Mechanisms underlying reduced natural killer cell cytotoxicity with age

by

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Abstract

Ageing is accompanied by a marked reduction in natural killer (NK) cell cytotoxicity (NKCC) at the single cell level that is thought to contribute to the accumulation of senescent cells in aged tissues. Although it is established that decreased killing results from reduced perforin secretion in response to co-culture with cancer cells, the mechanisms involved in reduced NKCC towards senescent cells remain unknown.

To investigate reduced NKCC towards senescent cells, we examined the composition of the circulating NK cell pool in healthy young (≤35 years) and older (≥65 years) adults, focussing primarily on highly differentiated $CD57^+$ NK cells as they show reduced function in killing assays. Additionally, we assessed perforin release and mitogen-activated protein kinase (MAPK) activation in NK cells following NKG2D and NKp30 receptor ligation given their role in granule polarisation and NKCC towards senescent cells.

Assessment of the composition of the circulating lymphocyte pool revealed a greater proportion of NK cells in older adults, with a marked increase in the percentage of $CD56^{DIM}$ NK cells and terminally differentiated $CD56^{DIM}CD57^+$ NK cells. Since CD57 expression is associated with immune cell dysfunction, an increased proportion of $CD57^+$ NK cells may contribute to impaired perforin release and NKCC. Whilst we were unable to show that NK cells from older adults secreted lower levels of perforin, though sample size was low, we found no age-associated difference in P38 MAPK activation in NK cells following receptor ligation. Thus, we propose that reduced NKCC with age towards senescent cells may be due to defects in the activation of other MAPK signalling pathways such as ERK.

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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell cytotoxicity
Ag	Antigen
ANOVA	Analysis of variance
APC	Allophycocyanin
BID	BH3-interacting domain
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
C/EBP-ß	CCAAT-enhancer-binding protein-beta
CMV	Cytomegalovirus
DAG	Diacylglycerol
DC	Dendritic cell
Dcr2	Decoy receptor 2
ddH_2O	Double distilled water
DDR	DNA damage response
DISC	Death-inducing signalling complex
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemoluminescence
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular signal-regulated protein kinase 1/2
FasL	Fas ligand
FasR	Fas receptor
FCS	Fetal calf serum
FS	Forward scatter
FITC	Fluorescein isothiocyanate
GH	Growth hormone
GPS	L-glutamine-penicillin-streptomycin
HIFCS	Heat inactivated fetal calf serum
HLA-E	Human leukocyte antigen-E

HRP	Horseradish peroxidase
iCAD	Inhibitor of caspase-activated deoxyribonuclease
IFN-I	Type I interferon
IFN-III	Type III interferon
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IP_3	Inositol triphosphate
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KIR	Killer immunoglobulin-like receptors
KLRG1	Killer cell lectin-like receptor subfamily G member 1
MAPK	Mitogen-activated protein kinase
mDC	Myeloid dendritic cell
MFI	Median fluorescence intensity
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II
MICA/B	MHC I chain related-proteins A and B
MTOC	Microtubule organising centre
NCR	Natural cytotoxicity receptor
NET	Neutrophil extracellular trap
NK-κB	Nuclear factor kappa B
NK	Natural killer
NKCC	NK cell cytotoxicity
NKT	Natural killer T cell
PARP	Poly ADP ribose polymerase
PBDC	Peripheral blood dendritic cell
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell

PE	Phycoerythrin
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidyl-inositol bisphosphate
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
pRB	Retinoblastoma protein
PRC	Polycomb repressive complex
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
RT	Room temperature
SA-ß-gal	Senescence-associated ß-galactosidase
SASP	Senesce-associated secretory phenotype
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
sMAC	Sestrin-dependent MAPK activation complex
SS	Side scatter
STAT	Signal transducer and activator of transcription
tBID	Truncated BID
TBS	Tris buffered saline
TBST	TBS Tween-20
TCR	T cell receptor
T_{FH}	T follicular helper cell
T_{FR}	T follicular regulatory cell
Th17	T helper cell 17
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-alpha
T_{reg}	Regulatory T cell
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TNF-related apoptosis-inducing ligand receptor
ULBP1-6	UL16-binding proteins 1-6
VEGF	Vascular endothelial growth factor

1.0 INTRODUCTION

1.1 Population ageing in the UK

The United Kingdom (UK), like many other countries, is experiencing rapid population ageing due to steadily declining birth and early-age mortality rates [1]. Improvements in public health and the emergence of preventative care have led to a significant increase in average life expectancy and the proportion of older people in society [1,2]. Since 1991, the number of individuals aged 65 and over has risen from 9.1 million to 11.8 million (currently 18% of the UK's population) [3]. A similar trend, although occurring at a much faster rate, has been observed in the 'oldest old' group, those aged 85 and over, who account for 2.4% of the population [3]. Recent estimates suggest that by 2046, the proportion of people aged 65 and over will exceed those aged 15 and under by 7%, and comprise 24.7% of the UK's population [4]. With both elderly age groups predicted to increase within the coming decades [3], population ageing has become a matter of concern and is expected to have profound implications for health and social care provisions due to the increasing prevalence of age-related diseases in older adults [2,3].

1.2 Ageing and immunity

Physiological ageing is accompanied by remodelling of the immune system that leads to immune dysfunction and loss of protective immunity [5]. This phenomenon termed immunesenescence is a multifactorial process affecting both arms of the immune system that manifests as loss of adaptive immunity and gain of nonspecific innate immunity [5-8]. In broad terms, immunesenescence is characterised by increased

reactivity, impaired antimicrobial activity, reduced immune surveillance of transformed cells and high serum levels of pro-inflammatory cytokines and chemokines. This not only reduces the capacity to mount rapid immune responses against foreign substances [6], but also creates a low-grade chronic inflammatory state termed inflammageing that contributes to the pathogenesis of several diseases including atherosclerosis, type II diabetes, sarcopenia, arthritis and Alzheimer's disease [9-12]. As well as an increased susceptibility to infection, immunesenescence results in loss of immune memory that reduces the prophylactic efficacy of vaccination with age; this is demonstrated by studies that report decreased antibody response post-vaccination and reduced vaccine longevity in older adults [13]. Current immune gerontology research aims to investigate the impact of immune ageing on health and more recently has included a focus on natural killer (NK) cells due to their role in early innate responses towards malignant cells, senescent cells and viral-infections.

