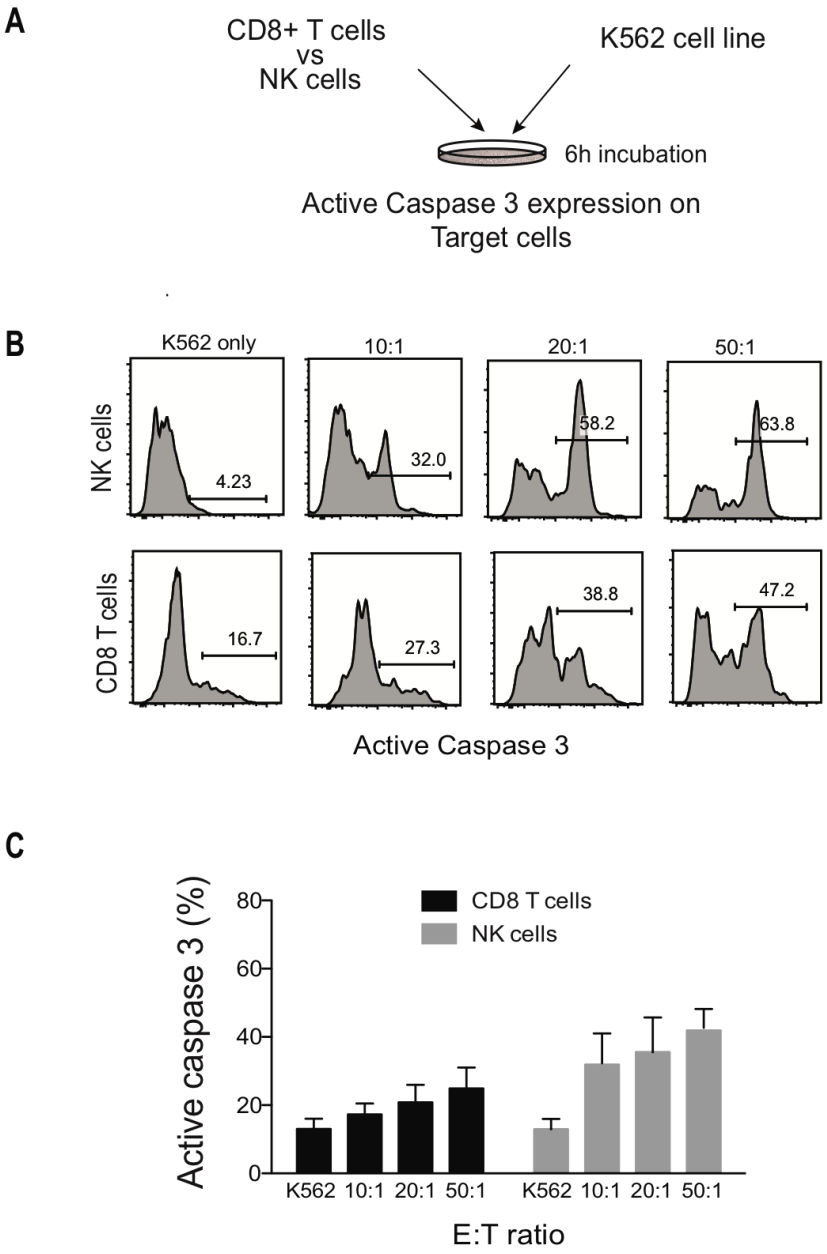

Figure 4.16 - CD8+ T cells can kill K562 cells, independently of the TCR.

A) Experimental design of active caspase 3 assay performed to compare the cytolytic activity of CD8+ T to NK cells, using the K562 cell line as target cells. NK and CD8+ T cells were purified from freshly isolated PBMCs using magnetic beads (negative selection). **B)** Representative FACS histograms of the expression of active caspase 3 on target cells after a 6-hour incubation period with NK cells (top panel) and CD8+ T cells (bottom), at the indicated effector to target (E:T) ratios. Numbers represent percentages of positive cells. **C)** Summary data of three independent experiments described as in A and represented as the percentage of cells positive for active caspase 3, assessed by flow cytometry as in B. K562 cells alone were used as negative controls.

To further investigate the cytolytic activity mediated by each CD8+ T cell subset, CD8+ T cells were sorted according to CD27/CD28 expression using flow cytometry-based cell sorting and used in cytotoxic assays with K562 cell line, at a fixed effector:target (E:T) ratio of 20:1. As seen in the representative FACS plots for CD107a expression (**Figure 4.17B)**, degranulation triggered by culturing with K562 cells was highest on highly differentiated CD8+ T cells, approaching the levels observed in NK cells (**Figure 4.17C**, $p < 0.01$). Collectively, these results indicate that highly differentiated CD27-CD28-CD8+ T cells exhibit a potent cytotoxic activity and can mediate killing of target cells independently of TCR-driven signals.

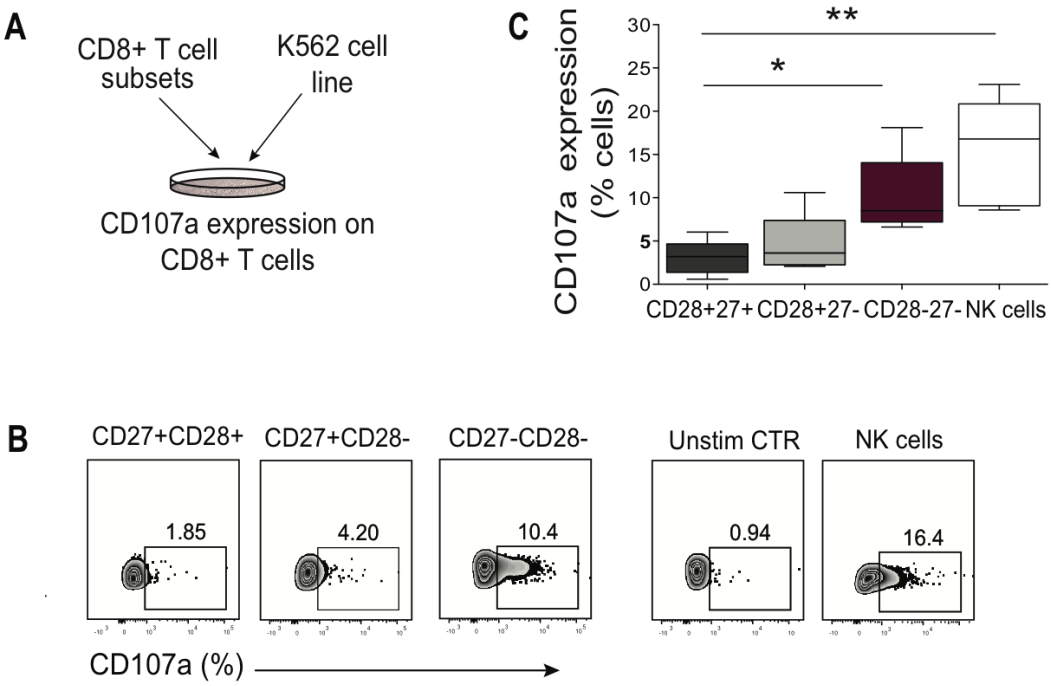

Figure 4.17 - The capacity to mediate cytotoxicity, independently of the TCR is highest in the terminal differentiated subset of CD8+ T cells.

A) Human CD8+ T cell subsets defined by the expression of CD27/CD28 were freshly isolated from PBMCs and cultured with K562 cells (E:T ratio 20:1) in a 6-hour incubation assay. **B)** The expression of CD107a, as a correlate of the cytotoxic activity, was assessed by flow cytometry on the indicated subsets of CD8+ T cells and on NK cells, as positive controls. An unstimulated control was included to assess spontaneous degranulation. **C)** Summary data comparing degranulation of the different subsets of CD8+ T cells and NK cells, acquired as in A (n=6). Statistical significance was calculated using the Kruskal-Wallis test with Bonferroni correction for multiple comparisons (*$p< 0,05$, **$p<0.01$).

4.3.6 TCR-independent, innate-like functions of highly differentiated CD8+ T cells are regulated by NKG2D

To explore the molecular mechanisms regulating TCR-independent functions of highly differentiated CD8+ T cells, the role of NKG2D, a master regulator of NK cell activity and effector function was investigated. The relevance of this receptor is supported by the high expression of NKG2D on CD8+ T cells **(Figure 4.6**) (Bauer et al., 1999). As described in previous chapters, NKG2D recognizes self-ligands induced by cellular stress, inflammation or transformation such as the MHC class I-related (MIC) A and B, and the UL-16 binding proteins (ULBPs 1-6) (Raulet, 2003, Lanier, 2015). To specifically address the role of NKG2D, a new set of *in vitro* killing assays were conducted using a target cell line transfected with the NKG2D ligand MICA*008 (C1R-MICA) and the respective control (C1R), lacking the expression of MICA. Target cells were co-cultured in a fixed E:T ratio with purified human CD8+ T cells effectively transfected with an siRNA for NKG2D or a scrambled siRNA control (**Figure 4.18A-C for experimental design).** CD8+ T cell degranulation in response to NKG2D engagement, as assessed by CD107a expression, was higher towards the MICA-transfectants compared to the control cell line (*p<0.001)***.** Moreover, silencing of NKG2D expression on CD8+ T cells significantly diminished this response (*p<0.01,* **Figure 4.18D**), suggesting that NKG2D is one of the regulators of the cytolytic function of these cells.

Collectively, these findings indicate that the expression of phenotypic NK-cell markers by CD8+ T cells is associated with the acquisition of innate-like functions, in particular potent cytotoxic functions mediated independently of TCR, in an NKG2D-dependent manner. Such MHC-unrestricted killing mediated by CD8+ T cells had already been anticipated in previous chapters, when it was shown that CD8+ T cells could effectively kill senescent fibroblasts, in an NKG2D-dependent mechanism.

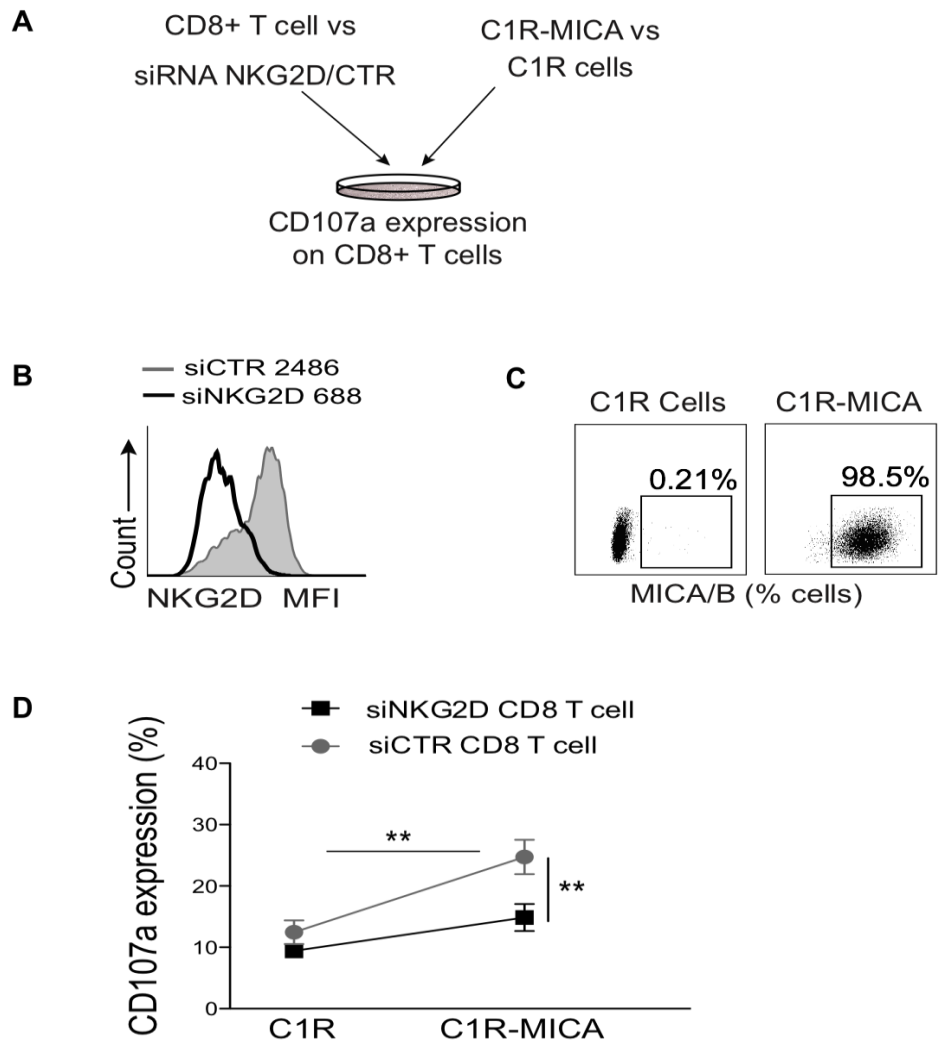

Figure 4.18 - Highly differentiated CD8+ T cells acquire NK-like functions, regulated by NKG2D.

A) Freshly isolated human CD28-CD8+ T cells were transfected with siRNA for NKG2D or a scramble control siRNA and cultured with a cell line expressing MICA*008 (C1R-MICA) or the control (C1R) cell line and the expression of CD107a was assessed by flow cytometry. **B)** Transfection efficiency was confirmed by flow cytometry 36 hours after transfection with siNKG2D/siCTR. Numbers represent mean fluorescence intensity (MFI). **C)** Representative dot plots of the expression of MICA on C1R-MICA and C1R control cell lines. Numbers indicate the percentage of MICA+ cells. **D)** Summary data (*n*=4) of degranulation assays after NKG2D silencing. Comparison between groups was determined with the non-parametric Mann-Whitney U test for independent samples (*$p< 0,05$, **$p<0.01$).

4.3.7 NKG2D signals through a functional complex with DAP12-Zap70 in highly differentiated CD8+ T cells

The previous findings indicate that, although NKG2D is widely expressed across all subsets of CD8+ T lymphocytes **(Figure 4.6**), the acquisition of TCR-independent, NKG2D-mediated cytotoxic functions is restricted to the highly differentiated population.

Expression of NKG2D receptors on the cell surface requires the association with adaptor proteins to stabilize the immunoreceptor complex and also to provide it with signaling activity (Lanier, 2015). Two adaptor molecules essential for NKG2D surface expression and signalling have been identified: DAP10 which has an YXXM-motif and activates Pi3K signaling (Wu et al., 1999, Groh et al., 2001) and DAP12 that carries an ITAM-motif in its cytoplasmic domain and thus is able to recruit ZAP70/Syk protein kinases and provide a direct stimulatory signal to the cell (Wu et al., 2000, Gilfillan et al., 2002). In human CD8+ T cells, NKG2D is thought to associate exclusively with the adaptor DAP10 (Groh et al., 2001). Nevertheless, NKG2D has been shown to couple with DAP12 in NK cells, directly triggering cytokine release and cytotoxicity (Diefenbach et al., 2002, Gilfillan et al., 2002).

It was therefore hypothesized that the distinct functional outcome of NKG2D stimulation in the highly differentiated CD8+ T cells would be due to the expression and association with the alternative adaptor DAP12. To assess this, the expression of DAP12 was analysed by intracellular flow cytometry (**Figure 4.19A,B**) and western blotting (**Figure 4.19C**). It was found that DAP12 was exclusively expressed on highly differentiated cells (either defined as CD27-CD28- or CD27-CD45RA+), whereas the expression of DAP10 was not substantially different across the subsets (**Figure 4.19D**).

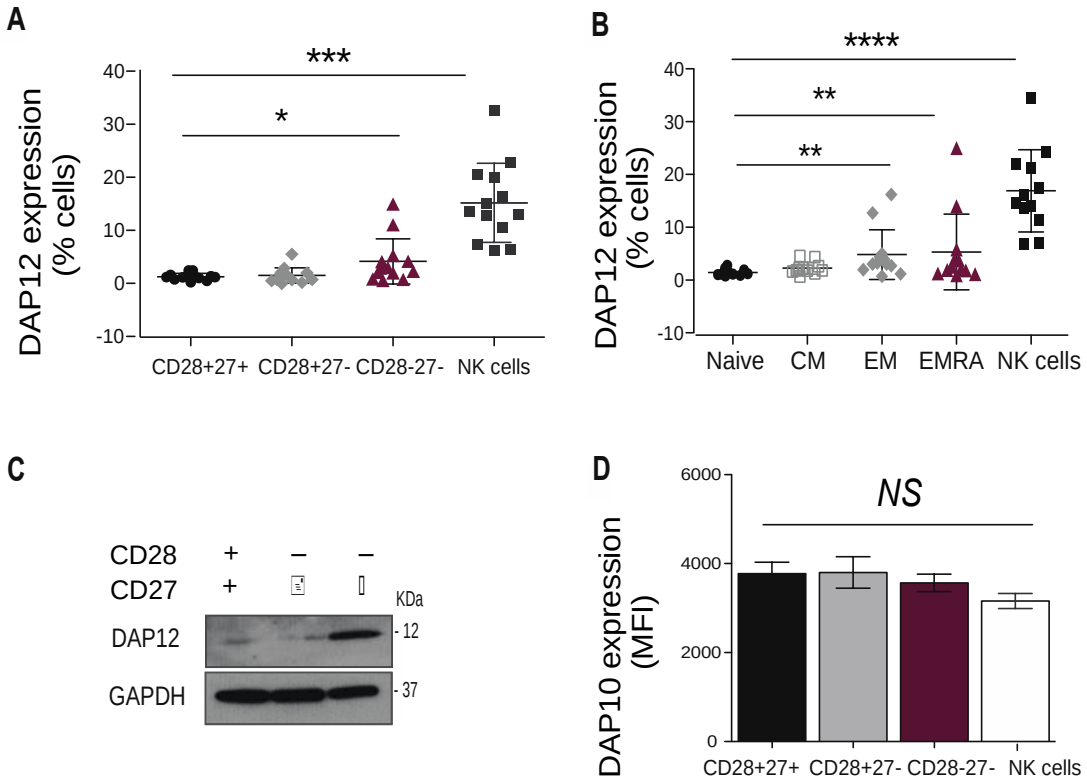

Figure 4.19 – Highly differentiated effector CD8+ T cells acquire the innate adaptor DAP12.

Ex-vivo expression of DAP12 on human CD8+ T cell subsets defined by CD27/CD28 (**A**) and by CD45RA/CD27 in (**B**), determined by intracellular flow cytomery (*n*=12). NK cells were used as positive controls for the expression of DAP12. **C)** Immunoblot analysis of the expression of DAP12 and GAPDH (loading control) in the indicated CD8+ T cell subsets, freshly isolated using magnetic beads. Data is representative of two independent experiments. **D)** Expression of DAP10 on CD8+ T cell subsets and NK cells analyzed by flow cytometry (*n*=12). Statistical significance was calculated using the Friedman test with correction for multiple comparisons (*p< 0,05*, ***p<0.01*, and ****p< 0.001)*.

To confirm the association of DAP12 with NKG2D in a functional complex immunoprecipitation studies were performed in freshly isolated human CD8+ T cell subsets after ligation of NKG2D. In order to obtain sufficient number of cells for analysis, freshly isolated CD8+ T cells were separated according to CD28 expression into CD28+ and CD28- populations, using magnetic beads (**Materials and Methods**). After crosslinking with an NKG2D antibody, cell lysates were obtained and immunoprecipitated with a mouse monoclonal anti-NKG2D antibody (clone 5C6, Santa Cruz) or an isotype-control antibody (IgG control) and blotted for DAP12 and Zap70 expression. DAP12 was only found in CD28- CD8+ T cells, confirming the association of DAP12 with NKG2D in highly differentiated CD8+ T cells and recruitment of Zap70/Syk to this complex (**Figure 4.20A**).

Furthermore, phosphorylation studies confirmed that NKG2D ligation resulted in a greater induction of Zap70/Syk phosphorylation in CD28- CD8+ T cells compared to TCR stimulation with anti-CD3 antibodies (**Figure 4.20B**), indicating that the defects on Zap70 phosphorylation initially observed after TCR crosslinking (**Figure 4.2A**) can be restored with NKG2D stimulation in terminally differentiated CD8+ T cells.

Finally, functional studies confirmed that NKG2D ligation was sufficient to induce direct activation of cytotoxicity ($p<0.001$) and IFNγ secretion ($p<0.05$, **Figure 4.20C**), mainly in highly differentiated CD8+ T cells.

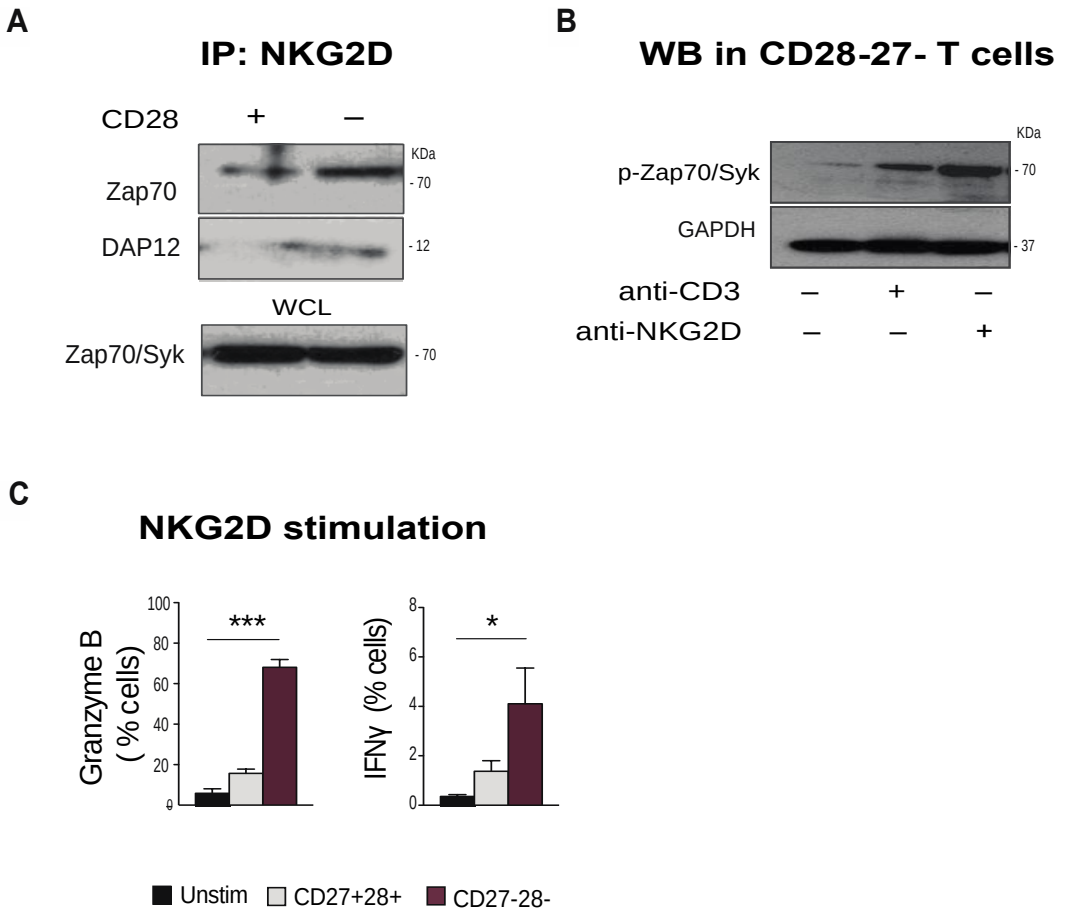

Figure 4.20 – Association of NKG2D with DAP12 converts NKG2D into a direct stimulatory receptor in highly differentiated CD8+ T cells.

A) MACS-purified human CD8+ CD28+ and CD28- T cells (purity > 95%) were crosslinked with anti-NKG2D (clone 1D11, 10 µg/mL) or an isotype control. Whole cell lysates were immunoprecipitated overnight with anti-NKG2D (clone 5C6) and blotted for DAP12 and ZAP70, confirming the association of NKG2D with DAP12 in CD28- T cells and recruitment of Zap70 to the complex. Immunoblot of Zap70 in whole-cell lysates (WCL) served as loading control. **B)** Phosphorylation of Zap70(Tyr319)/Syk(Tyr352) in freshly isolated CD27-CD28- CD8+ T cells after TCR crosslinking (OKT3, 10 µg/mL) and NKG2D crosslinking (1D11, 10 µg/mL), demonstrating an enhanced phosphorylation of Zap70/Syk upon NKG2D ligation. GAPDH was used as loading control. **C)** Granzyme B expression (left) and IFNγ secretion (right) after NKG2D ligation is predominantly induced in highly differentiated cells, which possess a functional NKG2D/DAP12 complex (*n*=15). Statistical significance was calculated using Friedman test with Dunn´s correction for multiple comparisons (*$p< 0,05$, **$p<0.01$ and ***$p< 0.001$).