1.3 Natural killer (NK) cells

Compromising approximately 10% of total circulating human lymphocytes, NK cells are large granular lymphocytes of the innate immune system that mediate anti-viral and anti-tumour responses [14-16]. In humans, NK cells are phenotypically defined as $CD3^{-}CD56^{+}$, and can be categorised into two functionally distinct subsets, $CD56^{DIM}$ and $CD56^{BRIGHT}$, based on differential surface expression of CD56 [14,16,17]. $CD56^{DIM}$ NK cells are cytotoxic effector cells that constitute the majority (~90%) of NK cells in peripheral blood [16-18]. These cells not only express high levels of perforin, but are also capable of cytokine and chemokine production following target cell recognition [15,19,20]. In contrast, $CD56^{BRIGHT}$ NK cells comprise only ~10% of the circulating NK cell pool, but predominate in lymph nodes and tonsils [15,18]. Functionally, $CD56^{BRIGHT}$

NK cells have low cytotoxic potential and lack perforin [21]. Instead, they are immunomodulatory and secrete a myriad of cytokines and chemokines such as interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) upon stimulation [21,22].

NK cells elicit direct cytotoxicity towards malignant and pathogen-infected cells via two mechanisms: granule exocytosis and death receptor ligation. Of the two, granule exocytosis is the predominant pathway by which NK cells induce target cell death via apoptosis, and involves directed secretion of lytic granules containing perforin and granzymes at the immunological synapse that forms at the NK-target cell interface [14,23]. After binding and incorporating itself into the target's plasma membrane [24], pore-forming perforin induces a membrane-repair response that results in co-endocytosis of perforin and granzymes [14,25]. Once inside, perforin induces endosomal lysis to facilitate the delivery of endocytosed granzymes into the cytosol [14,26]. Human NK cells express five granzymes: granzymes A, B, H, K and M [23,27]. Of these serine proteases, granzyme B is the most extensively studied and is primarily responsible for the induction of caspase-dependent apoptosis which occurs in one of two ways: (1) direct cleavage and activation of effector caspases 3 and 7 to induce apoptosis via degradation of DNA repair proteins (2) indirect activation of caspases 3 and 7 through cleavage of BH3-interacting domain (BID) into truncated BID (tBID), which translocates to the mitochondria to trigger cytochrome C release [14,27]. Granzyme B can also trigger apoptosis through a caspase-independent mechanism that involves direct cleavage of proteins involved in DNA repair and maintenance such as poly ADP ribose polymerase (PARP), inhibitor of caspase-activated deoxyribonuclease (iCAD) and laminin B [14].

Alongside granule exocytosis, NK cells eliminate target cells through death receptor ligation, which involves binding of Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) upregulated on the surface of activated NK cells to Fas and TRAIL receptors respectively on the target cell surface [14]. This leads to formation of the death-inducing signalling complex (DISC) and activation of caspases 8 and 10, which induce apoptosis directly via caspase 3 activation or indirectly through tBID-mediated release of cytochrome C from mitochondria [14].

NK cell activity is governed by the integration of signals received by a complex repertoire of germ-line encoded activating and inhibitory surface receptors [16,28,29]. Inhibitory receptors, which include killer immunoglobulin-like receptors (KIR) and C-type lectin family member CD94-NKG2A heterodimer receptor, negatively regulate NK cell activity by signalling through cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) upon interaction with major histocompatibility complex class I (MHC I)-expressing cells [16,28,29]. This results in a series of downstream signalling events, which disrupt NK cell cytotoxicity (NKCC) to prevent the destruction of healthy cells [28,30]. Conversely, most activating receptors (e.g. Fc receptor CD16 (FcγRIII) and natural cytotoxicity receptors (NCRs) NKp30, NKp44, NKp46 and NKp80), apart from C-type lectin family member NKG2D, lack intrinsic signalling activity and thus associate with immunoreceptor tyrosine-based activating motif (ITAM)-bearing adaptor proteins to induce NK cell activation and subsequent effector functions (e.g. cytokine production, proliferation, migration and target cell lysis) [31].

Indeed, co-stimulation of multiple activating receptors following target cell engagement is required for NK cell activation [30,32,33]. The combined signals are transmitted through several central signalling pathways (e.g. phosphoinositide 3-kinase (PI3K) and

janus kinase (JAK)/signal transducer and activator of transcription (STAT)) that converge on transcriptional activation of genes crucial for direct cytotoxicity [31,32,34]. Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases, consisting of P38, extracellular signal-regulated protein kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK) [35,36], whose activation is required for induction of lytic granule polarisation and ultimately NKCC [31,36,37]. Selective inhibition of P38, ERK and JNK MAPKs has been shown to reduce NK cell-mediated target cell lysis [36,38-40]. It is believed that MAPK signalling is essential for microtubule organising centre (MTOC) polarisation, which precedes lytic granule secretion [36,38-40]. Wei *et al*. demonstrated that inhibition of MAPK signalling prevents the delivery of secretory lysosomes to the NK cell synaptic cleft, thereby reducing perforin and granzyme B secretion [38].

1.4 Age-associated changes in NK cells

Like other immune cells, NK cells are subject to various age-related phenotypical and functional changes (Tables 1.1 and 1.2). Although ageing is associated with reduced NK cell proliferation and production [50-52], numerous studies have shown an increased percentage and/or absolute number of NK cells in older adults [51,53,54]. This suggests the existence of long-lived $CD57^+$ NK cells, whose frequency increases with age [53,54,89]. CD57 expression is a marker of terminal differentiation that is commonly associated with senescence in human T cells and mainly affects $CD56^{DIM}$ NK cells [89,90]. Despite retaining their cytotoxic potential, $CD56^{DIM}CD57^+$ NK cells have a lower proliferative capacity and are less responsive to cytokine stimulation *in vitro* [89,90]. With regards to the composition of the circulating NK cell pool, older adults display significantly fewer $CD56^{BRIGHT}$ NK cells and have a higher proportion of

$CD56^{DIM}$ NK cells, culminating in an increased $CD56^{DIM}$:$CD56^{BRIGHT}$ ratio [14,54,55,91].

Alongside redistribution of the NK cell subsets, ageing is associated with changes in the expression of activating and inhibitory NK cell receptors, with several studies reporting an age-related decrease in CD94, KLRG1 and NKp46 surface expression [54,56-58], whereas NKG2D, CD3 and CD16 expression is comparable with age [53,54,57,58]. However, the effect of age on NK cell receptor density remains controversial due to emerging evidence that the latent herpes virus cytomegalovirus (CMV) induces a shift in the NK cell surface phenotype to one that is associated with ageing [14,92]. Therefore, the apparent alterations in NK cell receptor expression previously observed may be due to a greater prevalence of CMV in the elderly population rather than age [92].

Whilst the effect of age on death-receptor-mediated apoptosis remains unknown, it is well established that ageing is accompanied by a decline in NKCC at the single cell level that is associated with decreased lytic activity and impaired granule exocytosis [5,14,54,93,94]. In contrast, ADCC, which occurs independently of NKCC and involves signalling of the Fc receptor CD16, appears to be unaltered with age [14]. Based on these findings, it is of no surprise that NK cell immunesenescence is associated with a higher incidence of viral infections and cancers among older adults [14,91,95,96].

Table 1.1: **Age-associated changes affecting the composition, phenotype and function of innate immune cells**

	Composition	Phenotype	Function	References
Monocytes/ macrophages	Non-classical monocytes ↑ Intermediate monocytes ↑ Classical monocytes ↓	CD38, CD62L & CD115 ↓ CD11b expression ↑ TLR1 expression ↓	Phagocytosis ↓ ROS production ↓ TLR1/2-induced IL-6 & TNF-α secretion ↓	[41-44]
Neutrophils	Immunosuppressive $CD16^{BRIGHT}CD62L^{DIM}$ neutrophils ↑	Unaltered CD11a & CD11b expression CD16 expression ↓	Chemotaxis ↓ Phagocytosis ↓ ROS production ↑/↓ NETosis ↓ Cytokine production ↓	[14,45-49]
Natural killer (NK) cells	Total NK cell proportion/frequency ↑ $CD56^{DIM}$ NK cell proportion/frequency ↑ $CD56^{BRIGHT}$ NK cell proportion/frequency ↓ $CD56^{DIM}$:$CD56^{BRIGHT}$ ratio ↑	Gain of CD57 expression by $CD56^{DIM}$ subset Comparable NKG2D, CD3 & CD16 expression CD94, KLRG1 & NKp46 expression ↓	Cytokine & chemokine production ↓ NKCC ↓ Impaired perforin release Unaltered ADCC	[50-58]
Natural killer T (NKT) cells	NKT cell proportion/frequency ↓ $CD8^{+}CD4^{-}$ NKT cell proportion/frequency ↑ $CD4^{+}CD8^{-}$ NKT cell proportion/frequency ↑	$Vα24^{+}/Vß11$ expression ↑	Unaltered/↓ proliferation IFN-γ secretion ↓	[59-62]
Dendritic cells (DCs)	Langerhans cell density ↓ Unaltered mDC frequency Unaltered/↓ pDC frequency	Comparable TLR7/9 expression on pDCs TLR1, 3 & 8 expression on mDCs ↓ MHC II expression on mDCs & pDCs ↓ Percentage of $CD83^{+}CD86^{+}$ PBDCs ↑	Impaired cytokine production (IL-6, IL-12, TNF-α, IFN-I & IFN-III) Migration ↓ Phagocytosis ↓ Ag presentation ↓	[63-69]

Abbreviations: ADCC, antibody-dependent cell cytotoxicity; Ag, antigen; IFN-I, type I interferon; IFN-III, type III interferon; IL, interleukin; KLRG1, killer cell lectin-like receptor subfamily G member 1; mDC, myeloid DC; MHC II, major histocompatibility complex class II; NET, neutrophil extracellular trap; PBDC, peripheral blood DC; pDC, plasmacytoid DC; ROS, reactive oxygen species; TLR, toll-like receptor.

Table 1.2: **Age-associated changes affecting the composition, phenotype and function of adaptive immune cells**

	Composition	Phenotype	Function	References
T cells	Naïve $CD4^+$ & $CD8^+$ T cell output ↓ Memory $CD4^+$ & $CD8^+$ T cell frequency ↑ Th17 cell differentiation ↑ T_{reg} frequency ↑ T_{FR}:T_{FH} ratio ↑	Loss of CD28 expression by $CD8^+$ T cells Gain of CD57 expression by $CD8^+$ T cells	TCR repertoire diversity ↓ $CD8^+$ T cell proliferative capacity ↓ $CD4^+$ T cell trafficking ↓ IFN-γ, TNF-α, granzyme B & IL-2 secretion ↓ Defective T_{FH} Ag-specific responses	[70-82]
B cells	Plasma cell frequency ↓ Naïve B cell percentage ↑ Memory B cell frequency ↓ Immunosuppressive $CD19^+CD24^{Hi}CD38^{Hi}$ B cell percentage/frequency ↓	IgM & IgD expression ↓ Comparable CD80, CD86 & CD40 expression on B cells	B cell diversity ↓ Defective Ig class switching Antibody production ↓ IL-10 production by $CD19^+CD24^{Hi}CD38^{Hi}$ B cells ↓	[83-88]

Abbreviations: Ig, immunoglobulin; TCR, T cell receptor; T_{FH}, T follicular helper cell; T_{FR}, T follicular regulatory cell; Th17, T helper cell 17; T_{reg}, regulatory T cell.