To investigate whether highly differentiated CD8+ T cells relied on the axis NKG2D-DAP12-Zap70 to mediate their effector functions, independently of the TCR, RNA interference was used to silence each of the components of this complex. Freshly isolated human CD28- CD8+ T cells were transfected with siRNA for DAP12, NKG2D or Zap70 or a scrambled siRNA control and co-cultured with C1R/C1R-MICA cell line in a 6-hour incubation degranulation assay, as previously described. Silencing of DAP12 significantly impaired the cytolytic function of highly differentiated CD8+ T cells towards C1R-MICA as compared to the scramble siRNA control (*p<0.01,* **Figure 4.21),** in a similar extent to what was observed after Zap70/Syk (*p<0.001)* and NKG2D silencing (*p<0.01*). These findings indicate that NKG2D regulates the cytolytic activity of highly differentiated CD8+ T cells in a DAP12/Zap70- dependent manner, independently of the TCR.

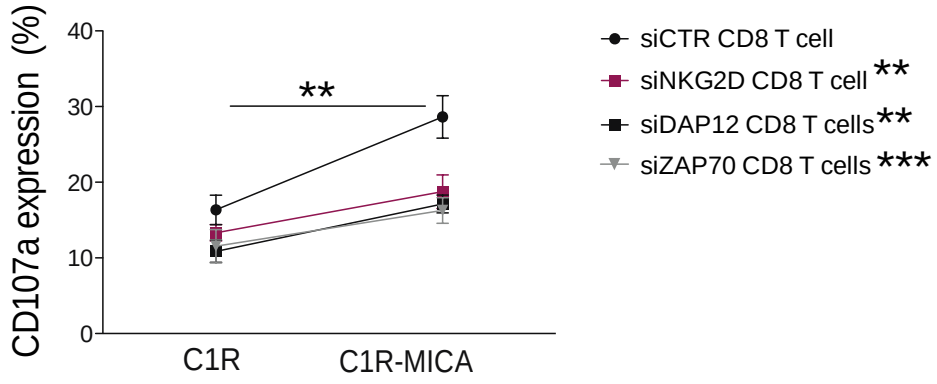

Figure 4.21 – NKG2D cytolytic activity in highly differentiated CD8+ T cells is mediated via DAP12/Zap70.

Human highly differentiated CD8+ T cells, transfected with siRNA for NKG2D, DAP12, Zap70/Syk or a scramble control siRNA were cultured with C1R-MICA*008 or the control (C1R) cell line and the expression of CD107a was assessed by flow cytometry (*n*= 3). Silencing of DAP12 (*p<0.01) and* Zap70/Syk (*p<0.001)* impaired cytolytic activity in a similar extent to NKG2D silencing (*p<0.01).* Transfection efficiency was confirmed by flow cytometry (**Fig 4.25 in Materials and Methods**). Statistical significance was calculated using a two-way ANOVA test with Bonferroni correction (**p< 0,05*, ***p<0.01* and ****p< 0.001).*

Together these observations indicate that DAP12 expression is necessary and sufficient to convert NKG2D into a direct stimulatory receptor and explain the acquisition of innate-like properties by highly differentiated T cells. Although NKG2D is constitutively expressed on all subsets of CD8+ T cells, the absence of DAP12 in less differentiated cells may explain why NKG2D ligation is insufficient to induce cytokine secretion and degranulation of these cells.

4.3.8 Genetic reprogramming of senescent CD8+ T cells into NK-like cells

To investigate whether the phenotypical and functional changes observed in terminally differentiated CD8+ T cells were associated with a specific transcriptional program, high-purity CD8+ T cell subsets were sorted according to CD27 and CD45RA expression, using a FACSAria flow cytometer and gene-expression profiles of sorted cells were obtained using Affymetrix U133 plus 2 microarrays.

A global comparative analysis between the terminally differentiated subset (T_{EMRA}) and naïve T cells (T_{naive}) identified 3139 differentially expressed genes, using an expression fold change of 2 with a *p* value cutoff of < 0.05 in the one-way ANOVA test and a Benjamini-Hochbert false-discovery rate (FDR) correction less than 5% (**Table 4.1**).

Differentially expressed genes	Number of genes
EMRA versus Naive	3139
Effector Memory versus Naive	3873
Central Memory versus Naive	2162

Naïve (CD45RA+CD27+);Central Memory (CD45RA-CD27+); Ef fector Memory (CD45RA-CD27-); Effector Memory Re-expressing CD45RA (EMRA:CD45RA+ CD2 7-)

Table 4.1 – Differentially expressed genes in CD8+ T cell subsets compared to naïve CD8+ T cells.

Global comparative analysis of differentially expressed genes between sorted populations of CD8+ T cells, according to CD45RA/CD27 expression, relative to naïve CD8+ T cells. Data was obtained from 6 different donors. Analysis performed with Transcriptome Analysis Console (Affymetrix), criteria (Fold change of expression ≥ 2, *p* value cutoff of < 0.05, FDR < 0.05).

In order to obtain a workable panel of genes, a selection of 80 genes of interest (GOI) from the whole-genome transcriptomic data was done, including surface receptors, signaling molecules, effector mediators, transcription factors and genes involved in proliferation (**Table 4.2)**. Hierarchical Clustering Analysis of these 80 genes of interest revealed a profile that clearly distinguished highly differentiated CD8+ T cells from the other subsets, in particular naïve CD8+ T cells (**Figure 4.22)**. As expected, one of the most striking changes in the gene expression profile of highly differentiated CD8+ T cells involved the upregulation of genes encoding NK cell receptors as well as signaling molecules traditionally linked to the NK cell receptor machinery. Amongst these, the transcript for DAP12 (*TYROBP*) was strongly induced only in highly differentiated cells (fold change relative to naive = 20.98, $p < 0.0001$, **Table 4.2**), confirming previous observations at the protein level. Similar findings were obtained for transcripts encoding other innate signaling molecules such as Syk, which is activated downstream of DAP12. This molecule is generally absent in peripheral T cells (Hauck et al., 2015, Mocsai et al., 2010) and was strongly upregulated in end-stage CD8+ T cells (fold change relative to naive = 17.33, $p < 0.0001$, **Table 4.2**). Similarly, transcripts encoding for DAP-12 coupled receptors (*KLRC1/KLRC2*, *KIR2DS* and *NCR2*) were overexpressed in highly differentiated CD8+ T cells. Collectively these findings corroborate a critical role for DAP12-associated signaling in terminally differentiated CD8+ T cells. Importantly, no significant changes were found in the gene coding for DAP10 across the subsets (*HCST* gene, fold change relative to naive 1.55, $p < 0.0001$, **Table 4.2**).

In parallel to NK-cell related genes, genes involved in cytotoxicity (*GZMA/B/H, PRF*) and cytokine signaling (in particular the cytokine receptors IL18R, IL12R, IL15R and IL2R) were induced in highly differentiated T cells, which is in relation to the functional maturation and increased responsiveness to cytokines in these cells.

In striking opposition to genes regulating effector functions, genes involved in proliferation and cell cycle control were downregulated (*CCNE1, CCND3* encoding cyclins D3 and E1), whereas cyclin-dependent kinase inhibitors such as p16 (*CDKN1A)* were induced, indicating an inhibition of proliferation. This comes in association with the suppression of genes involved in TCR signalling, including TCR/CD3 complex genes (*CD3E, CD3G, CD3E)* and protein tyrosine kinase genes (*LCK, LAT, PLCG1*).

Genes encoding transcription factors were also significantly altered in highly differentiated CD8+ T cells. Interestingly, two of the most differentially expressed transcriptions factors were *ZEB2* (fold change = 10.75, $p < 0.0001$) and PLZF (*ZBTB16* fold change = 6.48, $p < 0.0001$), which have been recently involved in the transcriptional control of terminal differentiation (Dominguez et al., 2015, Omilusik et al., 2015) and development of innate-like features in T cells (Raberger et al., 2008, Kovalovsky et al., 2008, Kovalovsky et al., 2010, Alonzo and Sant'Angelo, 2011), respectively. Of particular relevance, ZEB2 has been described to cooperate with Tbet to coordinately induce a terminal differentiation program in CTLs, while simultaneously repressing genes that are essential for T cell memory and function (Dominguez et al., 2015, Omilusik et al., 2015).

Overall, these findings indicate that the different subsets of CD8+ T cells are not only phenotypically but also functionally and genetically very distinct. Highly differentiated CD8+ T cells exhibit unique characteristics, determined by a genetic program that ensures the acquisition of cytotoxic effector functions, probably regulated by newly acquired NK receptors and signalling molecules, at the expenses of TCR-driven responses including proliferation.

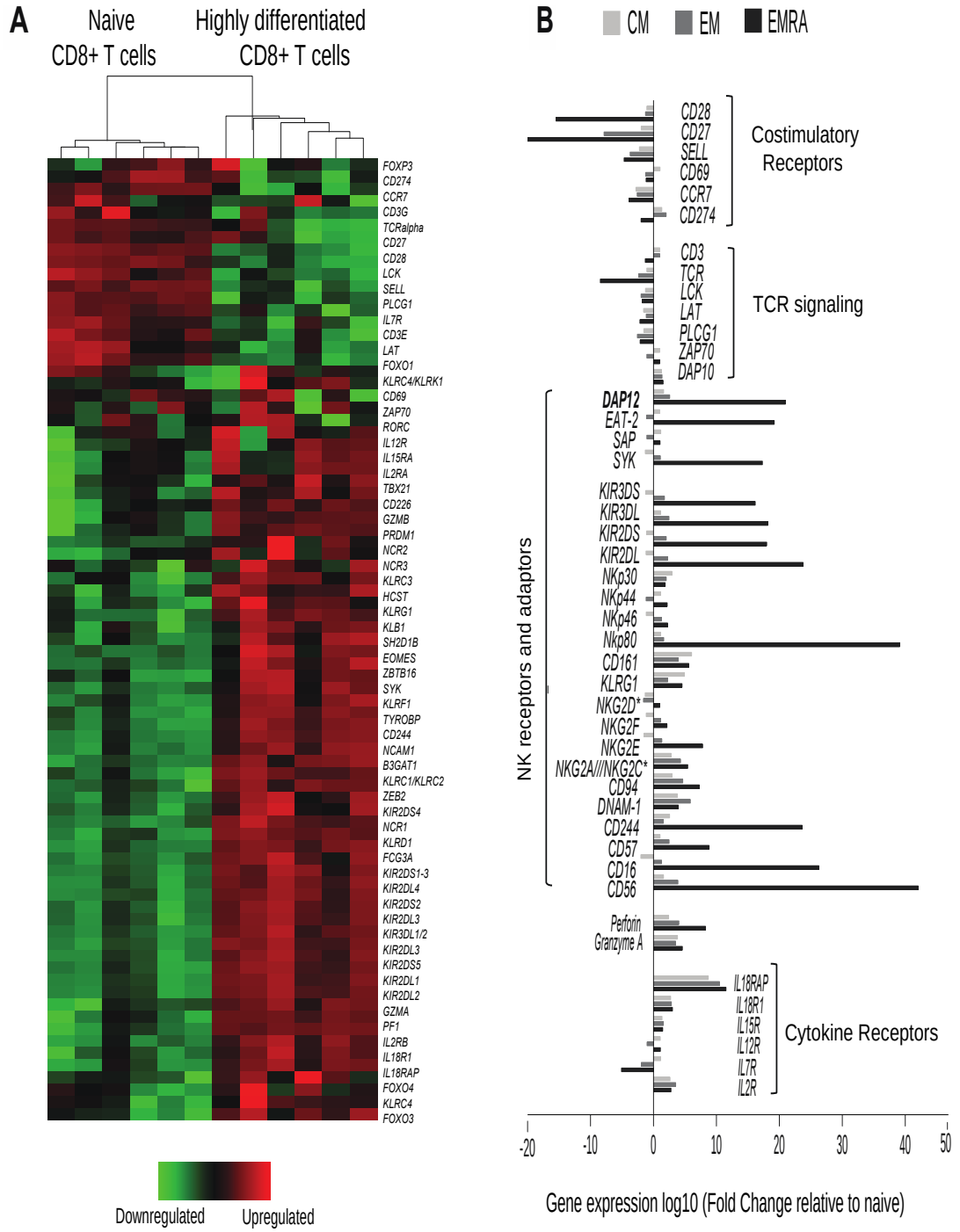

Figure 4.22 - Transcriptional signature of highly differentiated CD8+ T cells.

Gene-expression profiles of sorted central memory (T_{CM}; $CD45RA^{-}CD27^{+}$), effector memory (T_{EM}; $CD45RA^{-}CD27^{-}$) and effector memory re-expressing CD45RA (T_{EMRA}; $CD45RA^{+}CD27^{-}$) CD8+ T cells were compared to naïve (T_{N}; $CD45RA^{+}CD27^{+}$) cells. Data was obtained from 6 different donors. **A)** Hierarchical clustering analysis of 80

differentially expressed genes of interest between naïve CD8+ T cells and highly differentiated CD8+ T cells (T_{EMRA}; $CD45RA^{+}CD27^{-}$). Heat map key shows log-fold changes from baseline. **B)** Graph comparing the relative fold change (log10) for the selected genes of interest between the three indicated subsets (T_{CM}, T_{EM} and T_{EMRA} relative to T_N CD8+ cells), showing the overexpression of NK cell related genes and simultaneous downregulation of costimulatory receptors and TCR-related genes.

Gene Symbol	Name	Description	Fold Change CM/N	EM/N	EMRA/N	P value
NK cell receptors						
NCAM1	CD56	Neural cell adhesion molecule, NK cell marker	1.62	3.9	**42.07**	**0.000013**
FCGR3A /// FCGR3B *	CD16	Receptor for the Fc region of IgG; Antibody-dependent cellular cytotoxicity	-1.99	1.3	**26.31**	**1.73E-07**
B3GAT1	CD57	Marker of replicative senescence	1.05	2.5	8.86	0.000002
CD244 (2B4)	CD244 (2B4)	SLAM receptor; costimulation and inhibition in NK cells; co-inhibition in T cells	2.57	1.59	**23.64**	**0.000003**
CD226 (DNAM-1)	DNAM-1	Regulation of cytotoxicity in both NK and T cells	3.79	5.89	3.96	0.002281
KLRD1	CD94	C-type lectin NK cell receptor, dimerizes with NKG2 family of receptors	2.96	4.66	7.31	7.41E-07
KLRC1 /// KLRC2 *	NKG2A///NKG2C*	NKG2A inhibitory and NKG2C activating receptor, both recognize HLA-E	2.82	4.32	5.47	0.000027
KLRC3	NKG2E	Activating receptor	-1.5	1.34	7.82	0.000712
KLRC4	NKG2F	Activating receptor	-1.14	1.16	2.15	0.011495
*KLRC4-KLRK1 /// KLRK1**	NKG2D*	Activating receptor, regulation cytotoxocity and cytokine secretion	-1.32	-1.59	1.02	0.860846
KLRG1	KLRG1	Inhibitory receptor; marker of terminal differentiation	4.91	2.27	4.56	0.000391
KLRB1	CD161	Marker of Th17 phenotype; highly expressed on MAIT cells	6.06	3.94	5.63	0.002317
KLRF1	Nkp80	Activating receptor; marker of functional maturity in both NK and CD8 T cells	1.15	1.65	**39.13**	**0.000016**
NCR1 (NKp46)	NKp46	Natural Cytotoxicity Receptor	-1.15	1.29	2.25	2.79E-07
NCR2 (NKp44)	NKp44	Natural Cytotoxicity Receptor	1.16	-1.16	2.2	0.01141
NCR3 (NKp30)	NKp30	Natural Cytotoxicity Receptor	2.97	2.05	1.88	0.041509
KIR2DL1-3	KIR2DL	Killer cell Immunoglobulin-like receptor (inhibitory)	-1.18	2.26	**23.78**	**0.000001**
KIR2DS 1-5	KIR2DS	Killer cell Immunoglobulin-like receptor (stimulatory)	-1.1	2.05	**18.01**	**9.79E-07**
KIR3DL1-3	KIR3DL	Killer cell Immunoglobulin-like receptor (inhibitory)	1.15	2.48	**18.2**	**6.47E-07**
KIR3DS1-3	KIR3DS	Killer cell Immunoglobulin-like receptor (stimulatory)	-1.23	1.75	**16.19**	**0.000198**
Costimulatory and homing receptors						
CD27	CD27	Costimulatory Receptor	-1.93	-7.85	**-26.53**	**0.000001**
CD28	CD28	Costimulatory Receptor	-1.05	-1.26	**-15.48**	**0.000004**
CD274	PD-L1	T cell exhaustion	1.33	2.02	-1.95	0.01804
CCR7	CCR7	Secondary lymphoid organ homing receptor	-2.77	-2.59	-3.88	0.000568
CD69	CD69	Activation marker	1.06	-1.24	-1.17	0.271801
SELL	CD62	Adhesion molecule, migration to secondary lymphoid organs	-2.21	-3.71	-4.65	0.000024
T cell signaling transduction						
TCR-alpha	TCR-alpha	TCR complex	-1.05	-2.32	**-8.43**	**0.005636**
CD3D, CD3E, CD3G	CD3 complex	CD3 complex	1.02	1	-1.29	0.002293
LCK	LCK	Lymphocyte protein tyrosine kinase, TCR signaling	-1.25	-1.96	-1.78	0.000107
LAT	LAT	Lynker for activation of T cells, TCR signaling	-1.56	-1.14	-2.15	0.002927
PLCG1	PLCG1	Phospholipase gamma, TCR signaling	-1.52	-2.55	-2.11	0.000117
ZAP70	ZAP70	Syk-family protein tyrosine kinase, TCR signaling	1	-1.05	1	0.861184
HCST	DAP10	YxxM-Motif adaptor molecule, constitutive expression on CD8 T cells	1.28	1.39	1.55	0.000151
Innate signaling molecules						
TYROBP	DAP12	ITAM containing activating signaling adaptor, recruits Zap70/Syk kinases	1.64	2.59	**20.98**	**0.000004**
SYK	SYK	Syk-family protein tyrosine kinase, analogous of Zap70, BCR signaling	-1.28	1.1	**17.33**	**0.000099**
SH2D1A	SAP	SH2-domain containing molecule, binds SLAM receptors	1.17	-1.05	1.06	0.341073
SH2D1B	EAT-2	SH2-domain containing molecule, binds SLAM receptors	1.01	-1.08	19.2	0.000107
Transcription factors						
EOMES	EOMES	T-box transcription factor, cytotoxic effector differentiation and maturation	1.58	1.61	1.81	0.000789
TBX21	Tbet	T-box transcription factor, cytotoxic effector differentiation and maturation	-1.09	1.26	1.31	0.005497
FOXO1	FOXO1	Survival, proliferation and differentiation of T cells	-1.26	-1.75	-2.47	0.000031
FOXO3 /// FOXO3B	FOXO3	Survival, proliferation and differentiation of T cells	1.18	1.5	1.48	0.001226
FOXO4	FOXO4	Survival, proliferation and differentiation of T cells	-1.08	-1.03	1.43	0.065459
FOXP3	FOXP3	Development and function of regulatory T cells	2.78	2.36	-1.14	0.618522
PRDM1	Blimp-1	T cell effector differentiation and terminal maturation	2.97	3.61	3.16	0.002186
RORC	RORC	Th17 lineage differentiation	9.11	5.85	1.11	0.786225
ZBTB16	PLZF	NKT cell effector differentiation	7.65	4.78	**6.48**	**0.000081**
ZEB2	ZEB2	Cytotoxic T cell terminal differentiation	2.57	5.5	**10.75**	**0.000039**
Cytokine receptors						
IL2RA	IL2R	IL2 receptor	2.65	3.54	2.8	0.021623
IL7R	IL7R	IL7 receptor	1.16	-1.91	-5.06	0.001062
IL12RB1-2	IL12R	IL12 receptor	1.09	-1.01	1.58	0.173214
IL15RA	IL15R	IL15 receptor	1.39	1.6	1.47	0.066267
IL18R1	IL18R1	IL18 receptor	2.76	2.85	3.04	0.000252
IL18RAP	IL18RAP	IL18 receptor acessory protei	8.72	10.51	**11.54**	**0.000092**
Cytotoxicity						
GZMA	Granzyme A	Cyototoxicity	3.83	3.53	4.61	0.000159
GZMB	Granzyme B	Cyototoxicity	1.41	1.61	1.46	0.004983
GZMH	Granzyme H	Cyototoxicity	2.51	4.55	3.93	0.00122
GZMK	Granzyme K	Cyototoxicity	3.59	-1.04	-2.01	0.030512
PRF1	Perforin	Cyototoxicity	2.42	4.05	8.29	0.00001
Proliferation and cell cycle control						
CCND3	Cyclin D3	Cell cycle progression	-3.04	-3.43	-2.99	0.002381
CCNE1	Cyclin E1	Cell cycle progression	-2.09	-2.33	-3.21	0.000071
CDKN1A	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	3.63	3.99	4.52	0.000515
CDKN2A	CDKN2A	Cyclin-dependent kinase inhibitor 2A (p16)	2.48	2.73	2.53	0.000035
CDKN2B	CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	5.11	5.92	4.39	0.000601

Table 4.2– List of selected genes of interest with differential expression in CD8+ T cell subsets.

Numbers denote mean fold changes in gene expression compared to naïve T cells. The most significant changes are highlighted in bold. Asterisks (*) indicate the inability of Affymetrix microarrays to distinguish between two genes.

4.4 DISCUSSION

In this chapter a combination of phenotypic, functional and transcriptomic data was provided to support the hypothesis that aging is associated with an imprinting of several NK-cell related traits on highly differentiated CD8+ T cells leading to the expansion of a population with shared innate and adaptive features.

Firstly, it was demonstrated that highly differentiated or senescent CD8+ T cells are impaired in their capacity to respond to TCR stimulation due to a downmodulation of critical components of the proximal TCR signaling cascade. Concomitantly, these cells start expressing receptors that are normally attributed to NK cells and acquire the capacity to mediate cytotoxic functions, in an NK-cell like fashion, independently of the TCR. Although the acquisition of NK lineage receptors by T cells approaching terminal differentiation has been previously documented (Strauss-Albee et al., 2014, Tarazona et al., 2000, Abedin et al., 2005, Vallejo et al., 2011), here it was shown that the expression of NK-cell receptors and signalling adaptors is coordinately induced as T cells lose responsiveness to TCR signals. This may explain why highly differentiated T cells maintain their effector functions, despite TCR hyporesponsiveness. In line with this, the receptor NKG2D and the adaptor DAP12 were identified as key molecular players in the regulation of unconventional innate-like functions by these cells, mediated independently of the TCR.

NKG2D is an important regulator of NK cell effector functions and is a classical example of a NK cell receptor that is highly expressed on αβCD8+ T cells, where is thought to mediate mainly costimulatory functions (Bauer et al., 1999, Raulet, 2003, Groh et al., 2001). Here evidence is provided that NKG2D can act as a direct stimulatory receptor, inducing cytotoxicity and cytokine secretion independently of the TCR, in highly differentiated CD8+ T cells. Thus, despite being constitutively expressed across all subsets of CD8+ T cells, NKG2D appears to mediate a different

function in highly differentiated CD8+ T cells. Mechanistically, it was demonstrated that this functional switch in senescent CD8+ T cells depends on the association with the scaffold molecule DAP12, which triggers an alternative signaling cascade dependent on Zap70/Syk tyrosine kinases.

DAP12 is a signaling adaptor that is pivotal in mediating signals triggered by innate receptors and has been shown to couple with different activating receptors in NK cells, including activating KIRs and the CD94/NKG2C receptor complex (Lanier, 2009). Although to date, the association of DAP12 with NKG2D has been only demonstrated in activated mouse NK cells (Diefenbach et al., 2002, Gilfillan et al., 2002), herein we report the presence of a functional NKG2D/DAP12 receptor complex largely restricted to the highly differentiated subset of CD8+ T cells, thus explaining how NKG2D is functionally converted into a direct activating receptor only in this subset. Importantly its is shown, for the first time to our knowledge, that DAP12 is selectively induced only in the subsets with a terminally differentiated effector phenotype (evident at both the transcriptional and protein level) and that DAP12 is necessary for the acquisition of innate-like cytolytic functions by CD8+ T cells. By contrast, no significant changes in DAP10 expression were found across CD8+ T cell subsets, either at the mRNA level or protein level, corroborating that the association with DAP12 is critical for the assembly and function of NKG2D in senescent CD8+ T cells. Of note, several DAP12-coupled receptors (NKG2C, NKp44 and KIR2DS) are overexpressed in highly differentiated CD8+ T cells, thus a contribution of such receptors to mediating innate-like functions in these cells cannot be excluded.