1.5 Cellular senescence

In recent years, cellular senescence has gained considerable interest in the field of gerontology due to its involvement in normal tissue homeostasis and pathophysiology. Cellular senescence is a state of stable irreversible growth arrest that prevents the malignant transformation of stressed cells [97,98]. Originally thought to be foremost an anti-cancer mechanism, cellular senescence has since been shown to be implicated in many physiological processes such as tissue remodelling during embryogenesis and wound repair [97,98].

Senescent cells typically undergo extensive alterations in gene expression, chromatin organisation and metabolic programming and are thus characterised by an enlarged, flattened morphology, upregulation of senescence-associated ß-galactosidase (SA-ß-gal), persistent DNA damage foci and apoptosis resistance [97,99-101]. Cellular senescence can be induced in a number of mitotic cell types including fibroblasts and epithelial cells in response to severe genomic or epigenomic stress caused by telomere erosion, mitochondrial dysfunction, oxidative stress, irreparable DNA damage, oncogenic overexpression and aberrant mitogenic signalling [97,99,100,102,103]. Entry into senescence is mediated and maintained through activation of the p53/$p21^{CIP1}$ and $p16^{INK4a}$/retinoblastoma protein (pRB) tumour suppressor pathways following persistent DNA damage response (DDR) signalling (Figure 1.1) [97,103,104]. Ultimately, stabilisation of p53 and upregulation of $p16^{INK4a}$ lead to activation of master regulator pRB, which prevents progression from G_1 to S phase of the cell cycle by inhibiting E2F-dependent transcription [102,103].

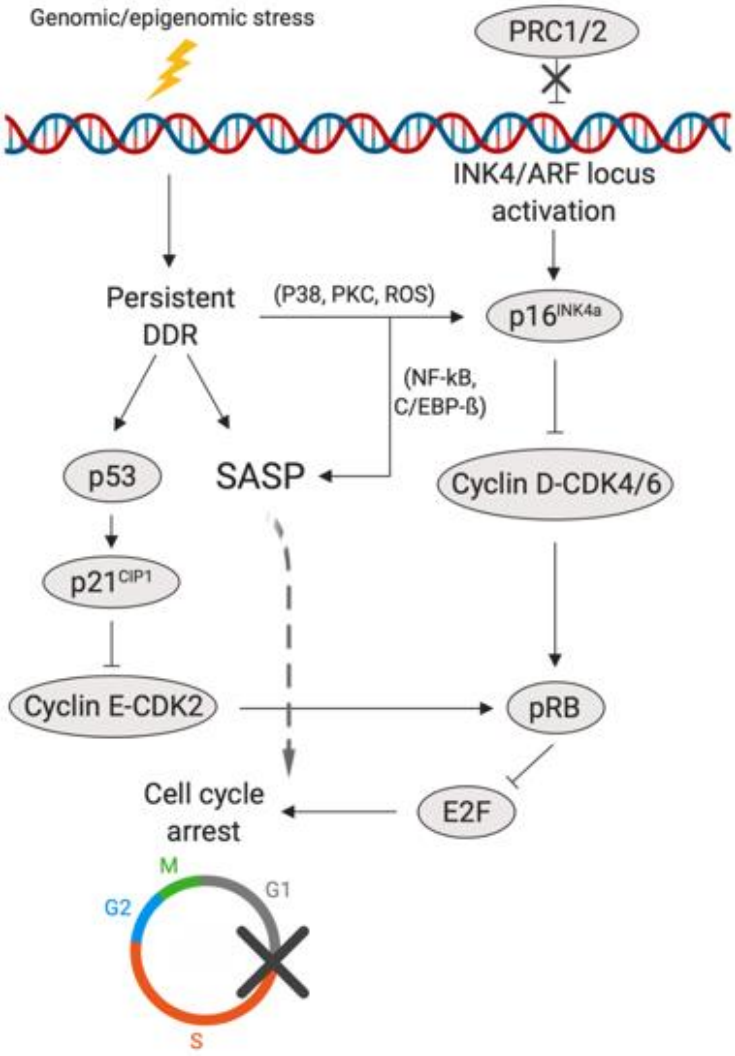

Figure 1.1: **Molecular pathways regulating cellular senescence and the SASP**

Cellular senescence is induced by severe genomic or epigenomic damage that leads to activation of the DDR and the INK4/ARF locus. Persistent DDR signalling causes stabilisation of p53, which induces expression of cyclin-dependent kinase (CDK) inhibitor p21. Inhibition of cyclinE-CDK2 by p21 results in dephosphorylation of pRB and repression of E2F-responsive genes, thereby preventing cell cycle progression. Concurrently, 16^{INK4a} expression is induced in response to persistent DDR signalling through increased ROS signalling and activation of P38 MAPK and protein kinase C (PKC), and activation of the INK4/ARK locus upon disruption of the Polycomb repressive complex (PRC). Ultimately, $p16^{INK4a}$ selectively inhibits cyclin D-CDK4/6, promoting pRB-mediated cell cycle arrest. In some forms of oncogene-induced senescence, growth arrest can be reinforced by the senescence-associated secretory phenotype (SASP), whose activity is primarily regulated by transcription factors nuclear factor-kappa B (NK-κB) and CCAAT-enhancer-binding protein-beta (C/EBP-ß), and p53. Figure adapted from review articles by Herranz and Gil [102], and Campisi and Roberts [103].

Despite losing their proliferative capacity upon senescence [103], senescent cells remain metabolically active and acquire a pro-inflammatory phenotype termed the senescence-associated secretory phenotype (SASP) [102,105]. As part of the SASP, senescent cells secrete a myriad of pro-inflammatory cytokines, chemokines, growth factors and extracellular matrix (ECM) components which have widespread paracrine and autocrine activities in tissues [105-109]. These effects can be either beneficial or deleterious depending on the biological context [108]. Beneficial aspects of the SASP appear to confer a potent tumour suppressive response driven by senescence reinforcement and immune surveillance [110-115]. Moreover, transient exposure to the SASP is known to direct tissue repair, limit fibrosis and induce cellular plasticity and "stemness" in neighbouring cells to promote tissue regeneration following acute injury [116-118]. In contrast, the SASP can have tumour-promoting activities in tissues, stimulating the proliferation and metastasis of transformed cells [108,119]. For example, increased secretion of SASP-derived factors IL-6 and IL-8 by senescent fibroblasts has been shown to induce epithelial-mesenchymal transition and enhanced invasion in nonaggressive carcinoma cells [108]. Moreover, upregulation of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) by the senescent cell secretome stimulates tumour vascularisation in mice and humans *in vitro* [120]. This apparent paradox confers dual functionality of the SASP in the context of physiology and pathology where a localised, time-limited SASP is beneficial for tumour clearance and tissue repair [121]. Whereas chronic exposure to the SASP is deleterious, creating a pro-inflammatory environment that disrupts tissue integrity and favours cancer evolution [108,121].

Recently, it has been postulated that senescent cells contribute to ageing and the development of age-related diseases. Evidence has shown that senescent cells accumulate in aged tissues and at sites of pathology despite being less abundant in healthy young individuals [107,122]. It is this increased presence and propagation of the SASP that are believed to drive pathogenicity in tissues. For instance, cognitive impairment and reduced neurogenesis in Alzheimer's and Parkinson's disease have been linked to an increased abundance of senescent glial cells and secretion of pro-inflammatory SASP-derived factors such as IL-6 in brain tissue [123,124]. Similarly, SASP-mediated ECM remodelling has been shown to drive atheroma formation and plaque instability in atherosclerosis [125,126]. These associative data are supported by two consecutive studies in BubR1 progeroid mice, in which selective deletion of $p16^{Ink4a}$-positive senescent cells in adipose, eye and skeletal muscle delayed the onset and attenuated the progression of already established age-related disorders, namely sarcopenia and cataracts [127]. Furthermore, drug-induced clearance of senescent cells extended lifespan in mice, delayed tumorigenesis and attenuated age-related deterioration of several organs including the kidney, heart and fat in this transgenic mouse model [128]. Such findings have prompted research into the use of senolytic drugs to reduce the senescent cell burden [129,130]. However, there are many unknowns and potential drawbacks to utilising these therapies due to the lack of human studies [98,131].

1.6 NK cell cytotoxicity towards senescent cells

Although there is causal evidence that lingering senescent cells are involved in the pathogenesis of age-related diseases, the mechanisms by which these cells accumulate have not been elucidated. Recent findings suggest that senescent cell

clearance may be impaired with age, contributing to their persistence in tissues. Several immune cell types including macrophages, neutrophils and $CD8^+$ T cells are known to eliminate senescent cells [115]. However, NK cells are thought to be the major innate cell involved in the removal of senescent cells, which they directly recognise through activating receptor NKG2D, which binds its respective ligands MHC I chain related-proteins A and B (MICA/B) and UL16-binding proteins 1-6 (ULBP1-6) expressed on senescent cells [132]. However, in order to NKCC following target cell engagement, co-stimulation of multiple activating receptors such as NKp30 which recognises MHC I molecules is required. Since upregulation of decoy receptor 2 (Dcr2) by senescent cells prevents death receptor ligation by competitively binding NK cell death receptor ligands (i.e. FasL and TRAIL) [133], the predominant mechanism by which NK cells eliminate senescent cells is contact-dependent granule exocytosis (Figure 1.2) [14,134].