While the general consensus is that NKG2D engagement serves as a co-stimulatory receptor in human CD8+ T cells, amplifying TCR signals in virus-specific responses (Groh et al., 2001) as well as antitumor immunity (Diefenbach et al., 2001, Groh et al., 2002), previous studies have reported that NKG2D can function as a direct

stimulatory receptor in human CD8+ T cells, particularly in pathological contexts associated with dysregulated inflammation and abnormal secretion of IL-15, such as in celiac disease (Meresse et al., 2004). Nevertheless, the authors could not find an explanation for this change in NKG2D function in the presence of high levels of IL-15 and the association of NKG2D with DAP12 in human intraepithelial CD8+ T cells could not been demonstrated. However, these studies were all done in total CD8+ T cells and thus did not account for the differential expression of genes within the different subsets of CD8+ T cells. Our results indicate that CD8+ T cells subsets are not only phenotypically but also functionally and transcriptionally distinct and thus should be analysed individually. Moreover, our observations come in agreement with studies done with cytokine-induced killer (CIK) cells, indicating that CD8+ T cell clones expanded and cultured *in vitro* in the presence of cytokines, such as IL-15 or high-dose IL-2, acquire TCR-independent cytotoxic activity, mediated by NKG2D and partially dependent of the presence of DAP12 (Verneris et al., 2004, Karimi et al., 2005). It would be interesting to explore whether TCR signaling is suppressed in these cells, suggesting a common mechanism to what is observed in highly differentiated CD8+ T cells.

By comparing gene-expression profiles of human CD8+ T cells at different stages of differentiation, a transcriptional signature associated with terminal differentiation or senescence in CD8+ T cells could be identified. This signature is characterized not only by a downregulation of costimulatory receptors and T cell receptor associated genes but also by an overrepresentation of genes coding for NK cell receptors and innate signaling molecules in parallel to genes involved in cytolytic functions. Overall, these findings suggest that the acquisition of NKR as CD8+ T cells differentiate is not a stochastic effect but rather a coordinated stepwise program that leads to functional maturation. This program most likely ensures that effector functions are privileged and mediated by newly acquired innate receptors, in detriment of TCR-mediated

responses such as proliferation and cell-cycle progression. In conclusion, these data suggests a novel aspect of T cell senescence where TCR suppression is associated with the acquisition of TCR-independent, innate-like functions and determined at the transcriptional level. Other recently published studies support the hypothesis that altered TCR signaling pathways may predispose cells to develop unconventional functions, not restricted by the TCR-MHC interaction (Broussard et al., 2006, Mingueneau et al., 2009, Wang et al., 2012).

Interestingly, Wencker *et al* have recently suggested that the association of peripheral suppression of canonical TCR signaling with acquired responsiveness to innate stimuli is a characteristic that defines innate-like T cells (Wencker et al., 2014). Collectively, our observations that senescent CD8+ T cells co-express both T and NK cell markers, have decreased TCR responsiveness and yet are able to mount robust effector responses, independently of the TCR, supports the hypothesis that senescent C8+ T cells resemble innate-like T cells (Pereira and Akbar, 2016).

In recent years, there has been a growing appreciation of the existence of subsets of T cells with features that bridge innate and adaptive immunity (Vermijlen and Prinz, 2014, Seyda et al., 2016, Paul, 2011). In humans, these innate-like T cells comprise the invariant NKT (iNKT) cells, CD1d-restricted NKT cells, mucosa-associated invariant T (MAIT) cells and γδT cells. Natural-killer T (NKT) cells are the prototypical example of innate T lymphocytes and the term NKT cell is sometimes misused to refer to other subsets of conventional αβT cells that express NK cell receptors, although there are fundamental differences between them. Classical NKT cells express an invariant TCR (Va24Ja18) that recognize glycolipids presented by the monomorphic CD1d molecule and they account for 0.1-1% of T cells in human peripheral blood (Brennan et al., 2013), whereas conventional αβT cells expressing NKR exhibit a diverse TCR repertoire and their frequency in peripheral blood is much higher, increasing with age and chronic inflammatory diseases (Vallejo et al., 2011).

In striking contrast, aging is associated with decreased frequency and function of iNKT cells (Jing et al., 2007, Peralbo et al., 2006). In addition, the origin and development of human senescent αβCD8+ T cells is distinct from that of classical innate T cells. While innate T cells are developmentally pre-programmed in the thymus (Vermijlen and Prinz, 2014), αβCD8+ T cells with NK-like features arise in the periphery, most likely driven by external environmental cues. The different origin may explain why aging is associated with decreased frequency and function of innate-like T cells (as a result of thymic involution) whereas the number of conventional αβT cells expressing NKR increases with age.

This work raises important questions regarding the factors that determine such changes in CD8 T cells with aging and perhaps, more importantly, about the biological significance of such alterations. As we age and the immune system matures, there is a contraction of the immune repertoire (Blackman and Woodland, 2011). The acquisition of a diverse set of NK receptors by T cells may contribute to broaden the repertoire, compensating for the decrease output of naïve T cells and the defects in T cell signaling with age.

Despite the capacity of latent viruses such as CMV and EBV to skew the immune repertoire (Fulop et al., 2013), a lower expression of NKR on virus-specific T cells than in global CD8+ T cells was unexpectedly found. This comes in disagreement with studies that have reported an up-regulation of NKR in CD8+ T cells in the acute phase after CMV seroconversion in transplant recipients, suggesting that antigenic stimulation may induce the expression of NKR on T cells (Gamadia et al., 2001, van Stijn et al., 2008) However, when the same authors compared the expression of NKR in CMV-tetramer+ cells during the latent phase (one year after infection), the expression was not raised compared to global CD8+ T cells. Of relevance, in the acute phase cells were sorted according to the expression of activation markers (CD8+ HLA-DR+ CD38+ cells), whereas in the latent phase CMV-specific tetramer+

cells were studied (van Stijn et al., 2008). In a previous study, the same group characterized the immunophenotype of CMV-specific CD8+ T cells (detected either by peptide-induced IFNγ secretion or CMV-specific tetramer binding) as displaying a terminally effector phenotype (CD27-CCR7-CD45RA+ and high expression of cytolytic mediators) but invariably low levels of NKR when compared to total CD8+ T cell population (Gamadia et al., 2001). This suggests that although CMV infection may have a contribution to the generation of NKR+ T cells, other factors must be involved in the maintenance and expansion of these cells.

We found an important role for inflammatory cytokines, such as IL-15 and IL-12 in promoting NKR expression and T cell differentiation. These cytokines have been previously associated with the bystander activation of T cells, independently of the TCR (Liu et al., 2002, Chu et al., 2013), particularly in pathological contexts such as in celiac disease (Meresse et al., 2004, Meresse et al., 2006), infection (Soudja et al., 2012) and in cancer (Strid et al., 2008). A recent study done in Prof. Akbar´s group indicates that IL-15 may induce the expansion of cells with a terminally differentiated phenotype after CMV infection but independently of antigen-specific signals (Griffiths et al., 2013). Other groups have shown that IL-15 and IL-12 modulate the expression of key transcription factors regulating effector T cell differentiation, particularly T-bet and Eomes (Takemoto et al., 2006, Joshi et al., 2007). The observation that highly differentiated CD8+ T cells have an increased expression of receptors for IL-15, IL-12 and IL-18 supports a role of these cytokines in driving functional maturation into NK-like T cells. Thus, it is plausible that the pro-inflammatory state associated with aging (“inflammaging”) may be critical to the development of NK-like CD8+ T cells.

Regarding the biological significance of these changes, my understanding is that the acquisition of innate sensors specialized in the recognition of “danger” signals by senescent CD8+ T cells put these cells in the first line of defence in potentially harmful situations. The ability to kill NKG2D-ligand expressing cells may be important

for the immune surveillance of transformed and stressed cells, which cannot be targeted by TCR-dependent mechanisms. This mechanism could be particularly relevant in the context of tumours and viral infections that induce a downregulation of MHC class I expression as a mechanism of immune evasion. Given the increased burden of tumors and infections with age, the contribution of highly differentiated CD8+ T cells with innate-like functions may be crucial and brings a clear advantage to the aged individual. The findings in previous chapters corroborate a role of these cells in targeting senescent fibroblasts expressing NKG2D ligands.

Nevertheless, the reversal of antigen-specific CD8+ T cells to an innate mode of function is not without consequence. The peripheral requirement for TCR engagement for T cell activation is an important control mechanism to prevent auto-reactivity. In conditions associated with chronic activation and inflammation, the balance between activating and inhibitory signals may thus favor the onset of autoimmune reactions. In line with this, recent reports have demonstrated a role of NKG2D in CD8+ T cell activation in inflammatory states and other stress conditions where NKG2D ligands are induced in normal tissues, such as celiac disease (Meresse et al., 2004), type I diabetes (Ogasawara et al., 2004) and transplantation (Li et al., 2012b, Karimi et al., 2015).

Interestingly, despite showing a decreased TCR responsiveness, studies indicate that these cells still retain the capacity to elicit specific TCR-dependent immune responses (Pievani et al., 2011). It would be important in the future to address whether these cells maintain the capacity to respond rapidly to antigen-specific and TCR-independent signals and therefore participate in both early and late phases of the immune response.

In conclusion, contrary to the current paradigm that senescent CD8+ T cells represent a population with compromised functionality, it is proposed that senescent

CD8+ T cells should not be seen as a dysfunctional population but instead a functionally distinct subset, which uses recently-acquired NK cell machinery to maintain rapid effector functions. It is likely that this is part of the remodeling of the immune system with aging, in an attempt to maintain effective immune responses, which could be beneficial in the responses to infections and cancer but may carry an increased risk of auto-immune and inflammatory diseases in the elderly (Sansoni et al., 2008).

4.5 MATERIALS AND METHODS

4.5.1 Ethical approval

The study protocol was approved by the Ethical Committee of the Royal Free and University College London Medical School. Written informed consent was obtained from all study participants. Donors did not have any co-morbidity, were not on any immunosuppressive drugs, and retained physical mobility and lifestyle independence.

4.5.2 Blood sample collection and processing

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll–Hypaque density gradient centrifugation (Amersham Biosciences, UK) from heparinized blood of healthy volunteers between the ages of 25 and 83 years (*n*= 50; median age 52, range 25-83) and resuspended in RPMI 1640 medium, supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 2 mM L-glutamine and 100 IU/ml penicillin/streptomycin (all from Sigma-Aldrich, UK), from now on referred as complete RPMI. Isolated PBMCs were either processed immediately, maintained in cell culture or cryopreserved in 10% DMSO/FCS for future use.

4.5.3 Cell sorting

For functional assays, NK and CD8+ T cells were freshly isolated by magnetic activated cell sorting (MACS, Miltenyi Biotec, UK) using a negative selection procedure (NK isolation kit and CD8 T cell isolation kit respectively; Miltenyi Biotec, UK), which provided better yields of untouched viable cells (> 95% purity).

Where indicated, CD8+ T cell subsets were separated from the above purified untouched CD8+ T cells, according to CD27/CD28 expression using a two-step procedure: briefly, negatively selected CD8+ T cells were sorted into CD28+/CD28–

fractions after incubation with CD28 biotin beads (130-092-955, Miltenyi Biotec) for 15 min at 4°C, washed in ice-cold MACS buffer (degassed phosphate-buffered saline containing 0.5% bovine serum albumin and 2 mm EDTA; all Sigma-Aldrich) and incubated with anti-biotin microbeads (130-093-196, Miltenyi Biotec) for 15 min at 4°C. Cells were washed again and resuspended in MACS buffer and passed through a magnetic separation (MS) column (Miltenyi Biotec). The columns were washed three times with 500 µl MACS buffer and removed from the magnetic field. Magnetically-labelled cells (CD28+ population) were eluted by pushing 1 ml MACS buffer through each column with a plunger. The CD8+CD28– fraction was further separated into CD28–27+ and CD28–27– using CD27 microbeads (130-093-196, Miltenyi Biotec), following a similar procedure. Cell viability was assessed by tripan bue exlusion test and the purity of isolated T cell subsets was confirmed by flow cytometry (**Figure 4.23).**

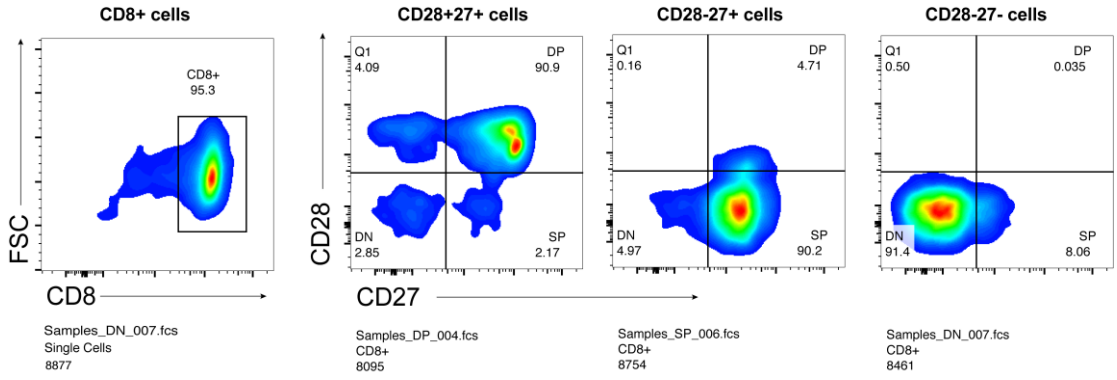

Figure 4.23 – Purity of MACS-sorted cells.

Human CD8+ T cells and subsets defined by CD27/C28 expression were purified using magnetic activated cell sorting (MACS). After selection, purity of each subset was confirmed using flow cytometry. Typically, purity of CD8+ cells is above 95% and purity of the subsets above 90% and shown in the representative plots.

Cells were ressuspended in complete RPMI and used for functional or signaling assays either directly ex vivo or upon genetic modifications, as described below. Of note, for immunoprecipitation studies and for transfection with small interfering RNA, subsets were separated into CD28-/CD28+ fractions only in order to obtain a sufficient number of cells for these assays.

For microarray analysis, high-purity subsets were sorted according to CD27 and CD45RA expression, using a FACS Aria flow cytometer (BD Biosciences). Magnetically purified CD8+ T cells were stained with antibodies against CD8, CD27 and CD45RA for 30 minutes at 4°C in PBS with 1%BSA. Cells were subsequently washed and resuspended in MACS buffer to avoid cell clumping and passed through a 35 µm nylon mesh into a new FACS tube (BD Biosciences). Cells were sorted into sterile tubes containing 50% FBS in RPMI corresponding to the four CD27/CD45RA subsets: naïve (NAIVE; $CD45RA^{+}CD27^{+}$), central memory (CM; $CD45RA^{-}CD27^{+}$), effector memory (EM; $CD45RA^{-}CD27^{-}$) and effector memory T cells that re-express CD45RA (EMRA; $CD45RA^{+}CD27^{-}$).

As CD8+ T cells sequentially lose CD28 followed by CD27 (Rufer et al., 2003) the subpopulation defined by the loss of CD27 and re-expression of CD45RA (EMRA; $CD45RA^{+}CD27^{-}$) is in its vast majority negative for both CD28 and CD27 (**Figure 4.24)** and therefore included within the subset of terminally differentiated CD8+ T cells defined by CD27/CD28 expression (CD28–27– subset).

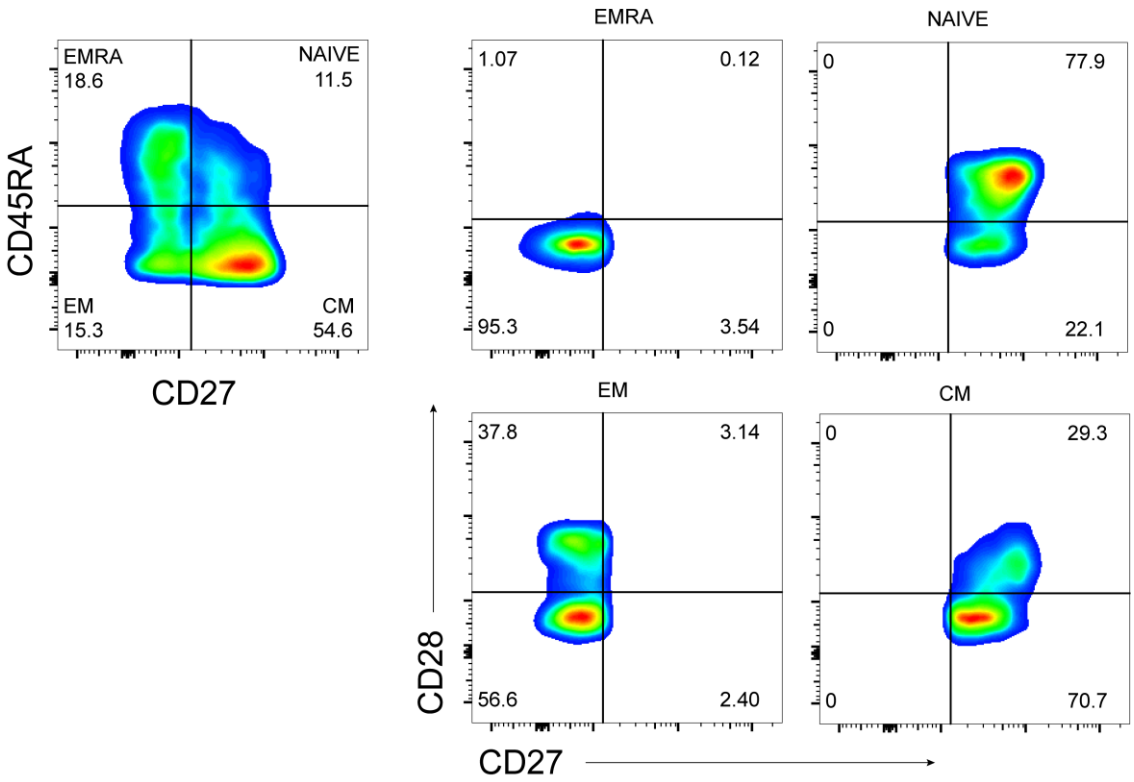

Figure 4.24 – CD8+ T cells re-expressing CD45RA (EMRA) are in their vast majority negative for both CD28 and CD27 receptors.

A) Representative FACS profile of the subsets of CD8+ T cells defined by CD27 and CD45RA expression as: naïve (CD27+ 45RA+), CM, central memory (CD27+ 45RA-), EM, effector memory (CD27- 45RA-) and EMRA, effector memory re-expressing CD45RA (CD27- 45RA+). Numbers represent percentages of cells within each compartment. **B)** Each subset was gated according to the expression of CD27/CD28 receptors and represented in the order shown in A), demonstrating how EMRA CD8+ T cells are mostly included in the CD28-27- subset (highly differentiated CD8+ T cells).

4.5.4 Determination of donor CMV and EBV status

Viral lysate stimulation as means of determining CMV and EBV carrier status is routinely used in our laboratory as it significantly correlates with seropositivity obtained from IgG serology from the diagnostic laboratory of University College London (Fletcher et al., 2005). Briefly, PBMCs were incubated overnight in complete

medium with CMV lysate (1:10, prepared in house) or EBV lysate (1:200, from Virusys, Taneytown, USA) at 37°C. The superantigen Staphylococcal Enterotoxin B (SEB; 1ng/ml; Sigma-Aldrich) was used as a positive control and no stimulant was added as a negative control. Brefeldin A (1 µg/ml, Sigma-Aldrich) was added 2 hours after the start of incubation. The following day, cells were harvested and IFNγ levels were measured via intracellular flow cytometry as detailed below. Participants were considered to be CMV positive if above background production of cytokines was at least 0.01% of the total CD4+ T cells.

4.5.5 Detection of virus-specific CD8+ T cells

Virus-specific CD8+ T cells were detected after labelling with HLA-specific dextramers, for 15 minutes at 37 °C in PBS before staining for surface markers, as described below. HLA-specific, APC-conjugated dextramers loaded with CMV- or EBV-specific peptides were obtained from Immudex (Denmark) and listed in **Table 4.2.** As a control for non-specific dextramer binding, a dextramer loaded with an irrelevant peptide was used in parallel.

HLA allele	Virus	Epitope	Tetramer	Protein
HLA-A*01:01	CMV	VTEHDTLLY	VTE	pp50
HLA-A*02:01	CMV	NLVPMVATV	NLV	pp65
HLA-B*08:01	EBV	RAKFKQLL	RAK	BZLF1
HLA-A*02:01	EBV	GLCTLVAML	GLC	BMLF1

Table 4.3– List of CMV and EBV-specific dextramers used for detection of virus-specific T cells.

4.5.6 Flow Cytometry

Multi-parametric flow cytometry was used for phenotypic and functional analysis of PBMCs. For analysis of expression of surface markers, staining was performed at 4°C for 30 min in the presence of saturating concentrations of antibodies listed in **Table 4.3** and a live/dead fixable Near-Infrared stain (Thermo Scientific, L10119). For the intracellular analysis of cytokine secretion, cytotoxic granule expression and DAP12/DAP10 expression, cells were fixed and permeabilized with the Fix & Perm® Kit (Invitrogen, Life Technologies, UK), before incubation with indicated antibodies or the respective isotype controls (**Table 4.3).** After staining, cells were washed in PBSA and resuspended in 2% paraformaldehyde until acquisition on a LSR II flow cytometer (BD Biosciences). Analysis was performed with FlowJo software (TreeStar, Ashland, OR).

4.5.7 Phosphocytometry

After staining for surface markers, CD8+ T cells were stimulated with anti-CD3 (n-house purified OKT3, 10 µg/mL) for 30 minutes on ice, followed by crosslinking with goat anti–mouse IgG antibody (during 30 minutes on ice. Cells were then transferred to an incubator at 37°C, and stimulation was terminated after 10 minutes, with immediate fixation with Cytofix Buffer (PBS containing 4% paraformaldehyde, BD Biosciences) followed by permeabilization with ice-cold Perm Buffer III (PBS containing 90% methanol, BD Biosciences) and staining with antibodies for phopho-proteins (listed in Supplemental Table 1) for 30 minutes at room temperature. Cells were finally washed in Stain Buffer (PBS, 0.2% BSA, 0.09% Sodium Azide, pH 7.4, BD Biosciences) and immediately analysed with a LSR II flow cytometer (BD Biosciences).

Antibody	Conjugate	Clone	Isotype	Manufacturer	Catalog #	Dilution
Surface markers						
CD3	BUV 395	UCHT1	Mouse IgG1	BD	563546	1:100
CD4	PercP Cy 5.5	SK3	Mouse IgG1	Biolegend	344608	1:50
CD8	BV 421	RPA-T8	Mouse IgG1	Biolegend	301036	1:50
CD27	BV 786	L128	Mouse IgG1	BD	563327	1:50
CD28	BV 510	T44	Mouse IgG1	Biolegend	302936	1:50
CD45RA	BV 605	HI100	Mouse IgG2b	Biolegend	304134	1:50
CD16	APC	VEP13	Mouse IgG1	Miltenyi Biotec	130-091-246	1:10
CD56	FITC	HCD56	Mouse IgG1	Biolegend	318304	1:20
CD57	FITC					
KLRG1	PE	2F1	Syrian Hamster	Biolegend	138408	1:100
NKG2A	AF 700	131411	Mouse IgG2a	R&D Systems	FAB 1059N	1:20
NKG2C	APC	134591	Mouse IgG1	R&D Systems	FAB138A	1:20
NKG2D	PE	149810	Mouse IgG1	R&D Systems	FAB139P	1:20
NKp30	PE	AF29	Mouse IgG1	Miltenyi Biotec	130-099-706	1:20
Nkp44	PE	2.29	Mouse IgG1	Miltenyi Biotec	130-092-480	1:20
CD244 (2B4)	PE	C1.7	Mouse IgG1	Biolegend	329508	1:20
CD161	PE	HP-3G10	Mouse IgG1	Biolegend	339904	1:20
KIR2DL2/3	PE	DX27	Mouse IgG1	Biolegend	312603	1:20
KIR2DL1/S1/S3/S5	PE	LB2	Mouse IgG1	Biolegend	339505	1:20
KIR3DL1 (NKB1)	PE	DX9	Mouse IgG1	Biolegend	312707	1:20
iNKT	APC	6B11	Mouse IgG1	Miltenyi Biotec	130-094-839	1:20
TCR αβ-1	FITC	WT31	Mouse IgG1	BD	347773	1:10
Intracellular markers						
IL2	FITC	MQ1-17H12	Mouse IgG1	BD	511408	1:10
IFNgamma	PE-Cy7	B27	Mouse IgG1	BD	557643	1:20
TNFalpha	APC	MAb11	Mouse IgG1	BD	340534	1:10
Granzyme B	AF 700	GB11	Mouse IgG1	BD	560213	1:10
Perforin	FITC	δG9	Mouse IgG2b	BD	65994X	1:25
DAP10	Unconjugated	FL-93	Rabbit IgG	Santa Cruz	sc-25623	1:100
DA12	PE	406288	Mouse IgG1	R&D Systems	IC5240P	1:50
PhosphoFlow						
P-CD3z (CD247) (pY142)	AF647	K5	Mouse IgG1	BD	558489	1:20
P-Zap70/Syk (pY319/pY352	PE	17A	Mouse IgG1	BD	557881	1:20

Table 4.4 – List of antibodies used in flow cytometry experiments.