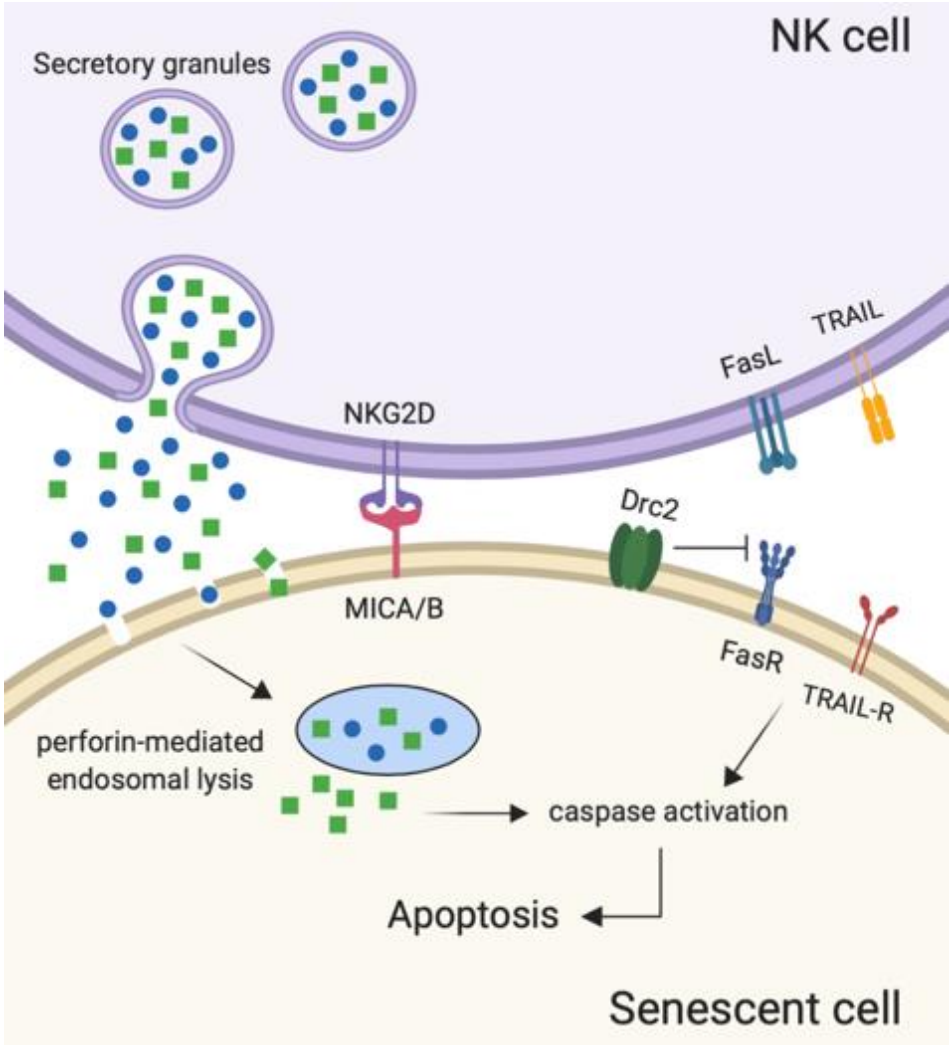

Figure 1.2: **NK cell-mediated killing of senescent cells via granule exocytosis** NK cells recognise transformed cells through activating receptor NKG2D, which binds stress-inducible glycoproteins (e.g. MICA/B) expressed on the senescent cell surface. Due to upregulation of Dcr2 on senescent cells, NK cell-mediated killing is biased towards granule exocytosis, which involves rapid expulsion of secretory granule contents perforin and granzymes into the immunological synapse. Upon degranulation, pore-forming perforin binds to and disrupts the target cell membrane, triggering co-endocytosis of perforin and granzymes. Through perforin-mediated endosomal lysis, granzyme B is delivered into the cytosol where it induces senescent cell apoptosis via various caspase-dependent and -independent mechanisms.

Despite being renowned for their ability to mediate direct cytotoxicity against transformed cells [135], NK cells show a marked reduction in killing ability with age that is thought to contribute to the accumulation of senescent cells. The reason for this is likely to be multifactorial. Pereira and colleagues proposed that senescent cells evade immune surveillance by upregulating surface expression of non-classical MHC molecule human leukocyte antigen-E (HLA-E), thus contributing to their persistence in tissues [136]. The group showed that by blocking interactions between HLA-E and inhibitory receptor NKG2A, NK and $CD8_+$ T cell-mediated surveillance of senescent cells was significantly improved *in vitro* [136]. Marked alterations in the phenotype and function of NK cells with age may also contribute to incomplete elimination of senescent cells. Despite effectively recognising and binding to tumour targets, NK cells from older adults exhibit an age-associated decline in NKCC that is indicative of a post-binding defect in NK cell activation and/or degranulation [5,53,93,94]. Groups that have investigated the effect of age on the expression of key NK cell receptors and lytic effector molecules needed for direct cytotoxicity have reported contradictory results regarding activating receptors NKG2D and NKp30 and perforin and granzyme B expression [14,53,56,137,138]. Despite these conflicting observations, Hazeldine *et al*. found no age-associated difference in perforin expression, but did report impaired perforin secretion and polarisation at the immunological synapse by NK cells from old subjects following co-culture with the K562 cancer cell line [54]. Interestingly, a reduction in perforin secretion has been shown to promote senescent cell accumulation and age-related disorders in perforin knockout mice [139], suggesting perforin is indispensable for NKCC-mediated cytotoxicity towards senescent cells. Importantly, M. Tariq has also found reduced NKCC towards senescent fibroblasts with

age [unpublished, personal communication], and this has formed the basis of this thesis.

Although it is well-documented that ageing is accompanied by a marked reduction in NKCC-mediated granule exocytosis [14,53], the mechanisms responsible remain unknown. However, there is mounting evidence to suggest that this intrinsic defect may be the result of aberrant intracellular signalling, a theme commonly associated with immune cell dysfunction in older adults [5,34,48]. Supporting this assumption is the work conducted by our laboratory which showed comparable amounts of perforin released by NK cells from young and old adults treated with phorbol 12-myristate 13-acetate (PMA) and ionomycin [unpublished findings by J. Hazeldine], compounds that bypass cell surface receptors to activate PKC directly and induce effector functions such as NK cell degranulation [5,14]. Similarly, Mariani *et al*. previously showed that NK cells from older adults have a lower capacity to generate second messenger inositol triphosphate (IP_3) following K562 stimulation due to an age-associated delay in phosphatidyl-inositol bisphosphate (PIP_2) hydrolysis [140]. This age-related decrease in calcium-mobilising IP_3 may interfere with downstream signalling events, such as phosphorylation of MAPKs or activation of PKC, involved in degranulation. In conclusion, these observations reinforce the theory that impaired perforin secretion may be due to compromised intracellular signalling required for perforin mobilisation at the immunological synapse [14].

As previously mentioned, the activation of MAPKs is crucial for lytic granule polarisation during granule exocytosis, and ultimately NKCC [31,36-40]. Whilst no study to date has investigated the impact of age on MAPK signalling in NK cells, Lanna *et al*. proposed constitutive activation of MAPKs by sestrins drives T cell

immunesenescence [141]. Interestingly, this group showed an increased frequency of sestrin-dependent MAPK activation complexes (sMACs) in stressed and aged T cells, which simultaneously coordinate autophosphorylation of P38, ERK and JNK MAPKs [141]. Disruption of the sMACs restored several antigen-specific functional responses *in vitro*, suggesting sestrins have pro-ageing activities in T lymphocytes [141]. More recently, our own data has found constitutive activation of ERK1/2 in resting NK cells from older adults and that this is associated with increased sestrin 2 expression [unpublished observations by M. Tariq]. Based on these findings, we propose that constitutive activation of MAPKs may also be implicated in NK cell immunesenescence, contributing to disrupted MAPK signalling and reduced NKCC-mediated perforin secretion with age.

1.7 Hypothesis and aims

Physiological ageing is accompanied by a decline in NKCC driven in part by reduced secretion and polarisation of perforin at the immune synapse. This age-associated impairment in granule exocytosis may contribute to incomplete senescent cell clearance with age. Given that MAPK signalling is crucial for NKCC and lytic granule polarisation, we hypothesised that following NKG2D and NKp30 receptor ligation, involved in the recognition of senescent cells, NK cells from older adults would exhibit aberrant activation of P38 and ERK1/2 MAPK signalling compared with younger subjects that would result in an age-associated decrease in perforin release. To address this hypothesis, the following aims were set:

- Determine the impact of ageing on the composition of the circulating lymphocyte pool;

- Determine the maturity, $CD57^+$ cell percentage, of the circulating NK cell pool;
- Investigate the effect of age on perforin release by NK cells following NKG2D and NKp30 receptor ligation;
- Investigate the effect of age on P38 and ERK1/2 activation in NK cells at rest and following receptor ligation of the activating receptors NKG2D and NKp30.

2.0 MATERIALS AND METHODS

2.1 Reagents and equipment

2.1.1 Reagents and chemicals

- Medium for PBMC isolation: RPMI-1640 media supplemented with 2% (v/v) L-glutamine-penicillin-streptomycin (GPS; Sigma-Aldrich, Missouri, USA)
- Ficoll-Paque™ Plus (GE Healthcare, Buckinghamshire, UK)
- autoMACS® Running buffer: 0.5% (w/v) bovine serum albumin (BSA), 2mM ethylenediaminetetraacetic acid (EDTA), phosphate-buffered saline (PBS), 0.09% (v/v) azide (Miltenyi Biotec, Bergisch Gladbach, Germany)
- Complete medium for NK cell resuspension: RPMI-1640 media supplemented with 10% fetal calf serum (FCS) and 2% GPS (Sigma-Aldrich, Missouri, USA)
- 50 µl of Fix & Perm® Medium A (Life Technologies, Carlsbad, CA)
- 25 nm PMA (Sigma-Aldrich, Missouri, USA)
- 1.6% (w/v) paraformaldehyde (PFA; Sigma-Aldrich, Missouri, USA)
- Carbonate-bicarbonate buffer (Sigma-Aldrich, Missouri, USA)
- 10% (v/v) heat-inactivated FCS (HIFCS; Sigma-Aldrich, Missouri, USA)
- 2x sodium dodecyl sulphate (SDS) sample buffer: 4% (v/v) SDS, 0.1M dithiothreitol (DTT), 20% (w/v) glycerol, 0.0625M Tris-HCL, 0.004% (w/v) bromophenol blue and double distilled water (ddH_2O) (Sigma-Aldrich, Missouri, USA)
- BSA (Fisher Scientific, New Hampshire, USA)
- TBST: 1x Tris buffered saline (TBS) (pH 7.5) containing 0.1% (v/v) Tween-20 (Fisher Scientific, New Hampshire, USA)
- Enhanced chemoluminescence (ECL) substrates (GE Healthcare, Buckinghamshire, UK)
- Mild stripping buffer: 1.5% (w/v) glycine, 0.1% (w/v) SDS, 1% (v/v) Tween-20 and ddH_2O (pH 2)

2.1.2 Antibodies

- 200 µg/ml Pacific Blue™-conjugated mouse anti-human CD3 antibody (Clone: UCHT1; BioLegend®, San Diego, CA)

- 50 µg/ml PE-conjugated monoclonal mouse anti-human CD56 antibody (Clone: AF12-7H3; Miltenyi Biotec, Bergisch Gladbach, Germany)
- 50 µg/ml FITC-conjugated mouse anti-human CD57 antibody (Clone: HCD577; BioLegend®, San Diego, CA)
- 2.5 µg/ml APC anti-ERK1/2 phosphospecific mouse antibody (Clone: 6B8B69; BioLegend®, San Diego, CA)
- 2.5 µg/ml APC isotype mouse IgG2α κ antibody (Clone: MOP; BioLegend®, San Diego, CA)
- 5 µg/ml NKG2D agonistic monoclonal antibody (Clone: 149810; R&D Systems, Minneapolis, MN)
- 5 µg/ml NKp30 agonistic monoclonal antibody (Clone: 210847; R&D Systems, Minneapolis, MN)
- Phospho-P38 rabbit monoclonal antibody (Cell Signalling Technology, Massachusetts, USA)
- Phospho-p44/42 MAPK (ERK1/2) rabbit monoclonal antibody (Cell Signalling Technology, Massachusetts, USA)
- HRP conjugated-anti-rabbit IgG antibody (GE Healthcare, Buckinghamshire, UK)
- P38 rabbit monoclonal antibody (Cell Signalling Technology, Massachusetts, USA)
- p44/42 MAPK (ERK1/2) rabbit monoclonal antibody (Cell Signalling Technology, Massachusetts, USA)