4.5.8 Cytotoxic assays - Active Caspase 3 assay

For determining the NK-like cytotoxicity of T cells, K562 cells were used as the target cells. Magnetic activated cell sorting (MACS)-purified NK and CD8+ T cells were co-cultured with K562 cells at various effector to target (E:T) ratios of 1:1. 10:1, 20:1 and 50:1. Cells were incubated in complete RPMI-1640 medium at 37°C, 5% CO2 for 6 hours, and then harvested, fixed and permeabilized for the analysis of active caspase 3 expression, a marker for cells undergoing apoptosis, using the PE Active Caspase-3 Apoptosis Kit (BD Biosciences, UK). Quantification of active caspase 3 expression in K562 cells incubated with medium only without effector cells was used as a negative control to assess spontaneous lysis. K562 cells treated with Camptothecin (8 µg/mL; Sigma-Aldrich) or incubated with NK cells for 6 hours, were used as positive controls.

4.5.9 Cytotoxic assays - CD107a degranulation assay

This assay was performed as described in Materials and Methods – Chapter II. Briefly, purified human NK and CD8+ T cell subsets were incubated at 37°C for 6 h with K562 or C1R-MICA/C1R cells, at a fixed effector to target (E:T) ratio of 2:1, in the presence of APC-conjugated CD107a antibody (BD Biosciences). Brefeldin A (1 µg/ml; Sigma-Aldrich) and Monensin (1 µg/ml; Sigma-Aldrich) were added in the final 5h-incubation period. Effector cells incubated alone in the presence phorbol-12-myristate-13-acetate (PMA, 50 ng/ml, Sigma-Aldrich) with Ionomycin, (250 ng/ml, Sigma-Aldrich) were used as positive control whereas medium alone served as unstimulated (US) control. After incubation, cells were stained for surface markers for 30 min on ice, followed by intracellular detection of cytokines (TNF-alpha and IFNγ) and CD107a expression and analysed by flow cytometry.

4.5.10 Cell lines

K562 (human erythroleukemic) cell line was purchased from the European Collection of Cell cultures (ECCAC, UK) and cultured in 25cm2 flasks (Nunc) in complete RPMI-1640. B-lymphoblastoid cell lines, C1R and C1R transfected with MICA*008 (C1R-MICA) were kindly provided by Professor Antoine Tubert (INSERM UMR1160, Paris) and maintained in compete RPMI-1640 in the presence of the aminoglycoside antibiotic G-418 (Sigma, G8168) for selection of transfected cells.

4.5.11 Cell transfection and RNA interference

Primary human CD28-CD8+ T cells were transfected with small interfering RNA (siRNA) by electroporation using the Amaxa Human T Cell Nucleofector Kit and Nucleofector technology (Lonza), according to the manufacturer's instructions. Briefly, 2-5x10^6 cells were resuspended in Nucleofector solution with 200 nmol of siRNA for NKG2D (sc-42948), DAP12 (sc-35172) or Zap70 (sc-29526, all from Santa Cruz Biotechnology). A scrambled control siRNA (sc-37007; Santa Cruz) was used throughout. Cells were transferred into 24-well cell culture plates containing pre-equilibrated complete medium (RPMI) and incubated at 37°C. Efficiency of siRNA transfection was confirmed by measuring the expression of the protein of interest using flow cytometry, typically 36-48 hours after transfection **(Figure 4.25).**

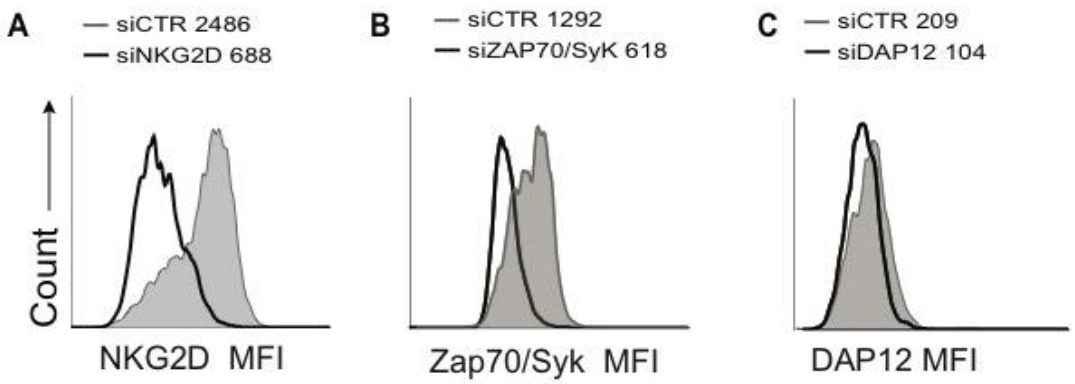

Figure 4.25 – Transfection efficiency after RNA interference.

Representative histograms of the expression of the indicated molecules measured by flow cytometry, 36-48 hours after transfection with the indicated siRNA. Numbers indicate MFI for each condition.

4.5.12 Western blotting

Human CD8+ T cell subsets purified using immunomagnetic separation (MACS) were stimulated with anti-CD3 (purified OKT3, 10 μg/mL) or anti-NKG2D (1D11, 10 μg/mL) before lysis. Cells were normalized by equal cell number and harvested by washing twice with ice-cold PBS followed by ultra-centrifugation at 13000 rpm for 2'. Cells were lysed in ice-cold Radio-Immunoprecipitation Assay (RIPA) buffer (Sigma-Aldrich, UK), supplemented with protease and phosphatase inhibitors (GE Healthcare, Amersham, UK), during 30 minutes on ice. Cell lysates were processed for immunoblot analysis as described in Materials and Methods, Chapter 2. All primary antibodies used are listed in **Table 4.4.**

Antibody	Isotype	MW	Manufacturer	Catalog #	Dilution
LCK	Rabbit	56 kDa	Cell Signaling	2787	1:1000
ZAP70	Rabbit	70 kDa	Cell Signaling	2705	1:1000
p-ZAP70/SYK (Y319/Y352)	Rabbit	70 kDa	Cell Signaling	2717	1:1000
LAT	Rabbit	36 kDa	Cell Signaling	9166	1:1000
SLP-76	Rabbit	76 kDa	Cell Signaling	4958	1:1000
PLCgamma1	Rabbit	150 kDa	Cell Signaling	5690	1:1000
DAP12	Rabbit	12 kDa	Cell Signaling	12492	1:1000
GAPDH	Rabbit	37 kDa	Cell Signaling	2118	1:4000

Table 4.5 – List of primary antibodies used for Western blotting.

4.5.13 Immunoprecipitation

Human CD8+ T cells were separated into CD28+/CD28- fractions (to obtain sufficient number of cells for analysis) and stimulated with anti-NKG2D (1D11, 10 µg/mL) or isotype control, for 30 minutes at 4 °C. Lysates from $10x10^7$ cells were prepared with ice-cold HNGT buffer (50 mM HEPES, pH 7.5, 150 mM EDTA, 10 mM sodium pyrophosphate, 100 mM sodium orthovanadate, 100 mM sodium fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride), for 30 minutes on ice. Cell lysates were incubated overnight at 4°C with anti-NKG2D antibody (clone 5C6, Santa Cruz) or control antibody, followed by precipitation with 10 µL of pre-washed protein A/G agarose beads (sc-2003, Santa Cruz) for 3 h at 4°C on a rotary shaker. After extensive washing in HGNT buffer, supernatants were recovered and processed for immunoblot analysis, as described above. Co-Immunoprecipitated proteins were detected after incubation with primary antibodies followed by incubation with mouse anti-rabbit IgG (conformation-specific antibody; L27A9; Cell Signaling) or mouse anti-rabbit IgG light chain (L57A3; Cell Signaling) and by a secondary anti-mouse IgG antibody (7076; Cell Signaling).

4.5.14 Microarrays

Gene expression studies were performed by Dr. Sian Henson (Prof.Akbar group) in collaboration with Dr. David Kipling (Cardiff University, UK). Microarrays data analysis was performed by Dr. Vitor Teixeira (Division of Respiratory Medicine, University College London).

High purity CD8+ T cells from 6 healthy donors were sorted using a FACSaria according to CD45RA and CD17 expression, as previously described. Total RNA was purified using miRNeasy Kits (Qiagen) and amplified using the Ovation RNA Amplification system V2 Kit (Nugen), following manufacturer recommendations. cDNA was hybridized with Affymetrix U133 plus 2 arrays and quality control performed with Microarray Suite version 5.0 (MAS 5.0) algorithm (Affymetrix). Normalized data (RMA) were used for calculating fold changes in expression for each probe. Differentially expressed genes were selected on the basis of a *p* value cutoff of < 0.05 in the one-way ANOVA test and a Benjamini-Hochbert false-discovery rate (FDR) correction less than 5%. Duplicate genes and genes of unknown function were removed. Data analysis and hierarchical clustering were performed with Transcriptome Analysis Console and Expression Console Software (Affymetrix). For microarray validation the expression of selected proteins with relevance to the experiment was confirmed by flow cytometry or immunoblot analysis.

4.5.15 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.00, as described in previous chapters. Values of $p < 0.05$ were considered significant for all tests. Data are presented as means ± standard error of the mean (SEM) unless otherwise stated.

5 GENERAL DISCUSSION

Aging is one of the most important risk factors for the development of disease and cancer and accumulating evidence now indicates that the accumulation of senescent cells in tissues promotes aging and age-related diseases (Campisi, 2013, Cerella et al., 2016).

The persistence of senescent cells in tissues is thought to contribute to the maintenance of a low-level chronic inflammatory state that underlies many age-related diseases (Campisi and d'Adda di Fagagna, 2007, Franceschi and Campisi, 2014, van Deursen, 2014). The assumption that the accumulation of senescent cells is responsible for aging phenotypes is supported by the identification of senescent cells at sites of age-related pathologies, including atherosclerosis, osteoarthritis, degenerating intervertebral discs and also in liver cirrhosis (Erusalimsky and Kurz, 2005, Price et al., 2002, Roberts et al., 2006, Wiemann et al., 2002, Sharpless and Sherr, 2015). The causality link between senescence and aging was finally proven when the removal of $p16^{INK4a}$ positive senescent cells in a progeroid mouse model effectively delayed the onset of age-related phenotypes. Strikingly, elimination of these cells later in life could also halt the progression of pre-established ageing phenotypes (Baker et al., 2011, Baker et al., 2016). Due to their potential involvement in many aging and disease processes, eliminating senescent cells and attenuating the senescence-associated secretory phenotype (SASP) have emerged as attractive therapeutic strategies (Provinciali and Malavolta, 2016). Strategies to improve the immune response against senescent cells, designated as senescence immune surveillance, have also provoked considerable interest not only for cancer therapy but also for the prevention of age-related diseases (Hoenicke and Zender, 2012, Naylor et al., 2013).

Although an increasing amount of evidence indicates that senescent cells can be targeted by the immune system (Hoenicke and Zender, 2012, Sagiv and Krizhanovsky, 2013), they fail to explain why these cells accumulate with age. Understanding the interactions between the immune system and senescent cells, with a particular interest on the effects of immunesenescence on the surveillance of senescent cells were the main objectives of this thesis.

We were surprised at the lack of studies describing a role for cytotoxic CD8+ T cells in senescence surveillance and therefore aimed to develop a model where the interactions of senescent cells with both NK and CD8+ T cells could be investigated. By employing an autologous *in vitro* coculture system with skin-derived human fibroblasts and *ex-vivo* isolated human PBMCs, we circumvented the confounding effect of allogeneic responses driven by MHC class I mismatch reactions.

We first investigated whether there were changes in expression of MHC molecules in fibroblasts upon the induction of senescence. The initial hypothesis was that senescent cells would downregulate the expression of classical MHC class I molecules and escape immune recognition by CD8+ T cells, using similar strategies to cancer and viral infected-cells (Campoli and Ferrone, 2008, Halenius et al., 2015). If confirmed, this hypothesis could also explain why senescent cells become targets of NK cell killing via the activation of “missing self” signals regulated by killer-cell immunoglobulin like receptors (KIRs). Unexpectedly, no consistent changes in classical MHC class I expression were found after the induction of senescence in primary human fibroblasts.

Interestingly though, the expression of non-classical MHC molecules was significantly altered upon the induction of senescence and the most consistent differences were related to the expression of HLA-E and MICA/B. As the expression of MICA/B and other NKG2D ligands had already been described in senescent cells

(Sagiv, 2016), we focused of our interest in studying the mechanisms regulating the expression of HLA-E on senescent cells. The expression of non-classical class Ib molecules, such as HLA-E is very uncommon and restricted to some tissues, such as the placental trophoblast (King et al., 2000) where it is though to play an important immunoregulatory role to ensure maternal tolerance to the fetus and protection from NK cell-mediated responses (Dahl and Hviid, 2012). In addition, an increased expression of HLA-E has been reported after viral infection and in several tumours as a mechanism of immune-evasion (Tomasec et al., 2000, Marin et al., 2003, Iwaszko and Bogunia-Kubik, 2011, Morandi and Pistoia, 2014). In line with this, it was hypothesized that HLA-E expression on senescent cells could have an important role in inhibiting immune responses to senescent cells.

Using *in vitro* antibody-blockade experiments, it was demonstrated that interaction of HLA-E with the inhibitory NKG2A receptor, expressed not only by NK cells but also CD8+ T cells impaired the lysis of senescent cells, mediated via NKG2D. Therefore, it was proposed that overexpression of HLA-E on senescent cells might represent an escape mechanism to an effective immune surveillance, favouring the persistence of senescent cells in tissues. This hypothesis was further supported by the findings that human melanocytic nevi, which are an example of human premalignant lesions typically enriched with cells with senescence markers, also expressed high levels of HLA-E.

Thus, the interactions of senescent cells with the immune system can be modulated by the expression of different ligands at the cell surface. Based on these findings, a model for the interplay between senescent cells and NK and CD8+ T cells was proposed, whereby a balance of activating and inhibitory signals seems to determine the final outcome of the immune responses. Whilst MICA/B recognition by NKG2D triggers both NK- and CD8+ T cell-mediated cytotoxicity, the simultaneous

expression of HLA-E may shift this balance in favour of the negative signals, by virtue of the interaction with the inhibitory receptor NKG2A.

Such regulation of immune responses determined by a fine-tuned balance of inhibitory and activating signals is typically described for NK cell-mediated responses (Long et al., 2013). However, this concept is not new to T cell biology either. Different immune checkpoints regulate T cell activation by inhibiting TCR-driven signals during immune responses (Schneider et al., 2006, Fife et al., 2009). This regulation is particularly important to ensure peripheral tolerance and prevent auto-reactivity, however under pathological conditions it may prevent effective immune responses against abnormal cells. In fact, accumulating evidence indicates that tumour cells have developed strategies to evade the host immune system by taking advantage of this mechanism (Dunn et al., 2002, Pardoll, 2012). Central to this, are the cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) and programmed death 1 (PD-1) immune checkpoint pathways (Buchbinder and Desai, 2016) and the use of immune checkpoint inhibitors has recently emerged as a promising therapy for cancer. Monoclonal antibodies targeting NKG2A are also part of this group of immune checkpoint inhibitors that act as immune modulators and restore the capacity of the immune system to eliminate harmful cells (Mahoney et al., 2015). Therefore, the results presented here indicating that *in vitro* NKG2A blockade can effectively enhance immune responses towards senescent cells could be seen as a proof of principle study demonstrating the potential benefit of immune modulators in ageing. Further studies using i*n vivo* models to test the effect of anti-NKG2A antibodies in boosting the host capacity to eliminate senescent cells would be necessary to confirm this hypothesis.

Importantly, the expression of the cognate receptors for HLA-E (NKG2A/NKG2C) and MICA/B (NKG2D) was found to be highest in cells with a terminally differentiated phenotype, which are expanded with age. This suggested that the immune system is

able to adapt to the aged microenvironment and to the increased number of senescent cells with age, through the increased expression of receptors that are able to recognize ligands expressed on senescent stromal cells. However, even if immune cells express high levels of the receptor NKG2D and are able to recognize MICA/B-expressing senescent cells, the increased expression of NKG2A on highly differentiated cells will prevent an effective clearance of these cells. As negative signals from inhibitory receptors tend to be dominant (Long et al., 2013), the functional outcome of engaging both activating and inhibitory receptors will result in the inhibition of immune responses and persistence of senescent cells.

In summary, the results presented in this thesis contribute to a better understanding of the interplay between the immune system and senescent cells and the mechanisms underlying the immune-mediated clearance of senescent cells.

In the context of the aforementioned results, the observation that differentiated CD8+ T cells expressed NK cell receptors and could mediate responses resembling NK cells raised our interest in understanding the biological significance of the expression of NK receptors on CD8+ T cells. Most importantly, we wanted to reconcile these observations with other described features of highly differentiated CD8+ T cells. Therefore, in the last part of this project, we extended the investigation in order to include a larger panel of NK cell receptors and explore their function in T cells. In keeping up with previous observations, it was found that highly differentiated CD8+ T cells express a diverse repertoire of NK receptors, both activating and inhibitory, and could mediate cytotoxic responses in a similar fashion to NK cells, that is independently of the TCR. Interestingly, these cells also displayed an impaired responsiveness to TCR stimulation suggesting that, as CD8+ T cells differentiate, they become less dependent on TCR-driven signals and more responsive to innate-like stimulation. Functionally, these results provided some insights into the reasons why these cells lack proliferative potential yet maintaining potent effector functions,

suggesting that cytotoxic effector functions of highly differentiated cells are regulated by distinct pathways to proliferation, independent of the TCR. Collectively, based on phenotypic, functional and transcriptomic data, it was proposed that terminal differentiated CD8+ T cells resemble NK cells in many aspects and represent a population with mixed innate and adaptive features. This hypothesis challenges once more the distinction between the innate and adaptive arms of the immune system and more importantly represents a paradigm shift in terms of how we regard terminally differentiated or senescent CD8+ T cells. Instead of being a population with limited functionality we suggest that these cells acquire new and unconventional functions in order to maintain effective immune responses throughout life and adapt to the aged microenvironment. Although not investigated here, it would be important in our perspective to elucidate whether these cells are able to maintain both antigen-specific and antigen-independent functions.

Overall, this work raises important questions on the biological significance of senescence surveillance. First, there has been a long-standing debate over the role of senescent cells in the organism and whether it is beneficial or not to eliminate them. Cellular senescence is an evolutionary conserved mechanism that is beneficial because it acts as a major barrier to cancer. Recently, evidence has pointed to beneficial effects of cellular senescence beyond tumour suppression, for instance in directing wound repair (Demaria et al., 2014) and in tissue regeneration (Ritschka et al., 2017). Despite being beneficial, senescence may have deleterious consequences later in life, particularly associated with the accumulation of senescent cells and their secretion of pro-inflammatory mediators (SASP), leading to tissue dysfunction. Cellular senescence is thus an example of antagonistic pleiotropy and the fact that most detrimental effects occur after reproductive age, determines that this mechanism has been conserved through evolution. Nevertheless, as it was pointed out before, accumulating evidence indicates that eliminating senescent cells from the

organism is in many aspects beneficial to the host, mainly through the prevention of age-related diseases.

Another emerging question regards the function of highly differentiated or senescent CD8+ T cells. What would be the benefit of expanding a population of T cells with overlapping features with NK cells? We believe that NK-like CD8+ T cells emerge as a result of homeostatic proliferation of CD8+ T cells induced by cytokines such as IL-15, most likely as a compensatory mechanism to the contraction of the immune repertoire with age. The acquisition of innate sensors specialized in the recognition of “danger” signals allows these cells to switch to a rapid and efficient mode of action, independently of the TCR and be part of the first line of defense against potentially harmful cells. This redundancy may be beneficial if we considered that conventional NK and NKT cells are reduced in number and have some aspects of their function impaired with age. Moreover, given the increased burden of tumors and infections with age, the contribution of such innate-like CD8+ T cells may be crucial to the organism. The demonstrated role of these cells in targeting senescent cells further supports the biological relevance of these cells. Although not investigated here, it would be important in our perspective to elucidate whether these cells are able to maintain both antigen-specific and antigen-independent functions, revealing more complex physiological roles to senescent cells that previously anticipated.

6 FUTURE PERSPECTIVES

I. Exploring additional mechanisms involved in the regulation of HLA-E expression by p38MAPK;

The regulation of HLA-E expression by p38MAPK represents an interesting finding, however the mechanisms involved in this regulation were not completely dissected. It has been demonstrated that p38MAPK exerts many of its functions by increasing NF-κB transcriptional activity and this has been implicated in the regulation of SASP (Freund et al., 2011). As NF-κB is an important transcriptional regulator of MHC gene expression (Molinero et al., 2004, van den Elsen, 2011), it may be speculated that p38MAPK regulates HLA-E expression through the activation of NF-κB.

The regulation of HLA-E expression by p38MAPK can also be exerted at a post-transcriptional level through the *de novo* generation of peptides to be presented by HLA-E and stabilize its expression at the cell surface. Recently it has been shown that HLA-E can present peptides derived from the leader sequence of heat-shock protein 60 (Hsp60), (Michaelsson et al., 2002). It would be interesting to investigate whether p38MAPK regulation of Hsp27 activity leads to the generation of stress-induced peptides that could be presented by HLA-E. Self-peptide presentation by HLA-E can be sensed by NKG2 receptors present in NK but is also thought to be central in the generation of suppressive CD8+ T cells. However, HLA-E may also lead to the activation of CD8+ T cells, through direct recognition by the TCR (Pietra et al., 2010, Joosten et al., 2016). The hypothesis that HLA-E can present senescent-associated antigens directly to CD8+ T cells or other immune cells, leading to specific immune responses against senescent cells would provide additional targets for boosting immune responses to senescent cells.

II. Identifying the source and role of NK-like T cells in antigen-specific immune responses;

Despite the accepted role of chronic latent viruses, such as CMV in driving T cell differentiation, one unexpected observation was that virus-specific CD8+ T cells expressed lower levels of NK cell receptors compared to the global CD8+ T cell compartment. Although an effect of homeostatic cytokines, such as IL-15 has been found, our understating of the exact source and main drivers of the expression of NK cell receptors on T cells is still incomplete.

Another standing question is whether NK-like CD8+ T cells still retain the capacity to elicit specific TCR-dependent immune responses. Although impaired in their capacity to respond to anti-CD3 cross-linking, these cells can still respond to TCR stimulation, suggesting that a higher threshold for activation may be needed. It would be important to understand whether the co-expression of NK cell receptors determines this threshold and contributes to the modulation of TCR responses.

III. Investigating the mechanistic link between TCR hyporesponsiveness and the acquisition of innate-like features.

It has been recently proposed that the suppression of TCR signaling is critical for the development of innate-like T cells. An elegant study done by Hayday and colleagues in mice models has brought some insights into how innate T cells down-modulate the TCR signaling machinery to allow an innate mode of activation in peripheral tissues, independent of the TCR (Wencker et al., 2014). The authors demonstrated that this mechanism of TCR tuning after development in the thymus, concomitant with acquisition of responsiveness to innate signals is a feature shared by diverse subsets of innate-like T cells. Nevertheless, it remains to be determined which factors control

the peripheral modulation of TCR signaling and whether there is a mechanistic link between the acquisition of NK cell receptors and the modulation of the TCR machinery. Although the data presented in this thesis suggests that it may be determined at the transcriptional level, the identification of the molecular mechanisms and the transcriptional regulators underpinning the development of innate features in T cells would be important.

7 BIBLIOGRAPHY

ABEDIN, S., MICHEL, J. J., LEMSTER, B. & VALLEJO, A. N. 2005. 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**,** 537-48.

ACOSTA, J. C., BANITO, A., WUESTEFELD, T., GEORGILIS, A., JANICH, P., MORTON, J. P., ATHINEOS, D., KANG, T. W., LASITSCHKA, F., ANDRULIS, M., PASCUAL, G., MORRIS, K. J., KHAN, S., JIN, H., DHARMALINGAM, G., SNIJDERS, A. P., CARROLL, T., CAPPER, D., PRITCHARD, C., INMAN, G. J., LONGERICH, T., SANSOM, O. J., BENITAH, S. A., ZENDER, L. & GIL, J. 2013. A complex secretory program orchestrated by the inflammasome controls paracrine senescence. *Nat Cell Biol,* 15**,** 978-90.

ACOSTA, J. C. & GIL, J. 2012. Senescence: a new weapon for cancer therapy. *Trends Cell Biol,* 22**,** 211-9.

ACOSTA, J. C., O'LOGHLEN, A., BANITO, A., GUIJARRO, M. V., AUGERT, A., RAGUZ, S., FUMAGALLI, M., DA COSTA, M., BROWN, C., POPOV, N., TAKATSU, Y., MELAMED, J., D'ADDA DI FAGAGNA, F., BERNARD, D., HERNANDO, E. & GIL, J. 2008. Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell,* 133**,** 1006-18.

AIRD, K. M., IWASAKI, O., KOSSENKOV, A. V., TANIZAWA, H., FATKHUTDINOV, N., BITLER, B. G., LE, L., ALICEA, G., YANG, T. L., JOHNSON, F. B., NOMA, K. I. & ZHANG, R. 2016. HMGB2 orchestrates the chromatin landscape of senescence-associated secretory phenotype gene loci. *J Cell Biol,* 215**,** 325-334.

AKBAR, A. N., BEVERLEY, P. C. & SALMON, M. 2004. Will telomere erosion lead to a loss of T-cell memory? *Nat Rev Immunol,* 4**,** 737-43.

AKBAR, A. N. & FLETCHER, J. M. 2005. Memory T cell homeostasis and senescence during aging. *Curr Opin Immunol,* 17**,** 480-5.

AKBAR, A. N. & HENSON, S. M. 2011. Are senescence and exhaustion intertwined or unrelated processes that compromise immunity? *Nat Rev Immunol,* 11**,** 289-95.

AKBAR, A. N., HENSON, S. M. & LANNA, A. 2016. Senescence of T Lymphocytes: Implications for Enhancing Human Immunity. *Trends Immunol,* 37**,** 866-876.

AKTAS, E., KUCUKSEZER, U. C., BILGIC, S., ERTEN, G. & DENIZ, G. 2009. Relationship between CD107a expression and cytotoxic activity. *Cell Immunol,* 254**,** 149-54.

ALAO, J. P. & SUNNERHAGEN, P. 2009. The ATM and ATR inhibitors CGK733 and caffeine suppress cyclin D1 levels and inhibit cell proliferation. *Radiat Oncol,* 4**,** 51.

ALGARRA, I., GARCIA-LORA, A., CABRERA, T., RUIZ-CABELLO, F. & GARRIDO, F. 2004. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. *Cancer Immunol Immunother,* 53**,** 904-10.

ALONZO, E. S. & SANT'ANGELO, D. B. 2011. Development of PLZF-expressing innate T cells. *Curr Opin Immunol,* 23**,** 220-7.

ALVES, N. L., HOOIBRINK, B., AROSA, F. A. & VAN LIER, R. A. 2003. IL-15 induces antigen-independent expansion and differentiation of human naive CD8+ T cells in vitro. *Blood,* 102**,** 2541-6.

AMIOT, L., FERRONE, S., GROSSE-WILDE, H. & SELIGER, B. 2011. Biology of HLA-G in cancer: a candidate molecule for therapeutic intervention? *Cell Mol Life Sci,* 68**,** 417-31.

AOSHIBA, K., TSUJI, T. & NAGAI, A. 2003. Bleomycin induces cellular senescence in alveolar epithelial cells. *Eur Respir J,* 22**,** 436-43.

APPAY, V., DUNBAR, P. R., CALLAN, M., KLENERMAN, P., GILLESPIE, G. M., PAPAGNO, L., OGG, G. S., KING, A., LECHNER, F., SPINA, C. A., LITTLE, S., HAVLIR, D. V., RICHMAN, D. D., GRUENER, N., PAPE, G., WATERS, A., EASTERBROOK, P., SALIO, M., CERUNDOLO, V., MCMICHAEL, A. J. & ROWLAND-JONES, S. L. 2002. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med,* 8**,** 379-85.

APPAY, V., VAN LIER, R. A., SALLUSTO, F. & ROEDERER, M. 2008. Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry A,* 73**,** 975-83.

ARNOLD, C. R., WOLF, J., BRUNNER, S., HERNDLER-BRANDSTETTER, D. & GRUBECK-LOEBENSTEIN, B. 2011. Gain and loss of T cell subsets in old age--age-related reshaping of the T cell repertoire. *J Clin Immunol,* 31**,** 137-46.

BAKER, D. J., CHILDS, B. G., DURIK, M., WIJERS, M. E., SIEBEN, C. J., ZHONG, J., SALTNESS, R. A., JEGANATHAN, K. B., VERZOSA, G. C., PEZESHKI, A., KHAZAIE, K., MILLER, J. D. & VAN DEURSEN, J. M. 2016. Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan. *Nature,* 530**,** 184-9.

BAKER, D. J., WIJSHAKE, T., TCHKONIA, T., LEBRASSEUR, N. K., CHILDS, B. G., VAN DE SLUIS, B., KIRKLAND, J. L. & VAN DEURSEN, J. M. 2011. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature,* 479**,** 232-6.

BARNES, T. C., ANDERSON, M. E. & MOOTS, R. J. 2011. The many faces of interleukin-6: the role of IL-6 in inflammation, vasculopathy, and fibrosis in systemic sclerosis. *Int J Rheumatol,* 2011**,** 721608.

BARTKOVA, J., REZAEI, N., LIONTOS, M., KARAKAIDOS, P., KLETSAS, D., ISSAEVA, N., VASSILIOU, L. V., KOLETTAS, E., NIFOROU, K., ZOUMPOURLIS, V. C., TAKAOKA, M., NAKAGAWA, H., TORT, F., FUGGER, K., JOHANSSON, F., SEHESTED, M., ANDERSEN, C. L., DYRSKJOT, L., ORNTOFT, T., LUKAS, J., KITTAS, C., HELLEDAY, T., HALAZONETIS, T. D., BARTEK, J. & GORGOULIS, V. G. 2006. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature,* 444**,** 633-7.

BAUER, J., CURTIN, J. A., PINKEL, D. & BASTIAN, B. C. 2007. Congenital melanocytic nevi frequently harbor NRAS mutations but no BRAF mutations. *J Invest Dermatol,* 127**,** 179-82.

BAUER, S., GROH, V., WU, J., STEINLE, A., PHILLIPS, J. H., LANIER, L. L. & SPIES, T. 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science,* 285**,** 727-9.

BEERMAN, I., BHATTACHARYA, D., ZANDI, S., SIGVARDSSON, M., WEISSMAN, I. L., BRYDER, D. & ROSSI, D. J. 2010. Functionally distinct hematopoietic

stem cells modulate hematopoietic lineage potential during aging by a mechanism of clonal expansion. *Proc Natl Acad Sci U S A,* 107**,** 5465-70.

BEN-PORATH, I. & WEINBERG, R. A. 2005. The signals and pathways activating cellular senescence. *Int J Biochem Cell Biol,* 37**,** 961-76.

BENNETT, D. C. 2016. Genetics of melanoma progression: the rise and fall of cell senescence. *Pigment Cell Melanoma Res,* 29**,** 122-40.

BEVONA, C., GOGGINS, W., QUINN, T., FULLERTON, J. & TSAO, H. 2003. Cutaneous melanomas associated with nevi. *Arch Dermatol,* 139**,** 1620-4; discussion 1624.

BEZIAT, V., HERVIER, B., ACHOUR, A., BOUTOLLEAU, D., MARFAIN-KOKA, A. & VIEILLARD, V. 2011. Human NKG2A overrides NKG2C effector functions to prevent autoreactivity of NK cells. *Blood,* 117**,** 4394-6.

BIANCHI, M. E. 2007. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol,* 81**,** 1-5.

BLACKBURN, S. D., SHIN, H., HAINING, W. N., ZOU, T., WORKMAN, C. J., POLLEY, A., BETTS, M. R., FREEMAN, G. J., VIGNALI, D. A. & WHERRY, E. J. 2009. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol,* 10**,** 29-37.

BLACKMAN, M. A. & WOODLAND, D. L. 2011. The narrowing of the CD8 T cell repertoire in old age. *Curr Opin Immunol,* 23**,** 537-42.

BLAND, F. A., LEMBERG, M. K., MCMICHAEL, A. J., MARTOGLIO, B. & BRAUD, V. M. 2003. Requirement of the proteasome for the trimming of signal peptide-derived epitopes presented by the nonclassical major histocompatibility complex class I molecule HLA-E. *J Biol Chem,* 278**,** 33747-52.

BLUWSTEIN, A., KUMAR, N., LEGER, K., TRAENKLE, J., OOSTRUM, J., REHRAUER, H., BAUDIS, M. & HOTTIGER, M. O. 2013. PKC signaling prevents irradiation-induced apoptosis of primary human fibroblasts. *Cell Death Dis,* 4**,** e498.

BOCCARDI, V., PELINI, L., ERCOLANI, S., RUGGIERO, C. & MECOCCI, P. 2015. From cellular senescence to Alzheimer's disease: The role of telomere shortening. *Ageing Res Rev,* 22**,** 1-8.

BODNAR, A. G., KIM, N. W., EFFROS, R. B. & CHIU, C. P. 1996. Mechanism of telomerase induction during T cell activation. *Exp Cell Res,* 228**,** 58-64.

BODNAR, A. G., OUELLETTE, M., FROLKIS, M., HOLT, S. E., CHIU, C. P., MORIN, G. B., HARLEY, C. B., SHAY, J. W., LICHTSTEINER, S. & WRIGHT, W. E. 1998. Extension of life-span by introduction of telomerase into normal human cells. *Science,* 279**,** 349-52.

BORREGO, F., MASILAMANI, M., KABAT, J., SANNI, T. B. & COLIGAN, J. E. 2005. The cell biology of the human natural killer cell CD94/NKG2A inhibitory receptor. *Mol Immunol,* 42**,** 485-8.

BORREGO, F., MASILAMANI, M., MARUSINA, A. I., TANG, X. & COLIGAN, J. E. 2006. The CD94/NKG2 family of receptors: from molecules and cells to clinical relevance. *Immunol Res,* 35**,** 263-78.

BRAIG, M., LEE, S., LODDENKEMPER, C., RUDOLPH, C., PETERS, A. H., SCHLEGELBERGER, B., STEIN, H., DORKEN, B., JENUWEIN, T. & SCHMITT, C. A. 2005. Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature,* 436**,** 660-5.

BRAUD, V. M., ALLAN, D. S., O'CALLAGHAN, C. A., SODERSTROM, K., D'ANDREA, A., OGG, G. S., LAZETIC, S., YOUNG, N. T., BELL, J. I., PHILLIPS, J. H., LANIER, L. L. & MCMICHAEL, A. J. 1998a. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature,* 391**,** 795-9.

BRAUD, V. M., ALLAN, D. S., WILSON, D. & MCMICHAEL, A. J. 1998b. TAP- and tapasin-dependent HLA-E surface expression correlates with the binding of an MHC class I leader peptide. *Curr Biol,* 8**,** 1-10.

BRENCHLEY, J. M., KARANDIKAR, N. J., BETTS, M. R., AMBROZAK, D. R., HILL, B. J., CROTTY, L. E., CASAZZA, J. P., KURUPPU, J., MIGUELES, S. A., CONNORS, M., ROEDERER, M., DOUEK, D. C. & KOUP, R. A. 2003. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. *Blood,* 101**,** 2711-20.

BRENNAN, P. J., BRIGL, M. & BRENNER, M. B. 2013. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol,* 13**,** 101-17.

BRONTE, V. & ZANOVELLO, P. 2005. Regulation of immune responses by L-arginine metabolism. *Nat Rev Immunol,* 5**,** 641-54.

BROUSSARD, C., FLEISCHACKER, C., HORAI, R., CHETANA, M., VENEGAS, A. M., SHARP, L. L., HEDRICK, S. M., FOWLKES, B. J. & SCHWARTZBERG, P. L. 2006. Altered development of CD8+ T cell lineages in mice deficient for the Tec kinases Itk and Rlk. *Immunity,* 25**,** 93-104.

BRUNNER, S., HERNDLER-BRANDSTETTER, D., WEINBERGER, B. & GRUBECK-LOEBENSTEIN, B. 2011. Persistent viral infections and immune aging. *Ageing Res Rev,* 10**,** 362-9.

BRUUNSGAARD, H. 2006. The clinical impact of systemic low-level inflammation in elderly populations. With special reference to cardiovascular disease, dementia and mortality. *Dan Med Bull,* 53**,** 285-309.

BRUUNSGAARD, H., LADELUND, S., PEDERSEN, A. N., SCHROLL, M., JORGENSEN, T. & PEDERSEN, B. K. 2003. Predicting death from tumour necrosis factor-alpha and interleukin-6 in 80-year-old people. *Clin Exp Immunol,* 132**,** 24-31.

BUBENIK, J. 2004. MHC class I down-regulation: tumour escape from immune surveillance? (review). *Int J Oncol,* 25**,** 487-91.

BUCHBINDER, E. I. & DESAI, A. 2016. CTLA-4 and PD-1 Pathways: Similarities, Differences, and Implications of Their Inhibition. *Am J Clin Oncol,* 39**,** 98-106.

CAMPISI, J. 1997. The biology of replicative senescence. *Eur J Cancer,* 33**,** 703-9.

CAMPISI, J. 2005. Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell,* 120**,** 513-22.

CAMPISI, J. 2013. Aging, cellular senescence, and cancer. *Annu Rev Physiol,* 75**,** 685-705.

CAMPISI, J. & D'ADDA DI FAGAGNA, F. 2007. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol,* 8**,** 729-40.

CAMPOLI, M. & FERRONE, S. 2008. HLA antigen changes in malignant cells: epigenetic mechanisms and biologic significance. *Oncogene,* 27**,** 5869-85.

CAWTHON, R. M., SMITH, K. R., O'BRIEN, E., SIVATCHENKO, A. & KERBER, R. A. 2003. Association between telomere length in blood and mortality in people aged 60 years or older. *Lancet,* 361**,** 393-5.

CELESTE, A., PETERSEN, S., ROMANIENKO, P. J., FERNANDEZ-CAPETILLO, O., CHEN, H. T., SEDELNIKOVA, O. A., REINA-SAN-MARTIN, B., COPPOLA, V., MEFFRE, E., DIFILIPPANTONIO, M. J., REDON, C., PILCH, D. R., OLARU, A., ECKHAUS, M., CAMERINI-OTERO, R. D., TESSAROLLO, L., LIVAK, F., MANOVA, K., BONNER, W. M., NUSSENZWEIG, M. C. & NUSSENZWEIG, A. 2002. Genomic instability in mice lacking histone H2AX. *Science,* 296**,** 922-7.

CERBONI, C., FIONDA, C., SORIANI, A., ZINGONI, A., DORIA, M., CIPPITELLI, M. & SANTONI, A. 2014. The DNA Damage Response: A Common Pathway in the Regulation of NKG2D and DNAM-1 Ligand Expression in Normal, Infected, and Cancer Cells. *Front Immunol,* 4**,** 508.

CERELLA, C., GRANDJENETTE, C., DICATO, M. & DIEDERICH, M. 2016. Roles of Apoptosis and Cellular Senescence in Cancer and Aging. *Curr Drug Targets,* 17**,** 405-15.

CHAKRABORTY, A. K. & WEISS, A. 2014. Insights into the initiation of TCR signaling. *Nat Immunol,* 15**,** 798-807.

CHAMPSAUR, M. & LANIER, L. L. 2010. Effect of NKG2D ligand expression on host immune responses. *Immunol Rev,* 235**,** 267-85.

CHANG, B. D., BROUDE, E. V., DOKMANOVIC, M., ZHU, H., RUTH, A., XUAN, Y., KANDEL, E. S., LAUSCH, E., CHRISTOV, K. & RONINSON, I. B. 1999. A senescence-like phenotype distinguishes tumor cells that undergo terminal proliferation arrest after exposure to anticancer agents. *Cancer Res,* 59**,** 3761-7.

CHANG, J., WANG, Y., SHAO, L., LABERGE, R. M., DEMARIA, M., CAMPISI, J., JANAKIRAMAN, K., SHARPLESS, N. E., DING, S., FENG, W., LUO, Y., WANG, X., AYKIN-BURNS, N., KRAGER, K., PONNAPPAN, U., HAUER-JENSEN, M., MENG, A. & ZHOU, D. 2016. Clearance of senescent cells by ABT263 rejuvenates aged hematopoietic stem cells in mice. *Nat Med,* 22**,** 78-83.

CHANG, L. & KARIN, M. 2001. Mammalian MAP kinase signalling cascades. *Nature,* 410**,** 37-40.

CHEENT, K. S., JAMIL, K. M., CASSIDY, S., LIU, M., MBIRIBINDI, B., MULDER, A., CLAAS, F. H., PURBHOO, M. A. & KHAKOO, S. I. 2013. Synergistic inhibition of natural killer cells by the nonsignaling molecule CD94. *Proc Natl Acad Sci U S A,* 110**,** 16981-6.

CHIU, W. K., FANN, M. & WENG, N. P. 2006. Generation and growth of CD28nullCD8+ memory T cells mediated by IL-15 and its induced cytokines. *J Immunol,* 177**,** 7802-10.

CHONG, L., VAN STEENSEL, B., BROCCOLI, D., ERDJUMENT-BROMAGE, H., HANISH, J., TEMPST, P. & DE LANGE, T. 1995. A human telomeric protein. *Science,* 270**,** 1663-7.

CHU, T., TYZNIK, A. J., ROEPKE, S., BERKLEY, A. M., WOODWARD-DAVIS, A., PATTACINI, L., BEVAN, M. J., ZEHN, D. & PRLIC, M. 2013. Bystander-activated memory CD8 T cells control early pathogen load in an innate-like, NKG2D-dependent manner. *Cell Rep,* 3**,** 701-8.

COLLADO, M., GIL, J., EFEYAN, A., GUERRA, C., SCHUHMACHER, A. J., BARRADAS, M., BENGURIA, A., ZABALLOS, A., FLORES, J. M., BARBACID, M., BEACH, D. & SERRANO, M. 2005. Tumour biology: senescence in premalignant tumours. *Nature,* 436**,** 642.

COLLADO, M. & SERRANO, M. 2006. The power and the promise of oncogene-induced senescence markers. *Nat Rev Cancer,* 6**,** 472-6.

COLLADO, M. & SERRANO, M. 2010. Senescence in tumours: evidence from mice and humans. *Nat Rev Cancer,* 10**,** 51-7.

COPPE, J. P., DESPREZ, P. Y., KRTOLICA, A. & CAMPISI, J. 2010a. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol,* 5**,** 99-118.

COPPE, J. P., PATIL, C. K., RODIER, F., KRTOLICA, A., BEAUSEJOUR, C. M., PARRINELLO, S., HODGSON, J. G., CHIN, K., DESPREZ, P. Y. & CAMPISI, J. 2010b. A human-like senescence-associated secretory phenotype is conserved in mouse cells dependent on physiological oxygen. *PLoS One,* 5**,** e9188.

COPPE, J. P., PATIL, C. K., RODIER, F., SUN, Y., MUNOZ, D. P., GOLDSTEIN, J., NELSON, P. S., DESPREZ, P. Y. & CAMPISI, J. 2008. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol,* 6**,** 2853-68.

CORREIA-MELO, C., HEWITT, G. & PASSOS, J. F. 2014. Telomeres, oxidative stress and inflammatory factors: partners in cellular senescence? *Longev Healthspan,* 3**,** 1.

COSMAN, D., MULLBERG, J., SUTHERLAND, C. L., CHIN, W., ARMITAGE, R., FANSLOW, W., KUBIN, M. & CHALUPNY, N. J. 2001. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity,* 14**,** 123-33.

COUPEL, S., MOREAU, A., HAMIDOU, M., HOREJSI, V., SOULILLOU, J. P. & CHARREAU, B. 2007. Expression and release of soluble HLA-E is an immunoregulatory feature of endothelial cell activation. *Blood,* 109**,** 2806-14.

D'ADDA DI FAGAGNA, F. 2008. Living on a break: cellular senescence as a DNA-damage response. *Nat Rev Cancer,* 8**,** 512-22.

D'ADDA DI FAGAGNA, F., REAPER, P. M., CLAY-FARRACE, L., FIEGLER, H., CARR, P., VON ZGLINICKI, T., SARETZKI, G., CARTER, N. P. & JACKSON, S. P. 2003. A DNA damage checkpoint response in telomere-initiated senescence. *Nature,* 426**,** 194-8.

DAHL, M. & HVIID, T. V. 2012. Human leucocyte antigen class Ib molecules in pregnancy success and early pregnancy loss. *Hum Reprod Update,* 18**,** 92-109.

DAVALOS, A. R., COPPE, J. P., CAMPISI, J. & DESPREZ, P. Y. 2010. Senescent cells as a source of inflammatory factors for tumor progression. *Cancer Metastasis Rev,* 29**,** 273-83.

DAVALOS, A. R., KAWAHARA, M., MALHOTRA, G. K., SCHAUM, N., HUANG, J., VED, U., BEAUSEJOUR, C. M., COPPE, J. P., RODIER, F. & CAMPISI, J. 2013. p53-dependent release of Alarmin HMGB1 is a central mediator of senescent phenotypes. *J Cell Biol,* 201**,** 613-29.

DAVIS, T., BAIRD, D. M., HAUGHTON, M. F., JONES, C. J. & KIPLING, D. 2005. Prevention of accelerated cell aging in Werner syndrome using a p38 mitogen-activated protein kinase inhibitor. *J Gerontol A Biol Sci Med Sci,* 60**,** 1386-93.

DAVIS, T. & KIPLING, D. 2009. Assessing the role of stress signalling via p38 MAP kinase in the premature senescence of ataxia telangiectasia and Werner syndrome fibroblasts. *Biogerontology,* 10**,** 253-66.

DE KRUIJF, E. M., SAJET, A., VAN NES, J. G., NATANOV, R., PUTTER, H., SMIT, V. T., LIEFERS, G. J., VAN DEN ELSEN, P. J., VAN DE VELDE, C. J. & KUPPEN, P. J. 2010. HLA-E and HLA-G expression in classical HLA class I-negative tumors is of prognostic value for clinical outcome of early breast cancer patients. *J Immunol,* 185**,** 7452-9.

DE LANGE, T. 2009. How telomeres solve the end-protection problem. *Science,* 326**,** 948-52.

DE MAGALHAES, J. P., CURADO, J. & CHURCH, G. M. 2009. Meta-analysis of age-related gene expression profiles identifies common signatures of aging. *Bioinformatics,* 25**,** 875-81.

DEGREGORI, J. 2011. Evolved tumor suppression: why are we so good at not getting cancer? *Cancer Res,* 71**,** 3739-44.

DEMARIA, M., OHTANI, N., YOUSSEF, S. A., RODIER, F., TOUSSAINT, W., MITCHELL, J. R., LABERGE, R. M., VIJG, J., VAN STEEG, H., DOLLE, M. E., HOEIJMAKERS, J. H., DE BRUIN, A., HARA, E. & CAMPISI, J. 2014. An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. *Dev Cell,* 31**,** 722-33.

DERRE, L., CORVAISIER, M., CHARREAU, B., MOREAU, A., GODEFROY, E., MOREAU-AUBRY, A., JOTEREAU, F. & GERVOIS, N. 2006. Expression and release of HLA-E by melanoma cells and melanocytes: potential impact on the response of cytotoxic effector cells. *J Immunol,* 177**,** 3100-7.

DERRE, L., CORVAISIER, M., PANDOLFINO, M. C., DIEZ, E., JOTEREAU, F. & GERVOIS, N. 2002. Expression of CD94/NKG2-A on human T lymphocytes is induced by IL-12: implications for adoptive immunotherapy. *J Immunol,* 168**,** 4864-70.

DI MITRI, D., AZEVEDO, R. I., HENSON, S. M., LIBRI, V., RIDDELL, N. E., MACAULAY, R., KIPLING, D., SOARES, M. V., BATTISTINI, L. & AKBAR, A. N. 2011. Reversible senescence in human CD4+CD45RA+CD27- memory T cells. *J Immunol,* 187**,** 2093-100.

DIEFENBACH, A., JAMIESON, A. M., LIU, S. D., SHASTRI, N. & RAULET, D. H. 2000. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat Immunol,* 1**,** 119-26.

DIEFENBACH, A., JENSEN, E. R., JAMIESON, A. M. & RAULET, D. H. 2001. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature,* 413**,** 165-71.

DIEFENBACH, A., TOMASELLO, E., LUCAS, M., JAMIESON, A. M., HSIA, J. K., VIVIER, E. & RAULET, D. H. 2002. Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. *Nat Immunol,* 3**,** 1142-9.

DIMRI, G. P., LEE, X., BASILE, G., ACOSTA, M., SCOTT, G., ROSKELLEY, C., MEDRANO, E. E., LINSKENS, M., RUBELJ, I., PEREIRA-SMITH, O. & ET AL. 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A,* 92**,** 9363-7.

DOMINGUEZ, C. X., AMEZQUITA, R. A., GUAN, T., MARSHALL, H. D., JOSHI, N. S., KLEINSTEIN, S. H. & KAECH, S. M. 2015. The transcription factors ZEB2

and T-bet cooperate to program cytotoxic T cell terminal differentiation in response to LCMV viral infection. *J Exp Med,* 212**,** 2041-56.

DUMITRIU, I. E., BARUAH, P., VALENTINIS, B., VOLL, R. E., HERRMANN, M., NAWROTH, P. P., ARNOLD, B., BIANCHI, M. E., MANFREDI, A. A. & ROVERE-QUERINI, P. 2005. Release of high mobility group box 1 by dendritic cells controls T cell activation via the receptor for advanced glycation end products. *J Immunol,* 174**,** 7506-15.

DUNN, G. P., BRUCE, A. T., IKEDA, H., OLD, L. J. & SCHREIBER, R. D. 2002. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol,* 3**,** 991-8.

DUNNE, P. J., BELARAMANI, L., FLETCHER, J. M., FERNANDEZ DE MATTOS, S., LAWRENZ, M., SOARES, M. V., RUSTIN, M. H., LAM, E. W., SALMON, M. & AKBAR, A. N. 2005. Quiescence and functional reprogramming of Epstein-Barr virus (EBV)-specific CD8+ T cells during persistent infection. *Blood,* 106**,** 558-65.

DUSSEAUX, M., MARTIN, E., SERRIARI, N., PEGUILLET, I., PREMEL, V., LOUIS, D., MILDER, M., LE BOURHIS, L., SOUDAIS, C., TREINER, E. & LANTZ, O. 2011. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. *Blood,* 117**,** 1250-9.

EFFROS, R. B. 1997. Loss of CD28 expression on T lymphocytes: a marker of replicative senescence. *Dev Comp Immunol,* 21**,** 471-8.

EFIMOVA, E. V., MAUCERI, H. J., GOLDEN, D. W., LABAY, E., BINDOKAS, V. P., DARGA, T. E., CHAKRABORTY, C., BARRETO-ANDRADE, J. C., CRAWLEY, C., SUTTON, H. G., KRON, S. J. & WEICHSELBAUM, R. R. 2010. Poly(ADP-ribose) polymerase inhibitor induces accelerated senescence in irradiated breast cancer cells and tumors. *Cancer Res,* 70**,** 6277-82.

ERICKSON, S., SANGFELT, O., HEYMAN, M., CASTRO, J., EINHORN, S. & GRANDER, D. 1998. Involvement of the Ink4 proteins p16 and p15 in T-lymphocyte senescence. *Oncogene,* 17**,** 595-602.

ERUSALIMSKY, J. D. & KURZ, D. J. 2005. Cellular senescence in vivo: its relevance in ageing and cardiovascular disease. *Exp Gerontol,* 40**,** 634-42.

FIFE, B. T., PAUKEN, K. E., EAGAR, T. N., OBU, T., WU, J., TANG, Q., AZUMA, M., KRUMMEL, M. F. & BLUESTONE, J. A. 2009. Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal. *Nat Immunol,* 10**,** 1185-92.

FLETCHER, J. M., VUKMANOVIC-STEJIC, M., DUNNE, P. J., BIRCH, K. E., COOK, J. E., JACKSON, S. E., SALMON, M., RUSTIN, M. H. & AKBAR, A. N. 2005. Cytomegalovirus-specific CD4+ T cells in healthy carriers are continuously driven to replicative exhaustion. *J Immunol,* 175**,** 8218-25.

FRANCESCHI, C., BONAFE, M., VALENSIN, S., OLIVIERI, F., DE LUCA, M., OTTAVIANI, E. & DE BENEDICTIS, G. 2000. Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann N Y Acad Sci,* 908**,** 244-54.

FRANCESCHI, C. & CAMPISI, J. 2014. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. *J Gerontol A Biol Sci Med Sci,* 69 Suppl 1**,** S4-9.

FREUND, A., PATIL, C. K. & CAMPISI, J. 2011. p38MAPK is a novel DNA damage response-independent regulator of the senescence-associated secretory phenotype. *EMBO J,* 30**,** 1536-48.

FULOP, T., LARBI, A. & PAWELEC, G. 2013. Human T cell aging and the impact of persistent viral infections. *Front Immunol,* 4**,** 271.

FUMAGALLI, M., ROSSIELLO, F., CLERICI, M., BAROZZI, S., CITTARO, D., KAPLUNOV, J. M., BUCCI, G., DOBREVA, M., MATTI, V., BEAUSEJOUR, C. M., HERBIG, U., LONGHESE, M. P. & D'ADDA DI FAGAGNA, F. 2012. Telomeric DNA damage is irreparable and causes persistent DNA-damage-response activation. *Nat Cell Biol,* 14**,** 355-65.

GAMADIA, L. E., RENTENAAR, R. J., BAARS, P. A., REMMERSWAAL, E. B., SURACHNO, S., WEEL, J. F., TOEBES, M., SCHUMACHER, T. N., TEN BERGE, I. J. & VAN LIER, R. A. 2001. Differentiation of cytomegalovirus-specific CD8(+) T cells in healthy and immunosuppressed virus carriers. *Blood,* 98**,** 754-61.

GARCIA, K. C., DEGANO, M., STANFIELD, R. L., BRUNMARK, A., JACKSON, M. R., PETERSON, P. A., TEYTON, L. & WILSON, I. A. 1996. An alphabeta T cell receptor structure at 2.5 A and its orientation in the TCR-MHC complex. *Science,* 274**,** 209-19.

GASSER, S., ORSULIC, S., BROWN, E. J. & RAULET, D. H. 2005. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature,* 436**,** 1186-90.

GEGINAT, J., LANZAVECCHIA, A. & SALLUSTO, F. 2003. Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood,* 101**,** 4260-6.

GILFILLAN, S., HO, E. L., CELLA, M., YOKOYAMA, W. M. & COLONNA, M. 2002. NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. *Nat Immunol,* 3**,** 1150-5.

GLADE, M. J. & MEGUID, M. M. 2015. A glance at ... telomeres, oxidative stress, antioxidants, and biological aging. *Nutrition,* 31**,** 1447-51.

GOBIN, S. J. & VAN DEN ELSEN, P. J. 2000. Transcriptional regulation of the MHC class Ib genes HLA-E, HLA-F, and HLA-G. *Hum Immunol,* 61**,** 1102-7.

GOLDSTEIN, S. 1990. Replicative senescence: the human fibroblast comes of age. *Science,* 249**,** 1129-33.

GOMES, N. M., RYDER, O. A., HOUCK, M. L., CHARTER, S. J., WALKER, W., FORSYTH, N. R., AUSTAD, S. N., VENDITTI, C., PAGEL, M., SHAY, J. W. & WRIGHT, W. E. 2011. Comparative biology of mammalian telomeres: hypotheses on ancestral states and the roles of telomeres in longevity determination. *Aging Cell,* 10**,** 761-8.

GORONZY, J. J., LI, G., YU, M. & WEYAND, C. M. 2012. Signaling pathways in aged T cells - a reflection of T cell differentiation, cell senescence and host environment. *Semin Immunol,* 24**,** 365-72.

GRAY-SCHOPFER, V. C., CHEONG, S. C., CHONG, H., CHOW, J., MOSS, T., ABDEL-MALEK, Z. A., MARAIS, R., WYNFORD-THOMAS, D. & BENNETT, D. C. 2006. Cellular senescence in naevi and immortalisation in melanoma: a role for p16? *Br J Cancer,* 95**,** 496-505.

GREIDER, C. W. & BLACKBURN, E. H. 1985. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell,* 43**,** 405-13.

GRIFFITHS, S. J., RIDDELL, N. E., MASTERS, J., LIBRI, V., HENSON, S. M., WERTHEIMER, A., WALLACE, D., SIMS, S., RIVINO, L., LARBI, A., KEMENY, D. M., NIKOLICH-ZUGICH, J., KERN, F., KLENERMAN, P.,

EMERY, V. C. & AKBAR, A. N. 2013. Age-associated increase of low-avidity cytomegalovirus-specific CD8+ T cells that re-express CD45RA. *J Immunol,* 190**,** 5363-72.

GROH, V., RHINEHART, R., RANDOLPH-HABECKER, J., TOPP, M. S., RIDDELL, S. R. & SPIES, T. 2001. Costimulation of CD8alphabeta T cells by NKG2D via engagement by MIC induced on virus-infected cells. *Nat Immunol,* 2**,** 255-60.

GROH, V., WU, J., YEE, C. & SPIES, T. 2002. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature,* 419**,** 734-8.

GUMA, M., ANGULO, A., VILCHES, C., GOMEZ-LOZANO, N., MALATS, N. & LOPEZ-BOTET, M. 2004. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood,* 104**,** 3664-71.

HALENIUS, A., GERKE, C. & HENGEL, H. 2015. Classical and non-classical MHC I molecule manipulation by human cytomegalovirus: so many targets-but how many arrows in the quiver? *Cell Mol Immunol,* 12**,** 139-53.

HANAHAN, D. & WEINBERG, R. A. 2011. Hallmarks of cancer: the next generation. *Cell,* 144**,** 646-74.

HARLEY, C. B., FUTCHER, A. B. & GREIDER, C. W. 1990. Telomeres shorten during ageing of human fibroblasts. *Nature,* 345**,** 458-60.

HASTIE, N. D., DEMPSTER, M., DUNLOP, M. G., THOMPSON, A. M., GREEN, D. K. & ALLSHIRE, R. C. 1990. Telomere reduction in human colorectal carcinoma and with ageing. *Nature,* 346**,** 866-8.

HAUCK, F., BLUMENTHAL, B., FUCHS, S., LENOIR, C., MARTIN, E., SPECKMANN, C., VRAETZ, T., MANNHARDT-LAAKMANN, W., LAMBERT, N., GIL, M., BORTE, S., AUDRAIN, M., SCHWARZ, K., LIM, A., SCHAMEL, W. W., FISCHER, A., EHL, S., RENSING-EHL, A., PICARD, C. & LATOUR, S. 2015. SYK expression endows human ZAP70-deficient CD8 T cells with residual TCR signaling. *Clin Immunol,* 161**,** 103-9.

HAYFLICK, L. 1965. The Limited in Vitro Lifetime of Human Diploid Cell Strains. *Exp Cell Res,* 37**,** 614-36.

HAYFLICK, L. & MOORHEAD, P. S. 1961. The serial cultivation of human diploid cell strains. *Exp Cell Res,* 25**,** 585-621.

HENNECKE, J. & WILEY, D. C. 2001. T cell receptor-MHC interactions up close. *Cell,* 104**,** 1-4.

HENRIQUES, C. M., CARNEIRO, M. C., TENENTE, I. M., JACINTO, A. & FERREIRA, M. G. 2013. Telomerase is required for zebrafish lifespan. *PLoS Genet,* 9**,** e1003214.

HENSON, S. M., FRANZESE, O., MACAULAY, R., LIBRI, V., AZEVEDO, R. I., KIANI-ALIKHAN, S., PLUNKETT, F. J., MASTERS, J. E., JACKSON, S., GRIFFITHS, S. J., PIRCHER, H. P., SOARES, M. V. & AKBAR, A. N. 2009. KLRG1 signaling induces defective Akt (ser473) phosphorylation and proliferative dysfunction of highly differentiated CD8+ T cells. *Blood,* 113**,** 6619-28.

HENSON, S. M., LANNA, A., RIDDELL, N. E., FRANZESE, O., MACAULAY, R., GRIFFITHS, S. J., PULESTON, D. J., WATSON, A. S., SIMON, A. K., TOOZE, S. A. & AKBAR, A. N. 2014. p38 signaling inhibits mTORC1-independent autophagy in senescent human CD8(+) T cells. *J Clin Invest,* 124**,** 4004-16.

HENSON, S. M., RIDDELL, N. E. & AKBAR, A. N. 2012. Properties of end-stage human T cells defined by CD45RA re-expression. *Curr Opin Immunol,* 24**,** 476-81.

HERBIG, U., FERREIRA, M., CONDEL, L., CAREY, D. & SEDIVY, J. M. 2006. Cellular senescence in aging primates. *Science,* 311**,** 1257.

HERBIG, U., JOBLING, W. A., CHEN, B. P., CHEN, D. J. & SEDIVY, J. M. 2004. Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a). *Mol Cell,* 14**,** 501-13.

HERRANZ, N., GALLAGE, S., MELLONE, M., WUESTEFELD, T., KLOTZ, S., HANLEY, C. J., RAGUZ, S., ACOSTA, J. C., INNES, A. J., BANITO, A., GEORGILIS, A., MONTOYA, A., WOLTER, K., DHARMALINGAM, G., FAULL, P., CARROLL, T., MARTINEZ-BARBERA, J. P., CUTILLAS, P., REISINGER, F., HEIKENWALDER, M., MILLER, R. A., WITHERS, D., ZENDER, L., THOMAS, G. J. & GIL, J. 2015. mTOR regulates MAPKAPK2 translation to control the senescence-associated secretory phenotype. *Nat Cell Biol,* 17**,** 1205-17.

HEWITT, G., JURK, D., MARQUES, F. D., CORREIA-MELO, C., HARDY, T., GACKOWSKA, A., ANDERSON, R., TASCHUK, M., MANN, J. & PASSOS, J. F. 2012. Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence. *Nat Commun,* 3**,** 708.

HICKSON, I., ZHAO, Y., RICHARDSON, C. J., GREEN, S. J., MARTIN, N. M., ORR, A. I., REAPER, P. M., JACKSON, S. P., CURTIN, N. J. & SMITH, G. C. 2004. Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. *Cancer Res,* 64**,** 9152-9.

HODES, R. J., HATHCOCK, K. S. & WENG, N. P. 2002. Telomeres in T and B cells. *Nat Rev Immunol,* 2**,** 699-706.

HOENICKE, L. & ZENDER, L. 2012. Immune surveillance of senescent cells--biological significance in cancer- and non-cancer pathologies. *Carcinogenesis,* 33**,** 1123-6.

HOROWITZ, A., STRAUSS-ALBEE, D. M., LEIPOLD, M., KUBO, J., NEMAT-GORGANI, N., DOGAN, O. C., DEKKER, C. L., MACKEY, S., MAECKER, H., SWAN, G. E., DAVIS, M. M., NORMAN, P. J., GUETHLEIN, L. A., DESAI, M., PARHAM, P. & BLISH, C. A. 2013. Genetic and environmental determinants of human NK cell diversity revealed by mass cytometry. *Sci Transl Med,* 5**,** 208ra145.

HUNTINGTON, N. D., TABARIAS, H., FAIRFAX, K., BRADY, J., HAYAKAWA, Y., DEGLI-ESPOSTI, M. A., SMYTH, M. J., TARLINTON, D. M. & NUTT, S. L. 2007. NK cell maturation and peripheral homeostasis is associated with KLRG1 up-regulation. *J Immunol,* 178**,** 4764-70.

IANNELLO, A., THOMPSON, T. W., ARDOLINO, M., LOWE, S. W. & RAULET, D. H. 2013. p53-dependent chemokine production by senescent tumor cells supports NKG2D-dependent tumor elimination by natural killer cells. *J Exp Med,* 210**,** 2057-69.

ISHITANI, A., SAGESHIMA, N., LEE, N., DOROFEEVA, N., HATAKE, K., MARQUARDT, H. & GERAGHTY, D. E. 2003. Protein expression and peptide binding suggest unique and interacting functional roles for HLA-E, F, and G in maternal-placental immune recognition. *J Immunol,* 171**,** 1376-84.

IWASA, H., HAN, J. & ISHIKAWA, F. 2003. Mitogen-activated protein kinase p38 defines the common senescence-signalling pathway. *Genes Cells,* 8**,** 131-44.

IWASZKO, M. & BOGUNIA-KUBIK, K. 2011. Clinical significance of the HLA-E and CD94/NKG2 interaction. *Arch Immunol Ther Exp (Warsz),* 59**,** 353-67.

JACKSON, S. P. & BARTEK, J. 2009. The DNA-damage response in human biology and disease. *Nature,* 461**,** 1071-8.

JENKINS, N. C., LIU, T., CASSIDY, P., LEACHMAN, S. A., BOUCHER, K. M., GOODSON, A. G., SAMADASHWILY, G. & GROSSMAN, D. 2011. The p16(INK4A) tumor suppressor regulates cellular oxidative stress. *Oncogene,* 30**,** 265-74.

JEROME, K. R., SLOAN, D. D. & AUBERT, M. 2003. Measurement of CTL-induced cytotoxicity: the caspase 3 assay. *Apoptosis,* 8**,** 563-71.

JING, Y., GRAVENSTEIN, S., CHAGANTY, N. R., CHEN, N., LYERLY, K. H., JOYCE, S. & DENG, Y. 2007. Aging is associated with a rapid decline in frequency, alterations in subset composition, and enhanced Th2 response in CD1d-restricted NKT cells from human peripheral blood. *Exp Gerontol,* 42**,** 719-32.

JOOSTEN, S. A., SULLIVAN, L. C. & OTTENHOFF, T. H. 2016. Characteristics of HLA-E Restricted T-Cell Responses and Their Role in Infectious Diseases. *J Immunol Res,* 2016**,** 2695396.

JOSHI, N. S., CUI, W., CHANDELE, A., LEE, H. K., URSO, D. R., HAGMAN, J., GAPIN, L. & KAECH, S. M. 2007. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity,* 27**,** 281-95.

JU, G. Z., WANG, X. M., FU, S. B. & LIU, S. Z. 2003. Effect of ionizing radiation on the expression of p16, cyclinD1 and CDK4 in mouse thymocytes and splenocytes. *Biomed Environ Sci,* 16**,** 47-52.

JUN, J. I. & LAU, L. F. 2010. The matricellular protein CCN1 induces fibroblast senescence and restricts fibrosis in cutaneous wound healing. *Nat Cell Biol,* 12**,** 676-85.

JURK, D., WILSON, C., PASSOS, J. F., OAKLEY, F., CORREIA-MELO, C., GREAVES, L., SARETZKI, G., FOX, C., LAWLESS, C., ANDERSON, R., HEWITT, G., PENDER, S. L., FULLARD, N., NELSON, G., MANN, J., VAN DE SLUIS, B., MANN, D. A. & VON ZGLINICKI, T. 2014. Chronic inflammation induces telomere dysfunction and accelerates ageing in mice. *Nat Commun,* 2**,** 4172.

KALATHUR, M., TOSO, A., CHEN, J., REVANDKAR, A., DANZER-BALTZER, C., GUCCINI, I., ALAJATI, A., SARTI, M., PINTON, S., BRAMBILLA, L., DI MITRI, D., CARBONE, G., GARCIA-ESCUDERO, R., PADOVA, A., MAGNONI, L., TARDITI, A., MACCARI, L., MALUSA, F., KALATHUR, R. K., L, A. P., COZZA, G., RUZZENE, M., DELALEU, N., CATAPANO, C. V., FREW, I. J. & ALIMONTI, A. 2015. A chemogenomic screening identifies CK2 as a target for pro-senescence therapy in PTEN-deficient tumours. *Nat Commun,* 6**,** 7227.

KANG, T. W., YEVSA, T., WOLLER, N., HOENICKE, L., WUESTEFELD, T., DAUCH, D., HOHMEYER, A., GEREKE, M., RUDALSKA, R., POTAPOVA, A., IKEN, M., VUCUR, M., WEISS, S., HEIKENWALDER, M., KHAN, S., GIL, J., BRUDER, D., MANNS, M., SCHIRMACHER, P., TACKE, F., OTT, M., LUEDDE, T., LONGERICH, T., KUBICKA, S. & ZENDER, L. 2011. Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. *Nature,* 479**,** 547-51.

KARIMI, M., CAO, T. M., BAKER, J. A., VERNERIS, M. R., SOARES, L. & NEGRIN, R. S. 2005. Silencing human NKG2D, DAP10, and DAP12 reduces cytotoxicity of activated CD8+ T cells and NK cells. *J Immunol,* 175**,** 7819-28.

KARIMI, M. A., BRYSON, J. L., RICHMAN, L. P., FESNAK, A. D., LEICHNER, T. M., SATAKE, A., VONDERHEIDE, R. H., RAULET, D. H., RESHEF, R. & KAMBAYASHI, T. 2015. NKG2D expression by CD8+ T cells contributes to GVHD and GVT effects in a murine model of allogeneic HSCT. *Blood,* 125**,** 3655-63.

KHAN, N., SHARIFF, N., COBBOLD, M., BRUTON, R., AINSWORTH, J. A., SINCLAIR, A. J., NAYAK, L. & MOSS, P. A. 2002. Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. *J Immunol,* 169**,** 1984-92.

KING, A., ALLAN, D. S., BOWEN, M., POWIS, S. J., JOSEPH, S., VERMA, S., HIBY, S. E., MCMICHAEL, A. J., LOKE, Y. W. & BRAUD, V. M. 2000. HLA-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells. *Eur J Immunol,* 30**,** 1623-31.

KOCH, S., LARBI, A., DERHOVANESSIAN, E., OZCELIK, D., NAUMOVA, E. & PAWELEC, G. 2008. Multiparameter flow cytometric analysis of CD4 and CD8 T cell subsets in young and old people. *Immun Ageing,* 5**,** 6.

KOCHAN, G., ESCORS, D., BRECKPOT, K. & GUERRERO-SETAS, D. 2013. Role of non-classical MHC class I molecules in cancer immunosuppression. *Oncoimmunology,* 2**,** e26491.

KOKKOLA, R., ANDERSSON, A., MULLINS, G., OSTBERG, T., TREUTIGER, C. J., ARNOLD, B., NAWROTH, P., ANDERSSON, U., HARRIS, R. A. & HARRIS, H. E. 2005. RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages. *Scand J Immunol,* 61**,** 1-9.

KOVALOVSKY, D., ALONZO, E. S., UCHE, O. U., EIDSON, M., NICHOLS, K. E. & SANT'ANGELO, D. B. 2010. PLZF induces the spontaneous acquisition of memory/effector functions in T cells independently of NKT cell-related signals. *J Immunol,* 184**,** 6746-55.

KOVALOVSKY, D., UCHE, O. U., ELADAD, S., HOBBS, R. M., YI, W., ALONZO, E., CHUA, K., EIDSON, M., KIM, H. J., IM, J. S., PANDOLFI, P. P. & SANT'ANGELO, D. B. 2008. The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nat Immunol,* 9**,** 1055-64.

KRISHNAMURTHY, J., RAMSEY, M. R., LIGON, K. L., TORRICE, C., KOH, A., BONNER-WEIR, S. & SHARPLESS, N. E. 2006. p16INK4a induces an age-dependent decline in islet regenerative potential. *Nature,* 443**,** 453-7.

KRISHNAMURTHY, J., TORRICE, C., RAMSEY, M. R., KOVALEV, G. I., AL-REGAIEY, K., SU, L. & SHARPLESS, N. E. 2004. Ink4a/Arf expression is a biomarker of aging. *J Clin Invest,* 114**,** 1299-307.

KRIZHANOVSKY, V., YON, M., DICKINS, R. A., HEARN, S., SIMON, J., MIETHING, C., YEE, H., ZENDER, L. & LOWE, S. W. 2008. Senescence of activated stellate cells limits liver fibrosis. *Cell,* 134**,** 657-67.

KRTOLICA, A., PARRINELLO, S., LOCKETT, S., DESPREZ, P. Y. & CAMPISI, J. 2001. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc Natl Acad Sci U S A,* 98**,** 12072-7.

KUILMAN, T., MICHALOGLOU, C., VREDEVELD, L. C., DOUMA, S., VAN DOORN, R., DESMET, C. J., AARDEN, L. A., MOOI, W. J. & PEEPER, D. S. 2008.

Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell,* 133**,** 1019-31.

KUILMAN, T. & PEEPER, D. S. 2009. Senescence-messaging secretome: SMS-ing cellular stress. *Nat Rev Cancer,* 9**,** 81-94.

KUMARI, N., DWARAKANATH, B. S., DAS, A. & BHATT, A. N. 2016. Role of interleukin-6 in cancer progression and therapeutic resistance. *Tumour Biol,* 37**,** 11553-11572.

KURANDA, K., VARGAFTIG, J., DE LA ROCHERE, P., DOSQUET, C., CHARRON, D., BARDIN, F., TONNELLE, C., BONNET, D. & GOODHARDT, M. 2011. Age-related changes in human hematopoietic stem/progenitor cells. *Aging Cell,* 10**,** 542-6.

KURZ, D. J., DECARY, S., HONG, Y. & ERUSALIMSKY, J. D. 2000. Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J Cell Sci,* 113 (Pt 20)**,** 3613-22.

LABERGE, R. M., AWAD, P., CAMPISI, J. & DESPREZ, P. Y. 2012. Epithelial-mesenchymal transition induced by senescent fibroblasts. *Cancer Microenviron,* 5**,** 39-44.

LABERGE, R. M., SUN, Y., ORJALO, A. V., PATIL, C. K., FREUND, A., ZHOU, L., CURRAN, S. C., DAVALOS, A. R., WILSON-EDELL, K. A., LIU, S., LIMBAD, C., DEMARIA, M., LI, P., HUBBARD, G. B., IKENO, Y., JAVORS, M., DESPREZ, P. Y., BENZ, C. C., KAPAHI, P., NELSON, P. S. & CAMPISI, J. 2015. MTOR regulates the pro-tumorigenic senescence-associated secretory phenotype by promoting IL1A translation. *Nat Cell Biol,* 17**,** 1049-61.

LACHMANN, R., BAJWA, M., VITA, S., SMITH, H., CHEEK, E., AKBAR, A. & KERN, F. 2012. Polyfunctional T cells accumulate in large human cytomegalovirus-specific T cell responses. *J Virol,* 86**,** 1001-9.

LACKNER, D. H., HAYASHI, M. T., CESARE, A. J. & KARLSEDER, J. 2014. A genomics approach identifies senescence-specific gene expression regulation. *Aging Cell,* 13**,** 946-50.

LANE, D. P. 1992. Cancer. p53, guardian of the genome. *Nature,* 358**,** 15-6.

LANIER, L. L. 2005. NK cell recognition. *Annu Rev Immunol,* 23**,** 225-74.

LANIER, L. L. 2009. DAP10- and DAP12-associated receptors in innate immunity. *Immunol Rev,* 227**,** 150-60.

LANIER, L. L. 2015. NKG2D Receptor and Its Ligands in Host Defense. *Cancer Immunol Res,* 3**,** 575-82.

LANIER, L. L., CORLISS, B., WU, J. & PHILLIPS, J. H. 1998a. Association of DAP12 with activating CD94/NKG2C NK cell receptors. *Immunity,* 8**,** 693-701.

LANIER, L. L., CORLISS, B. C., WU, J., LEONG, C. & PHILLIPS, J. H. 1998b. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature,* 391**,** 703-7.

LANNA, A., COUTAVAS, E., LEVATI, L., SEIDEL, J., RUSTIN, M. H., HENSON, S. M., AKBAR, A. N. & FRANZESE, O. 2013. IFN-alpha inhibits telomerase in human CD8(+) T cells by both hTERT downregulation and induction of p38 MAPK signaling. *J Immunol,* 191**,** 3744-52.

LANNA, A., HENSON, S. M., ESCORS, D. & AKBAR, A. N. 2014. The kinase p38 activated by the metabolic regulator AMPK and scaffold TAB1 drives the senescence of human T cells. *Nat Immunol,* 15**,** 965-72.

LARBI, A., DOUZIECH, N., DUPUIS, G., KHALIL, A., PELLETIER, H., GUERARD, K. P. & FULOP, T., JR. 2004. Age-associated alterations in the recruitment of signal-transduction proteins to lipid rafts in human T lymphocytes. *J Leukoc Biol,* 75**,** 373-81.

LE GARFF-TAVERNIER, M., BEZIAT, V., DECOCQ, J., SIGURET, V., GANDJBAKHCH, F., PAUTAS, E., DEBRE, P., MERLE-BERAL, H. & VIEILLARD, V. 2010. Human NK cells display major phenotypic and functional changes over the life span. *Aging Cell,* 9**,** 527-35.

LE SAUX, S., WEYAND, C. M. & GORONZY, J. J. 2012. Mechanisms of immunosenescence: lessons from models of accelerated immune aging. *Ann N Y Acad Sci,* 1247**,** 69-82.

LEE, B. Y., HAN, J. A., IM, J. S., MORRONE, A., JOHUNG, K., GOODWIN, E. C., KLEIJER, W. J., DIMAIO, D. & HWANG, E. S. 2006. Senescence-associated beta-galactosidase is lysosomal beta-galactosidase. *Aging Cell,* 5**,** 187-95.

LEE, N., GOODLETT, D. R., ISHITANI, A., MARQUARDT, H. & GERAGHTY, D. E. 1998. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J Immunol,* 160**,** 4951-60.

LEVINE, A. J. 1997. p53, the cellular gatekeeper for growth and division. *Cell,* 88**,** 323-31.

LEVY, M. Z., ALLSOPP, R. C., FUTCHER, A. B., GREIDER, C. W. & HARLEY, C. B. 1992. Telomere end-replication problem and cell aging. *J Mol Biol,* 225**,** 951-60.

LI, G., YU, M., LEE, W. W., TSANG, M., KRISHNAN, E., WEYAND, C. M. & GORONZY, J. J. 2012a. Decline in miR-181a expression with age impairs T cell receptor sensitivity by increasing DUSP6 activity. *Nat Med,* 18**,** 1518-24.

LI, J., ZHU, H., WANG, S., YE, P., LIU, C., WU, J. & XIA, J. 2012b. Blockade of NKG2D synergized with CTLA4-Ig in promoting long-term graft survival in murine models of cardiac transplantation. *Transplantation,* 93**,** 356-63.

LI, Y., LI, F., JIANG, F., LV, X., ZHANG, R., LU, A. & ZHANG, G. 2016. A Mini-Review for Cancer Immunotherapy: Molecular Understanding of PD-1/PD-L1 Pathway & Translational Blockade of Immune Checkpoints. *Int J Mol Sci,* 17.

LIBRI, V., AZEVEDO, R. I., JACKSON, S. E., DI MITRI, D., LACHMANN, R., FUHRMANN, S., VUKMANOVIC-STEJIC, M., YONG, K., BATTISTINI, L., KERN, F., SOARES, M. V. & AKBAR, A. N. 2011. Cytomegalovirus infection induces the accumulation of short-lived, multifunctional CD4+CD45RA+CD27+ T cells: the potential involvement of interleukin-7 in this process. *Immunology,* 132**,** 326-39.

LINTON, P. J. & DORSHKIND, K. 2004. Age-related changes in lymphocyte development and function. *Nat Immunol,* 5**,** 133-9.

LIU, K., CATALFAMO, M., LI, Y., HENKART, P. A. & WENG, N. P. 2002. IL-15 mimics T cell receptor crosslinking in the induction of cellular proliferation, gene expression, and cytotoxicity in CD8+ memory T cells. *Proc Natl Acad Sci U S A,* 99**,** 6192-7.

LIU, Y., JOHNSON, S. M., FEDORIW, Y., ROGERS, A. B., YUAN, H., KRISHNAMURTHY, J. & SHARPLESS, N. E. 2011. Expression of p16(INK4a) prevents cancer and promotes aging in lymphocytes. *Blood,* 117**,** 3257-67.

LIU, Y., SANOFF, H. K., CHO, H., BURD, C. E., TORRICE, C., IBRAHIM, J. G., THOMAS, N. E. & SHARPLESS, N. E. 2009. Expression of p16(INK4a) in peripheral blood T-cells is a biomarker of human aging. *Aging Cell,* 8**,** 439-48.

LLANO, M., LEE, N., NAVARRO, F., GARCIA, P., ALBAR, J. P., GERAGHTY, D. E. & LOPEZ-BOTET, M. 1998. HLA-E-bound peptides influence recognition by inhibitory and triggering CD94/NKG2 receptors: preferential response to an HLA-G-derived nonamer. *Eur J Immunol,* 28**,** 2854-63.

LONG, E. O., KIM, H. S., LIU, D., PETERSON, M. E. & RAJAGOPALAN, S. 2013. Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annu Rev Immunol,* 31**,** 227-58.

LOPEZ-BOTET, M., LLANO, M., NAVARRO, F. & BELLON, T. 2000. NK cell recognition of non-classical HLA class I molecules. *Semin Immunol,* 12**,** 109-19.

LOPEZ-VERGES, S., MILUSH, J. M., PANDEY, S., YORK, V. A., ARAKAWA-HOYT, J., PIRCHER, H., NORRIS, P. J., NIXON, D. F. & LANIER, L. L. 2010. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. *Blood,* 116**,** 3865-74.

LUJAMBIO, A. 2016. To clear, or not to clear (senescent cells)? That is the question. *Bioessays,* 38 Suppl 1**,** S56-64.

LUJAMBIO, A., AKKARI, L., SIMON, J., GRACE, D., TSCHAHARGANEH, D. F., BOLDEN, J. E., ZHAO, Z., THAPAR, V., JOYCE, J. A., KRIZHANOVSKY, V. & LOWE, S. W. 2013. Non-cell-autonomous tumor suppression by p53. *Cell,* 153**,** 449-60.

LYNCH, H. E., GOLDBERG, G. L., CHIDGEY, A., VAN DEN BRINK, M. R., BOYD, R. & SEMPOWSKI, G. D. 2009. Thymic involution and immune reconstitution. *Trends Immunol,* 30**,** 366-73.

MACCALLI, C., NONAKA, D., PIRIS, A., PENDE, D., RIVOLTINI, L., CASTELLI, C. & PARMIANI, G. 2007. NKG2D-mediated antitumor activity by tumor-infiltrating lymphocytes and antigen-specific T-cell clones isolated from melanoma patients. *Clin Cancer Res,* 13**,** 7459-68.

MAGGI, L., SANTARLASCI, V., CAPONE, M., PEIRED, A., FROSALI, F., CROME, S. Q., QUERCI, V., FAMBRINI, M., LIOTTA, F., LEVINGS, M. K., MAGGI, E., COSMI, L., ROMAGNANI, S. & ANNUNZIATO, F. 2010. CD161 is a marker of all human IL-17-producing T-cell subsets and is induced by RORC. *Eur J Immunol,* 40**,** 2174-81.

MAGGIO, M., GURALNIK, J. M., LONGO, D. L. & FERRUCCI, L. 2006. Interleukin-6 in aging and chronic disease: a magnificent pathway. *J Gerontol A Biol Sci Med Sci,* 61**,** 575-84.

MAHNKE, Y. D., BRODIE, T. M., SALLUSTO, F., ROEDERER, M. & LUGLI, E. 2013. The who's who of T-cell differentiation: human memory T-cell subsets. *Eur J Immunol,* 43**,** 2797-809.

MAHONEY, K. M., RENNERT, P. D. & FREEMAN, G. J. 2015. Combination cancer immunotherapy and new immunomodulatory targets. *Nat Rev Drug Discov,* 14**,** 561-84.

MALISSEN, B., GREGOIRE, C., MALISSEN, M. & RONCAGALLI, R. 2014. Integrative biology of T cell activation. *Nat Immunol,* 15**,** 790-7.

MARIN, R., RUIZ-CABELLO, F., PEDRINACI, S., MENDEZ, R., JIMENEZ, P., GERAGHTY, D. E. & GARRIDO, F. 2003. Analysis of HLA-E expression in human tumors. *Immunogenetics,* 54**,** 767-75.

MATZINGER, P. 2002. The danger model: a renewed sense of self. *Science,* 296**,** 301-5.

MCELHANEY, J. E., ZHOU, X., TALBOT, H. K., SOETHOUT, E., BLEACKLEY, R. C., GRANVILLE, D. J. & PAWELEC, G. 2012. The unmet need in the elderly: how immunosenescence, CMV infection, co-morbidities and frailty are a challenge for the development of more effective influenza vaccines. *Vaccine,* 30**,** 2060-7.

MENIER, C., SAEZ, B., HOREJSI, V., MARTINOZZI, S., KRAWICE-RADANNE, I., BRUEL, S., LE DANFF, C., REBOUL, M., HILGERT, I., RABREAU, M., LARRAD, M. L., PLA, M., CAROSELLA, E. D. & ROUAS-FREISS, N. 2003. Characterization of monoclonal antibodies recognizing HLA-G or HLA-E: new tools to analyze the expression of nonclassical HLA class I molecules. *Hum Immunol,* 64**,** 315-26.

MERESSE, B., CHEN, Z., CISZEWSKI, C., TRETIAKOVA, M., BHAGAT, G., KRAUSZ, T. N., RAULET, D. H., LANIER, L. L., GROH, V., SPIES, T., EBERT, E. C., GREEN, P. H. & JABRI, B. 2004. Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity,* 21**,** 357-66.

MERESSE, B., CURRAN, S. A., CISZEWSKI, C., ORBELYAN, G., SETTY, M., BHAGAT, G., LEE, L., TRETIAKOVA, M., SEMRAD, C., KISTNER, E., WINCHESTER, R. J., BRAUD, V., LANIER, L. L., GERAGHTY, D. E., GREEN, P. H., GUANDALINI, S. & JABRI, B. 2006. Reprogramming of CTLs into natural killer-like cells in celiac disease. *J Exp Med,* 203**,** 1343-55.

MICHAELSSON, J., TEIXEIRA DE MATOS, C., ACHOUR, A., LANIER, L. L., KARRE, K. & SODERSTROM, K. 2002. A signal peptide derived from hsp60 binds HLA-E and interferes with CD94/NKG2A recognition. *J Exp Med,* 196**,** 1403-14.

MICHALOGLOU, C., VREDEVELD, L. C., SOENGAS, M. S., DENOYELLE, C., KUILMAN, T., VAN DER HORST, C. M., MAJOOR, D. M., SHAY, J. W., MOOI, W. J. & PEEPER, D. S. 2005. BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature,* 436**,** 720-4.

MICHIE, C. A., MCLEAN, A., ALCOCK, C. & BEVERLEY, P. C. 1992. Lifespan of human lymphocyte subsets defined by CD45 isoforms. *Nature,* 360**,** 264-5.

MINGUENEAU, M., RONCAGALLI, R., GREGOIRE, C., KISSENPFENNIG, A., MIAZEK, A., ARCHAMBAUD, C., WANG, Y., PERRIN, P., BERTOSIO, E., SANSONI, A., RICHELME, S., LOCKSLEY, R. M., AGUADO, E., MALISSEN, M. & MALISSEN, B. 2009. Loss of the LAT adaptor converts antigen-responsive T cells into pathogenic effectors that function independently of the T cell receptor. *Immunity,* 31**,** 197-208.

MIYAJI, C., WATANABE, H., MINAGAWA, M., TOMA, H., KAWAMURA, T., NOHARA, Y., NOZAKI, H., SATO, Y. & ABO, T. 1997. Numerical and functional characteristics of lymphocyte subsets in centenarians. *J Clin Immunol,* 17**,** 420-9.

MOCSAI, A., RULAND, J. & TYBULEWICZ, V. L. 2010. The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat Rev Immunol,* 10**,** 387-402.

MOISEEVA, O., MALLETTE, F. A., MUKHOPADHYAY, U. K., MOORES, A. & FERBEYRE, G. 2006. DNA damage signaling and p53-dependent senescence after prolonged beta-interferon stimulation. *Mol Biol Cell,* 17**,** 1583-92.

MOLINERO, L. L., FUERTES, M. B., GIRART, M. V., FAINBOIM, L., RABINOVICH, G. A., COSTAS, M. A. & ZWIRNER, N. W. 2004. NF-kappa B regulates expression of the MHC class I-related chain A gene in activated T lymphocytes. *J Immunol,* 173**,** 5583-90.

MONDAL, A. M., HORIKAWA, I., PINE, S. R., FUJITA, K., MORGAN, K. M., VERA, E., MAZUR, S. J., APPELLA, E., VOJTESEK, B., BLASCO, M. A., LANE, D. P. & HARRIS, C. C. 2013. p53 isoforms regulate aging- and tumor-associated replicative senescence in T lymphocytes. *J Clin Invest,* 123**,** 5247-57.

MONTEIRO, J., BATLIWALLA, F., OSTRER, H. & GREGERSEN, P. K. 1996. Shortened telomeres in clonally expanded CD28-CD8+ T cells imply a replicative history that is distinct from their CD28+CD8+ counterparts. *J Immunol,* 156**,** 3587-90.

MOOI, W. J. & PEEPER, D. S. 2006. Oncogene-induced cell senescence--halting on the road to cancer. *N Engl J Med,* 355**,** 1037-46.

MORANDI, F. & PISTOIA, V. 2014. Interactions between HLA-G and HLA-E in Physiological and Pathological Conditions. *Front Immunol,* 5**,** 394.

MOYZIS, R. K., BUCKINGHAM, J. M., CRAM, L. S., DANI, M., DEAVEN, L. L., JONES, M. D., MEYNE, J., RATLIFF, R. L. & WU, J. R. 1988. A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. *Proc Natl Acad Sci U S A,* 85**,** 6622-6.

MULLER-DUROVIC, B., LANNA, A., POLACO COVRE, L., MILLS, R. S., HENSON, S. M. & AKBAR, A. N. 2016. Killer Cell Lectin-like Receptor G1 Inhibits NK Cell Function through Activation of Adenosine 5'-Monophosphate-Activated Protein Kinase. *J Immunol*.

MUNTASELL, A., PUPULEKU, A., CISNEROS, E., VERA, A., MORARU, M., VILCHES, C. & LOPEZ-BOTET, M. 2016. Relationship of NKG2C Copy Number with the Distribution of Distinct Cytomegalovirus-Induced Adaptive NK Cell Subsets. *J Immunol,* 196**,** 3818-27.

MURNANE, J. P. 2012. Telomere dysfunction and chromosome instability. *Mutat Res,* 730**,** 28-36.

NARDELLA, C., CLOHESSY, J. G., ALIMONTI, A. & PANDOLFI, P. P. 2011. Pro-senescence therapy for cancer treatment. *Nat Rev Cancer,* 11**,** 503-11.

NAUSCH, N. & CERWENKA, A. 2008. NKG2D ligands in tumor immunity. *Oncogene,* 27**,** 5944-58.

NAYLOR, R. M., BAKER, D. J. & VAN DEURSEN, J. M. 2013. Senescent cells: a novel therapeutic target for aging and age-related diseases. *Clin Pharmacol Ther,* 93**,** 105-16.

NEEFJES, J., JONGSMA, M. L., PAUL, P. & BAKKE, O. 2011. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol,* 11**,** 823-36.

NEEFJES, J. & OVAA, H. 2013. A peptide's perspective on antigen presentation to the immune system. *Nat Chem Biol,* 9**,** 769-75.

NELSON, G., WORDSWORTH, J., WANG, C., JURK, D., LAWLESS, C., MARTIN-RUIZ, C. & VON ZGLINICKI, T. 2012. A senescent cell bystander effect: senescence-induced senescence. *Aging Cell,* 11**,** 345-9.

NICE, T. J., COSCOY, L. & RAULET, D. H. 2009. Posttranslational regulation of the NKG2D ligand Mult1 in response to cell stress. *J Exp Med,* 206**,** 287-98.

O'SULLIVAN, R. J. & KARLSEDER, J. 2010. Telomeres: protecting chromosomes against genome instability. *Nat Rev Mol Cell Biol,* 11**,** 171-81.

OGASAWARA, K., HAMERMAN, J. A., EHRLICH, L. R., BOUR-JORDAN, H., SANTAMARIA, P., BLUESTONE, J. A. & LANIER, L. L. 2004. NKG2D blockade prevents autoimmune diabetes in NOD mice. *Immunity,* 20**,** 757-67.

OMILUSIK, K. D., BEST, J. A., YU, B., GOOSSENS, S., WEIDEMANN, A., NGUYEN, J. V., SEUNTJENS, E., STRYJEWSKA, A., ZWEIER, C., ROYCHOUDHURI, R., GATTINONI, L., BIRD, L. M., HIGASHI, Y., KONDOH, H., HUYLEBROECK, D., HAIGH, J. & GOLDRATH, A. W. 2015. Transcriptional repressor ZEB2 promotes terminal differentiation of CD8+ effector and memory T cell populations during infection. *J Exp Med,* 212**,** 2027-39.

OUYANG, Q., WAGNER, W. M., VOEHRINGER, D., WIKBY, A., KLATT, T., WALTER, S., MULLER, C. A., PIRCHER, H. & PAWELEC, G. 2003. Age-associated accumulation of CMV-specific CD8+ T cells expressing the inhibitory killer cell lectin-like receptor G1 (KLRG1). *Exp Gerontol,* 38**,** 911-20.

PANDA, A., ARJONA, A., SAPEY, E., BAI, F., FIKRIG, E., MONTGOMERY, R. R., LORD, J. M. & SHAW, A. C. 2009. Human innate immunosenescence: causes and consequences for immunity in old age. *Trends Immunol,* 30**,** 325-33.

PARDOLL, D. M. 2012. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer,* 12**,** 252-64.

PARHAM, P. & MOFFETT, A. 2013. Variable NK cell receptors and their MHC class I ligands in immunity, reproduction and human evolution. *Nat Rev Immunol,* 13**,** 133-44.

PARK, J. S., SVETKAUSKAITE, D., HE, Q., KIM, J. Y., STRASSHEIM, D., ISHIZAKA, A. & ABRAHAM, E. 2004. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *J Biol Chem,* 279**,** 7370-7.

PARRINELLO, S., COPPE, J. P., KRTOLICA, A. & CAMPISI, J. 2005. Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation. *J Cell Sci,* 118**,** 485-96.

PASSOS, J. F. & VON ZGLINICKI, T. 2006. Oxygen free radicals in cell senescence: are they signal transducers? *Free Radic Res,* 40**,** 1277-83.

PAUL, W. E. 2011. Bridging innate and adaptive immunity. *Cell,* 147**,** 1212-5.

PAWELEC, G. & GOUTTEFANGEAS, C. 2006. T-cell dysregulation caused by chronic antigenic stress: the role of CMV in immunosenescence? *Aging Clin Exp Res,* 18**,** 171-3.

PEGRAM, H. J., ANDREWS, D. M., SMYTH, M. J., DARCY, P. K. & KERSHAW, M. H. 2011. Activating and inhibitory receptors of natural killer cells. *Immunol Cell Biol,* 89**,** 216-24.

PERALBO, E., DELAROSA, O., GAYOSO, I., PITA, M. L., TARAZONA, R. & SOLANA, R. 2006. Decreased frequency and proliferative response of

invariant Valpha24Vbeta11 natural killer T (iNKT) cells in healthy elderly. *Biogerontology,* 7**,** 483-92.

PEREIRA, B. I. & AKBAR, A. N. 2016. Convergence of Innate and Adaptive Immunity during Human Aging. *Front Immunol,* 7**,** 445.

PETRIE, E. J., CLEMENTS, C. S., LIN, J., SULLIVAN, L. C., JOHNSON, D., HUYTON, T., HEROUX, A., HOARE, H. L., BEDDOE, T., REID, H. H., WILCE, M. C., BROOKS, A. G. & ROSSJOHN, J. 2008. CD94-NKG2A recognition of human leukocyte antigen (HLA)-E bound to an HLA class I leader sequence. *J Exp Med,* 205**,** 725-35.

PIEPKORN, M. 2000. The expression of p16(INK4a), the product of a tumor suppressor gene for melanoma, is upregulated in human melanocytes by UVB irradiation. *J Am Acad Dermatol,* 42**,** 741-5.

PIETRA, G., ROMAGNANI, C., MANZINI, C., MORETTA, L. & MINGARI, M. C. 2010. The emerging role of HLA-E-restricted CD8+ T lymphocytes in the adaptive immune response to pathogens and tumors. *J Biomed Biotechnol,* 2010**,** 907092.

PIEVANI, A., BORLERI, G., PENDE, D., MORETTA, L., RAMBALDI, A., GOLAY, J. & INTRONA, M. 2011. Dual-functional capability of CD3+CD56+ CIK cells, a T-cell subset that acquires NK function and retains TCR-mediated specific cytotoxicity. *Blood,* 118**,** 3301-10.

PITTET, M. J., SPEISER, D. E., VALMORI, D., CEROTTINI, J. C. & ROMERO, P. 2000. Cutting edge: cytolytic effector function in human circulating CD8+ T cells closely correlates with CD56 surface expression. *J Immunol,* 164**,** 1148-52.

PLOEGH, H. L. 1998. Viral strategies of immune evasion. *Science,* 280**,** 248-53.

PLUNKETT, F. J., FRANZESE, O., FINNEY, H. M., FLETCHER, J. M., BELARAMANI, L. L., SALMON, M., DOKAL, I., WEBSTER, D., LAWSON, A. D. & AKBAR, A. N. 2007. The loss of telomerase activity in highly differentiated CD8+CD28-CD27- T cells is associated with decreased Akt (Ser473) phosphorylation. *J Immunol,* 178**,** 7710-9.

POLLOCK, P. M., HARPER, U. L., HANSEN, K. S., YUDT, L. M., STARK, M., ROBBINS, C. M., MOSES, T. Y., HOSTETTER, G., WAGNER, U., KAKAREKA, J., SALEM, G., POHIDA, T., HEENAN, P., DURAY, P., KALLIONIEMI, O., HAYWARD, N. K., TRENT, J. M. & MELTZER, P. S. 2003. High frequency of BRAF mutations in nevi. *Nat Genet,* 33**,** 19-20.

PRICE, J. S., WATERS, J. G., DARRAH, C., PENNINGTON, C., EDWARDS, D. R., DONELL, S. T. & CLARK, I. M. 2002. The role of chondrocyte senescence in osteoarthritis. *Aging Cell,* 1**,** 57-65.

PROVINCIALI, M. & MALAVOLTA, M. C. A. N.-C. M. I. M. M. B. H. C. S. M. T. B. H. M. A. I. T. Q. 2016. Editorial: Therapeutic Modulators of Cellular Senescence: Common Targets in Cancer And Aging. *Curr Drug Targets,* 17**,** 376.

PURCELL, M., KRUGER, A. & TAINSKY, M. A. 2014. Gene expression profiling of replicative and induced senescence. *Cell Cycle,* 13**,** 3927-37.

RABERGER, J., SCHEBESTA, A., SAKAGUCHI, S., BOUCHERON, N., BLOMBERG, K. E., BERGLOF, A., KOLBE, T., SMITH, C. I., RULICKE, T. & ELLMEIER, W. 2008. The transcriptional regulator PLZF induces the development of CD44 high memory phenotype T cells. *Proc Natl Acad Sci U S A,* 105**,** 17919-24.

RAKHRA, K., BACHIREDDY, P., ZABUAWALA, T., ZEISER, R., XU, L., KOPELMAN, A., FAN, A. C., YANG, Q., BRAUNSTEIN, L., CROSBY, E., RYEOM, S. & FELSHER, D. W. 2010. CD4(+) T cells contribute to the remodeling of the microenvironment required for sustained tumor regression upon oncogene inactivation. *Cancer Cell,* 18**,** 485-98.

RAMIREZ, R. D., MORALES, C. P., HERBERT, B. S., ROHDE, J. M., PASSONS, C., SHAY, J. W. & WRIGHT, W. E. 2001. Putative telomere-independent mechanisms of replicative aging reflect inadequate growth conditions. *Genes Dev,* 15**,** 398-403.

RAULET, D. H. 2003. Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol,* 3**,** 781-90.

RAULET, D. H., GASSER, S., GOWEN, B. G., DENG, W. & JUNG, H. 2013. Regulation of ligands for the NKG2D activating receptor. *Annu Rev Immunol,* 31**,** 413-41.

RAZIORROUH, B., SCHRAUT, W., GERLACH, T., NOWACK, D., GRUNER, N. H., ULSENHEIMER, A., ZACHOVAL, R., WACHTLER, M., SPANNAGL, M., HAAS, J., DIEPOLDER, H. M. & JUNG, M. C. 2010. The immunoregulatory role of CD244 in chronic hepatitis B infection and its inhibitory potential on virus-specific CD8+ T-cell function. *Hepatology,* 52**,** 1934-47.

REIMANN, M., LEE, S., LODDENKEMPER, C., DORR, J. R., TABOR, V., AICHELE, P., STEIN, H., DORKEN, B., JENUWEIN, T. & SCHMITT, C. A. 2010. Tumor stroma-derived TGF-beta limits myc-driven lymphomagenesis via Suv39h1-dependent senescence. *Cancer Cell,* 17**,** 262-72.

RESSLER, S., BARTKOVA, J., NIEDEREGGER, H., BARTEK, J., SCHARFFETTER-KOCHANEK, K., JANSEN-DURR, P. & WLASCHEK, M. 2006. p16INK4A is a robust in vivo biomarker of cellular aging in human skin. *Aging Cell,* 5**,** 379-89.

RITSCHKA, B., STORER, M., MAS, A., HEINZMANN, F., ORTELLS, M. C., MORTON, J. P., SANSOM, O. J., ZENDER, L. & KEYES, W. M. 2017. The senescence-associated secretory phenotype induces cellular plasticity and tissue regeneration. *Genes Dev,* 31**,** 172-183.

ROBERT H. TE POELE, A. L. O., LESLEY JARDINE, JEFFREY CUMMINGS, AND SIMON P. JOEL 2002. DNA Damage Is Able to Induce Senescence in Tumor Cells in Vitro and in Vivo. *Cancer Research***,** 1876-83.

ROBERTS, S., EVANS, E. H., KLETSAS, D., JAFFRAY, D. C. & EISENSTEIN, S. M. 2006. Senescence in human intervertebral discs. *Eur Spine J,* 15 Suppl 3**,** S312-6.

RODIER, F., COPPE, J. P., PATIL, C. K., HOEIJMAKERS, W. A., MUNOZ, D. P., RAZA, S. R., FREUND, A., CAMPEAU, E., DAVALOS, A. R. & CAMPISI, J. 2009. Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nat Cell Biol,* 11**,** 973-9.

ROMERO, P., ZIPPELIUS, A., KURTH, I., PITTET, M. J., TOUVREY, C., IANCU, E. M., CORTHESY, P., DEVEVRE, E., SPEISER, D. E. & RUFER, N. 2007. Four functionally distinct populations of human effector-memory CD8+ T lymphocytes. *J Immunol,* 178**,** 4112-9.

ROTH, A., YSSEL, H., PENE, J., CHAVEZ, E. A., SCHERTZER, M., LANSDORP, P. M., SPITS, H. & LUITEN, R. M. 2003. Telomerase levels control the lifespan of human T lymphocytes. *Blood,* 102**,** 849-57.

RUFER, N., ZIPPELIUS, A., BATARD, P., PITTET, M. J., KURTH, I., CORTHESY, P., CEROTTINI, J. C., LEYVRAZ, S., ROOSNEK, E., NABHOLZ, M. & ROMERO, P. 2003. Ex vivo characterization of human CD8+ T subsets with distinct replicative history and partial effector functions. *Blood,* 102**,** 1779-87.

SAEZ-BORDERIAS, A., ROMO, N., MAGRI, G., GUMA, M., ANGULO, A. & LOPEZ-BOTET, M. 2009. IL-12-dependent inducible expression of the CD94/NKG2A inhibitory receptor regulates CD94/NKG2C+ NK cell function. *J Immunol,* 182**,** 829-36.

SAGIV, A. 2016. NKG2D ligands mediate immunosurveillance of senescent cells. *Aging* 8**,**

.

SAGIV, A. & KRIZHANOVSKY, V. 2013. Immunosurveillance of senescent cells: the bright side of the senescence program. *Biogerontology,* 14**,** 617-28.

SALLUSTO, F., GEGINAT, J. & LANZAVECCHIA, A. 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol,* 22**,** 745-63.

SALMINEN, A., OJALA, J., KAARNIRANTA, K., HAAPASALO, A., HILTUNEN, M. & SOININEN, H. 2011. Astrocytes in the aging brain express characteristics of senescence-associated secretory phenotype. *Eur J Neurosci,* 34**,** 3-11.

SAMANI, N. J., BOULTBY, R., BUTLER, R., THOMPSON, J. R. & GOODALL, A. H. 2001. Telomere shortening in atherosclerosis. *Lancet,* 358**,** 472-3.

SAMASSEKOU, O., GADJI, M., DROUIN, R. & YAN, J. 2010. Sizing the ends: normal length of human telomeres. *Ann Anat,* 192**,** 284-91.

SANSONI, P., VESCOVINI, R., FAGNONI, F., BIASINI, C., ZANNI, F., ZANLARI, L., TELERA, A., LUCCHINI, G., PASSERI, G., MONTI, D., FRANCESCHI, C. & PASSERI, M. 2008. The immune system in extreme longevity. *Exp Gerontol,* 43**,** 61-5.

SCHAFER, M. J., WHITE, T. A., IIJIMA, K., HAAK, A. J., LIGRESTI, G., ATKINSON, E. J., OBERG, A. L., BIRCH, J., SALMONOWICZ, H., ZHU, Y., MAZULA, D. L., BROOKS, R. W., FUHRMANN-STROISSNIGG, H., PIRTSKHALAVA, T., PRAKASH, Y. S., TCHKONIA, T., ROBBINS, P. D., AUBRY, M. C., PASSOS, J. F., KIRKLAND, J. L., TSCHUMPERLIN, D. J., KITA, H. & LEBRASSEUR, N. K. 2017. Cellular senescence mediates fibrotic pulmonary disease. *Nat Commun,* 8**,** 14532.

SCHEURING, U. J., SABZEVARI, H. & THEOFILOPOULOS, A. N. 2002. Proliferative arrest and cell cycle regulation in CD8(+)CD28(-) versus CD8(+)CD28(+) T cells. *Hum Immunol,* 63**,** 1000-9.

SCHMIDT, D., GORONZY, J. J. & WEYAND, C. M. 1996. CD4+ CD7- CD28- T cells are expanded in rheumatoid arthritis and are characterized by autoreactivity. *J Clin Invest,* 97**,** 2027-37.

SCHMITT, C. A., FRIDMAN, J. S., YANG, M., LEE, S., BARANOV, E., HOFFMAN, R. M. & LOWE, S. W. 2002. A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. *Cell,* 109**,** 335-46.

SCHNEIDER, H., DOWNEY, J., SMITH, A., ZINSELMEYER, B. H., RUSH, C., BREWER, J. M., WEI, B., HOGG, N., GARSIDE, P. & RUDD, C. E. 2006. Reversal of the TCR stop signal by CTLA-4. *Science,* 313**,** 1972-5.

SERRANO, M., LIN, A. W., MCCURRACH, M. E., BEACH, D. & LOWE, S. W. 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell,* 88**,** 593-602.

SEVERINO, J., ALLEN, R. G., BALIN, S., BALIN, A. & CRISTOFALO, V. J. 2000. Is beta-galactosidase staining a marker of senescence in vitro and in vivo? *Exp Cell Res,* 257**,** 162-71.

SEYDA, M., ELKHAL, A., QUANTE, M., FALK, C. S. & TULLIUS, S. G. 2016. T Cells Going Innate. *Trends Immunol,* 37**,** 546-56.

SHARPLESS, N. E. & SHERR, C. J. 2015. Forging a signature of in vivo senescence. *Nat Rev Cancer,* 15**,** 397-408.

SHERR, C. J. & MCCORMICK, F. 2002. The RB and p53 pathways in cancer. *Cancer Cell,* 2**,** 103-12.

SHERR, C. J. & ROBERTS, J. M. 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev,* 13**,** 1501-12.

SINGH, T. & NEWMAN, A. B. 2011. Inflammatory markers in population studies of aging. *Ageing Res Rev,* 10**,** 319-29.

SNYDER, M. R., WEYAND, C. M. & GORONZY, J. J. 2004. The double life of NK receptors: stimulation or co-stimulation? *Trends Immunol,* 25**,** 25-32.

SORIANI, A., ZINGONI, A., CERBONI, C., IANNITTO, M. L., RICCIARDI, M. R., DI GIALLEONARDO, V., CIPPITELLI, M., FIONDA, C., PETRUCCI, M. T., GUARINI, A., FOA, R. & SANTONI, A. 2009. ATM-ATR-dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype. *Blood,* 113**,** 3503-11.

SOUDJA, S. M., RUIZ, A. L., MARIE, J. C. & LAUVAU, G. 2012. Inflammatory monocytes activate memory CD8(+) T and innate NK lymphocytes independent of cognate antigen during microbial pathogen invasion. *Immunity,* 37**,** 549-62.

STRAUSS-ALBEE, D. M., HOROWITZ, A., PARHAM, P. & BLISH, C. A. 2014. Coordinated regulation of NK receptor expression in the maturing human immune system. *J Immunol,* 193**,** 4871-9.

STRID, J., ROBERTS, S. J., FILLER, R. B., LEWIS, J. M., KWONG, B. Y., SCHPERO, W., KAPLAN, D. H., HAYDAY, A. C. & GIRARDI, M. 2008. Acute upregulation of an NKG2D ligand promotes rapid reorganization of a local immune compartment with pleiotropic effects on carcinogenesis. *Nat Immunol,* 9**,** 146-54.

SURAM, A., KAPLUNOV, J., PATEL, P. L., RUAN, H., CERUTTI, A., BOCCARDI, V., FUMAGALLI, M., DI MICCO, R., MIRANI, N., GURUNG, R. L., HANDE, M. P., D'ADDA DI FAGAGNA, F. & HERBIG, U. 2012. Oncogene-induced telomere dysfunction enforces cellular senescence in human cancer precursor lesions. *EMBO J,* 31**,** 2839-51.

TAKAI, H., SMOGORZEWSKA, A. & DE LANGE, T. 2003. DNA damage foci at dysfunctional telomeres. *Curr Biol,* 13**,** 1549-56.

TAKASHIMA, A. 2001. Establishment of fibroblast cultures. *Curr Protoc Cell Biol,* Chapter 2**,** Unit 2 1.

TAKEMOTO, N., INTLEKOFER, A. M., NORTHRUP, J. T., WHERRY, E. J. & REINER, S. L. 2006. Cutting Edge: IL-12 inversely regulates T-bet and

eomesodermin expression during pathogen-induced CD8+ T cell differentiation. *J Immunol,* 177**,** 7515-9.

TARAZONA, R., DELAROSA, O., ALONSO, C., OSTOS, B., ESPEJO, J., PENA, J. & SOLANA, R. 2000. Increased expression of NK cell markers on T lymphocytes in aging and chronic activation of the immune system reflects the accumulation of effector/senescent T cells. *Mech Ageing Dev,* 121**,** 77-88.

TASDEMIR, N., BANITO, A., ROE, J. S., ALONSO-CURBELO, D., CAMIOLO, M., TSCHAHARGANEH, D. F., HUANG, C. H., AKSOY, O., BOLDEN, J. E., CHEN, C. C., FENNELL, M., THAPAR, V., CHICAS, A., VAKOC, C. R. & LOWE, S. W. 2016. BRD4 Connects Enhancer Remodeling to Senescence Immune Surveillance. *Cancer Discov,* 6**,** 612-29.

TESAR, B. M., DU, W., SHIRALI, A. C., WALKER, W. E., SHEN, H. & GOLDSTEIN, D. R. 2009. Aging augments IL-17 T-cell alloimmune responses. *Am J Transplant,* 9**,** 54-63.

TIVEY, H. S., BROOK, A. J., ROKICKI, M. J., KIPLING, D. & DAVIS, T. 2013. p38 (MAPK) stress signalling in replicative senescence in fibroblasts from progeroid and genomic instability syndromes. *Biogerontology,* 14**,** 47-62.

TOMASEC, P., BRAUD, V. M., RICKARDS, C., POWELL, M. B., MCSHARRY, B. P., GADOLA, S., CERUNDOLO, V., BORYSIEWICZ, L. K., MCMICHAEL, A. J. & WILKINSON, G. W. 2000. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. *Science,* 287**,** 1031.

TOSO, A., DI MITRI, D. & ALIMONTI, A. 2015. Enhancing chemotherapy efficacy by reprogramming the senescence-associated secretory phenotype of prostate tumors: A way to reactivate the antitumor immunity. *Oncoimmunology,* 4**,** e994380.

TOSO, A., REVANDKAR, A., DI MITRI, D., GUCCINI, I., PROIETTI, M., SARTI, M., PINTON, S., ZHANG, J., KALATHUR, M., CIVENNI, G., JARROSSAY, D., MONTANI, E., MARINI, C., GARCIA-ESCUDERO, R., SCANZIANI, E., GRASSI, F., PANDOLFI, P. P., CATAPANO, C. V. & ALIMONTI, A. 2014. Enhancing chemotherapy efficacy in Pten-deficient prostate tumors by activating the senescence-associated antitumor immunity. *Cell Rep,* 9**,** 75-89.

UHRBERG, M., VALIANTE, N. M., YOUNG, N. T., LANIER, L. L., PHILLIPS, J. H. & PARHAM, P. 2001. The repertoire of killer cell Ig-like receptor and CD94:NKG2A receptors in T cells: clones sharing identical alpha beta TCR rearrangement express highly diverse killer cell Ig-like receptor patterns. *J Immunol,* 166**,** 3923-32.

ULBRECHT, M., COUTURIER, A., MARTINOZZI, S., PLA, M., SRIVASTAVA, R., PETERSON, P. A. & WEISS, E. H. 1999. Cell surface expression of HLA-E: interaction with human beta2-microglobulin and allelic differences. *Eur J Immunol,* 29**,** 537-47.

UPSHAW, J. L., ARNESON, L. N., SCHOON, R. A., DICK, C. J., BILLADEAU, D. D. & LEIBSON, P. J. 2006. NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells. *Nat Immunol,* 7**,** 524-32.

UYTTENHOVE, C., PILOTTE, L., THEATE, I., STROOBANT, V., COLAU, D., PARMENTIER, N., BOON, T. & VAN DEN EYNDE, B. J. 2003. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. *Nat Med,* 9**,** 1269-74.

VALLEJO, A. N. 2007. Immune remodeling: lessons from repertoire alterations during chronological aging and in immune-mediated disease. *Trends Mol Med,* 13**,** 94-102.

VALLEJO, A. N., MUELLER, R. G., HAMEL, D. L., JR., WAY, A., DVERGSTEN, J. A., GRIFFIN, P. & NEWMAN, A. B. 2011. Expansions of NK-like alphabetaT cells with chronologic aging: novel lymphocyte effectors that compensate for functional deficits of conventional NK cells and T cells. *Ageing Res Rev,* 10**,** 354-61.

VAN DEN ELSEN, P. J. 2011. Expression regulation of major histocompatibility complex class I and class II encoding genes. *Front Immunol,* 2**,** 48.

VAN DEURSEN, J. M. 2014. The role of senescent cells in ageing. *Nature,* 509**,** 439-46.

VAN HALL, T., OLIVEIRA, C. C., JOOSTEN, S. A. & OTTENHOFF, T. H. 2010. The other Janus face of Qa-1 and HLA-E: diverse peptide repertoires in times of stress. *Microbes Infect,* 12**,** 910-8.

VAN LIER, R. A., TEN BERGE, I. J. & GAMADIA, L. E. 2003. Human CD8(+) T-cell differentiation in response to viruses. *Nat Rev Immunol,* 3**,** 931-9.

VAN STIJN, A., ROWSHANI, A. T., YONG, S. L., BAAS, F., ROOSNEK, E., TEN BERGE, I. J. & VAN LIER, R. A. 2008. Human cytomegalovirus infection induces a rapid and sustained change in the expression of NK cell receptors on CD8+ T cells. *J Immunol,* 180**,** 4550-60.

VANGIPURAM, M., TING, D., KIM, S., DIAZ, R. & SCHULE, B. 2013. Skin punch biopsy explant culture for derivation of primary human fibroblasts. *J Vis Exp***,** e3779.

VERMIJLEN, D. & PRINZ, I. 2014. Ontogeny of Innate T Lymphocytes - Some Innate Lymphocytes are More Innate than Others. *Front Immunol,* 5**,** 486.

VERNERIS, M. R., KARIMI, M., BAKER, J., JAYASWAL, A. & NEGRIN, R. S. 2004. Role of NKG2D signaling in the cytotoxicity of activated and expanded CD8+ T cells. *Blood,* 103**,** 3065-72.

VIJAYACHANDRA, K., LEE, J. & GLICK, A. B. 2003. Smad3 regulates senescence and malignant conversion in a mouse multistage skin carcinogenesis model. *Cancer Res,* 63**,** 3447-52.

VIVIER, E. & ANFOSSI, N. 2004. Inhibitory NK-cell receptors on T cells: witness of the past, actors of the future. *Nat Rev Immunol,* 4**,** 190-8.

VIVIER, E., TOMASELLO, E. & PAUL, P. 2002. Lymphocyte activation via NKG2D: towards a new paradigm in immune recognition? *Curr Opin Immunol,* 14**,** 306-11.

VOEHRINGER, D., KOSCHELLA, M. & PIRCHER, H. 2002. Lack of proliferative capacity of human effector and memory T cells expressing killer cell lectinlike receptor G1 (KLRG1). *Blood,* 100**,** 3698-702.

VON ZGLINICKI, T. 2002. Oxidative stress shortens telomeres. *Trends Biochem Sci,* 27**,** 339-44.

VON ZGLINICKI, T., PETRIE, J. & KIRKWOOD, T. B. 2003. Telomere-driven replicative senescence is a stress response. *Nat Biotechnol,* 21**,** 229-30.

WAAIJER, M. E., PARISH, W. E., STRONGITHARM, B. H., VAN HEEMST, D., SLAGBOOM, P. E., DE CRAEN, A. J., SEDIVY, J. M., WESTENDORP, R.

G., GUNN, D. A. & MAIER, A. B. 2012. The number of p16INK4a positive cells in human skin reflects biological age. *Aging Cell,* 11**,** 722-5.

WADA, H., MATSUMOTO, N., MAENAKA, K., SUZUKI, K. & YAMAMOTO, K. 2004. The inhibitory NK cell receptor CD94/NKG2A and the activating receptor CD94/NKG2C bind the top of HLA-E through mostly shared but partly distinct sets of HLA-E residues. *Eur J Immunol,* 34**,** 81-90.

WANG, C., JURK, D., MADDICK, M., NELSON, G., MARTIN-RUIZ, C. & VON ZGLINICKI, T. 2009. DNA damage response and cellular senescence in tissues of aging mice. *Aging Cell,* 8**,** 311-23.

WANG, X., BISHOP, K. A., HEGDE, S., RODENKIRCH, L. A., PIKE, J. W. & GUMPERZ, J. E. 2012. Human invariant natural killer T cells acquire transient innate responsiveness via histone H4 acetylation induced by weak TCR stimulation. *J Exp Med,* 209**,** 987-1000.

WEI, X. H. & ORR, H. T. 1990. Differential expression of HLA-E, HLA-F, and HLA-G transcripts in human tissue. *Hum Immunol,* 29**,** 131-42.

WENCKER, M., TURCHINOVICH, G., DI MARCO BARROS, R., DEBAN, L., JANDKE, A., COPE, A. & HAYDAY, A. C. 2014. Innate-like T cells straddle innate and adaptive immunity by altering antigen-receptor responsiveness. *Nat Immunol,* 15**,** 80-7.

WENG, N. P., AKBAR, A. N. & GORONZY, J. 2009. CD28(-) T cells: their role in the age-associated decline of immune function. *Trends Immunol,* 30**,** 306-12.

WIEMANN, S. U., SATYANARAYANA, A., TSAHURIDU, M., TILLMANN, H. L., ZENDER, L., KLEMPNAUER, J., FLEMMING, P., FRANCO, S., BLASCO, M. A., MANNS, M. P. & RUDOLPH, K. L. 2002. Hepatocyte telomere shortening and senescence are general markers of human liver cirrhosis. *FASEB J,* 16**,** 935-42.

WONG, E. S., LE GUEZENNEC, X., DEMIDOV, O. N., MARSHALL, N. T., WANG, S. T., KRISHNAMURTHY, J., SHARPLESS, N. E., DUNN, N. R. & BULAVIN, D. V. 2009. p38MAPK controls expression of multiple cell cycle inhibitors and islet proliferation with advancing age. *Dev Cell,* 17**,** 142-9.

WRIGHT, W. E., PIATYSZEK, M. A., RAINEY, W. E., BYRD, W. & SHAY, J. W. 1996. Telomerase activity in human germline and embryonic tissues and cells. *Dev Genet,* 18**,** 173-9.

WU, J., CHERWINSKI, H., SPIES, T., PHILLIPS, J. H. & LANIER, L. L. 2000. DAP10 and DAP12 form distinct, but functionally cooperative, receptor complexes in natural killer cells. *J Exp Med,* 192**,** 1059-68.

WU, J., SONG, Y., BAKKER, A. B., BAUER, S., SPIES, T., LANIER, L. L. & PHILLIPS, J. H. 1999. An activating immunoreceptor complex formed by NKG2D and DAP10. *Science,* 285**,** 730-2.

XU, Y., LI, N., XIANG, R. & SUN, P. 2014. Emerging roles of the p38 MAPK and PI3K/AKT/mTOR pathways in oncogene-induced senescence. *Trends Biochem Sci,* 39**,** 268-76.

XUE, W., ZENDER, L., MIETHING, C., DICKINS, R. A., HERNANDO, E., KRIZHANOVSKY, V., CORDON-CARDO, C. & LOWE, S. W. 2007. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature,* 445**,** 656-60.

YAMAKOSHI, K., TAKAHASHI, A., HIROTA, F., NAKAYAMA, R., ISHIMARU, N., KUBO, Y., MANN, D. J., OHMURA, M., HIRAO, A., SAYA, H., ARASE, S.,

HAYASHI, Y., NAKAO, K., MATSUMOTO, M., OHTANI, N. & HARA, E. 2009. Real-time in vivo imaging of p16Ink4a reveals cross talk with p53. *J Cell Biol,* 186**,** 393-407.

YANG, N. C. & HU, M. L. 2005. The limitations and validities of senescence associated-beta-galactosidase activity as an aging marker for human foreskin fibroblast Hs68 cells. *Exp Gerontol,* 40**,** 813-9.

YUN, M. H., DAVAAPIL, H. & BROCKES, J. P. 2015. Recurrent turnover of senescent cells during regeneration of a complex structure. *Elife,* 4.

ZARITSKAYA, L., SHURIN, M. R., SAYERS, T. J. & MALYGUINE, A. M. 2010. New flow cytometric assays for monitoring cell-mediated cytotoxicity. *Expert Rev Vaccines,* 9**,** 601-16.

ZARUBIN, T. & HAN, J. 2005. Activation and signaling of the p38 MAP kinase pathway. *Cell Res,* 15**,** 11-8.

ZEESTRATEN, E. C., REIMERS, M. S., SAADATMAND, S., GOOSSENS-BEUMER, I. J., DEKKER, J. W., LIEFERS, G. J., VAN DEN ELSEN, P. J., VAN DE VELDE, C. J. & KUPPEN, P. J. 2014. Combined analysis of HLA class I, HLA-E and HLA-G predicts prognosis in colon cancer patients. *Br J Cancer,* 110**,** 459-68.

ZHU, Y., TCHKONIA, T., FUHRMANN-STROISSNIGG, H., DAI, H. M., LING, Y. Y., STOUT, M. B., PIRTSKHALAVA, T., GIORGADZE, N., JOHNSON, K. O., GILES, C. B., WREN, J. D., NIEDERNHOFER, L. J., ROBBINS, P. D. & KIRKLAND, J. L. 2016. Identification of a novel senolytic agent, navitoclax, targeting the Bcl-2 family of anti-apoptotic factors. *Aging Cell,* 15**,** 428-35.

ZHU, Y., XU, L., ZHANG, J., HU, X., LIU, Y., YIN, H., LV, T., ZHANG, H., LIU, L., AN, H., LIU, H., XU, J. & LIN, Z. 2013. Sunitinib induces cellular senescence via p53/Dec1 activation in renal cell carcinoma cells. *Cancer Sci,* 104**,** 1052-61.

ZITVOGEL, L., TESNIERE, A. & KROEMER, G. 2006. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat Rev Immunol,* 6**,** 715-27.