2.1.3 Equipment and software

- NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany)
- LS columns and a QuadroMACS™ Separator (Miltenyi Biotec, Bergisch Gladbach, Germany)
- CyAn$_{ADP}$ cytometer (Dako Ltd, Cambridge, UK)
- Summit v4.3 software (Beckman Coulter, California, USA)
- Commercial perforin (PRF1) human *in vitro* ELISA kit (Abcam, Cambridge, UK)
- Spectrophotometer (BioTek, Vermont, USA)
- Gen5 software (BioTek, Vermont, USA)

- GraphPad Prism® software (GraphPad Software Inc., California, USA)
- Polyvinylidene difluoride (PVDF) membranes (Bio-Rad, California, USA)
- ChemiDoc technology (Bio-Rad, California, USA)
- ImageLab software (Bio-Rad, California, USA)
- ImageJ software (National Institutes of Health, Maryland, USA)

2.2 Participants and blood sampling

This study was approved by the University of Birmingham Research Ethics Committee (ref REN 14-1342). A total of 16 healthy young (mean age 28 years, range 18-35 years, 6 female, 10 male) and 23 healthy old (mean age 74 years, range 65-82 years, 15 female, 8 male) volunteers were recruited into this study. Individuals taking any medication known to influence function were excluded from this study. Prior to blood sampling, written consent was obtained from all volunteers, who at the time were free from infection. Approximately 20-30 ml of Venous blood was collected into heparinised vacutainers (BD Biosciences, Oxford, UK) by qualified healthcare professionals under sterile conditions using a standard venepuncture technique.

2.3 Immune cell isolation

2.3.1 Peripheral blood mononuclear cell (PBMC) isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood samples using Ficoll-Paque density gradient centrifugation. To begin, heparinised blood was diluted at a 1:1 ratio with RPMI-1640 media supplemented with 2% (v/v) GPS. To establish a Ficoll-Paque density gradient, 10-15 ml of blood was gently layered on top of 6 ml of Ficoll-Paque™ Plus. Gradients were centrifuged at 400 x *g* for 30 minutes at 22°C with no break. Post-spin, PBMCs, which resided at the plasma-Ficoll-Paque interface, were transferred into 25 ml universals containing autoMACS® Running buffer. Cells were washed twice with autoMACS® Running buffer at 300 x *g*

for 10 minutes at 22°C. Supernatants were discarded and cells re-suspended in 10 ml of autoMACS® Running buffer, after which PBMC numbers were determined using a haemocytometer.

2.3.2 NK cell isolation

NK cells were separated from PBMC suspensions via negative selection using a NK cell isolation kit and MACS® technology (Miltenyi Biotec, Bergisch Gladbach, Germany). Freshly isolated PBMCs were spun at 300 x *g* for 10 minutes at 22°C, after which supernatants were discarded and cells were re-suspended in 40 µl of autoMACS® Running buffer per 10^7 total cells. PBMCs were incubated with 10 µl of human NK cell biotin-antibody cocktail (Miltenyi Biotec, Bergisch Gladbach, Germany) per 10^7 total cells for 10 minutes at 4°C with occasional mixing. Post incubation, 30 µl of autoMACS® Running buffer per 10^7 total cells was added to the mixture, which was incubated with 20 µl of NK cell MicroBeads cocktail (Miltenyi Biotec, Bergisch Gladbach, Germany) per 10^7 total cells for 10 minutes at 4°C with occasional mixing. Cell suspensions were made up to 500 µl with autoMACS® Running buffer in preparation for magnetic separation.

To separate non-magnetically labelled NK cells from the magnetically labelled cell fraction, LS columns were placed in the magnetic field of a QuadroMACS™ Separator and washed with 3 ml of autoMACS® Running buffer. PBMC suspensions were passed through the LS columns, followed by 3 ml of autoMACS® Running buffer to flush any remaining non-magnetic NK cells. Purified NK cells were collected and centrifuged at 300 x *g* for 10 minutes at 22°C. Supernatants were discarded and cells were re-suspended in RPMI-1640 media supplemented with 10% FCS and 2% GPS (hereafter

referred to as complete medium). NK cell numbers were determined using a haemocytometer.

2.4 Immunostaining

2.4.1 NK cell phenotyping

Freshly isolated PBMCs were stained with CD3, CD56 and CD57 antibodies to analyse the composition of the NK cell pool in both young and old volunteers. In brief, 250,000 PBMCs were plated into wells of a 96-well round bottom-plate (BD Falcon™, New Jersey, USA), which was spun at 250 x *g* for 5 minutes at 4°C. Following centrifugation, supernatants were discarded and cells re-suspended in 92 µl of PBS prior to immunostaining. Cells were stained with 1 µl of Pacific Blue™-conjugated mouse anti-human CD3 antibody, 2 µl of PE-conjugated monoclonal mouse anti-human CD56 antibody and 5 µl of FITC-conjugated mouse anti-human CD57 antibody for 15 minutes on ice in the dark. Post incubation, cells were washed with 100 µl of PBS at 250 x *g* for 5 minutes at 4°C. Cells were fixed with 50 µl of Fix & Perm® Medium A for 20 minutes in the dark at room temperature (RT), after which PBMCs were washed with 100 µl of PBS at 250 x *g* for 5 minutes at 4°C. Subsequent pellets were re-suspended in 300 µl of PBS and placed in 5ml polypropylene round-bottom tubes (BD Falcon™) for flow cytometric analysis on a CyAn$_{ADP}$ cytometer using Summit v4.3 software.

To determine lymphocyte proportions, 10,000 lymphocytes were gated based on a forward scatter (FS)/side scatter (SS) properties and the percentage of cells displaying a $CD3^+$, $CD3^+CD56^+$, $CD3^-CD56^+$, $CD3^-CD56^+CD57^-$ and $CD3^-CD56^+CD57^+$ surface phenotype recorded (Figure 2.1).

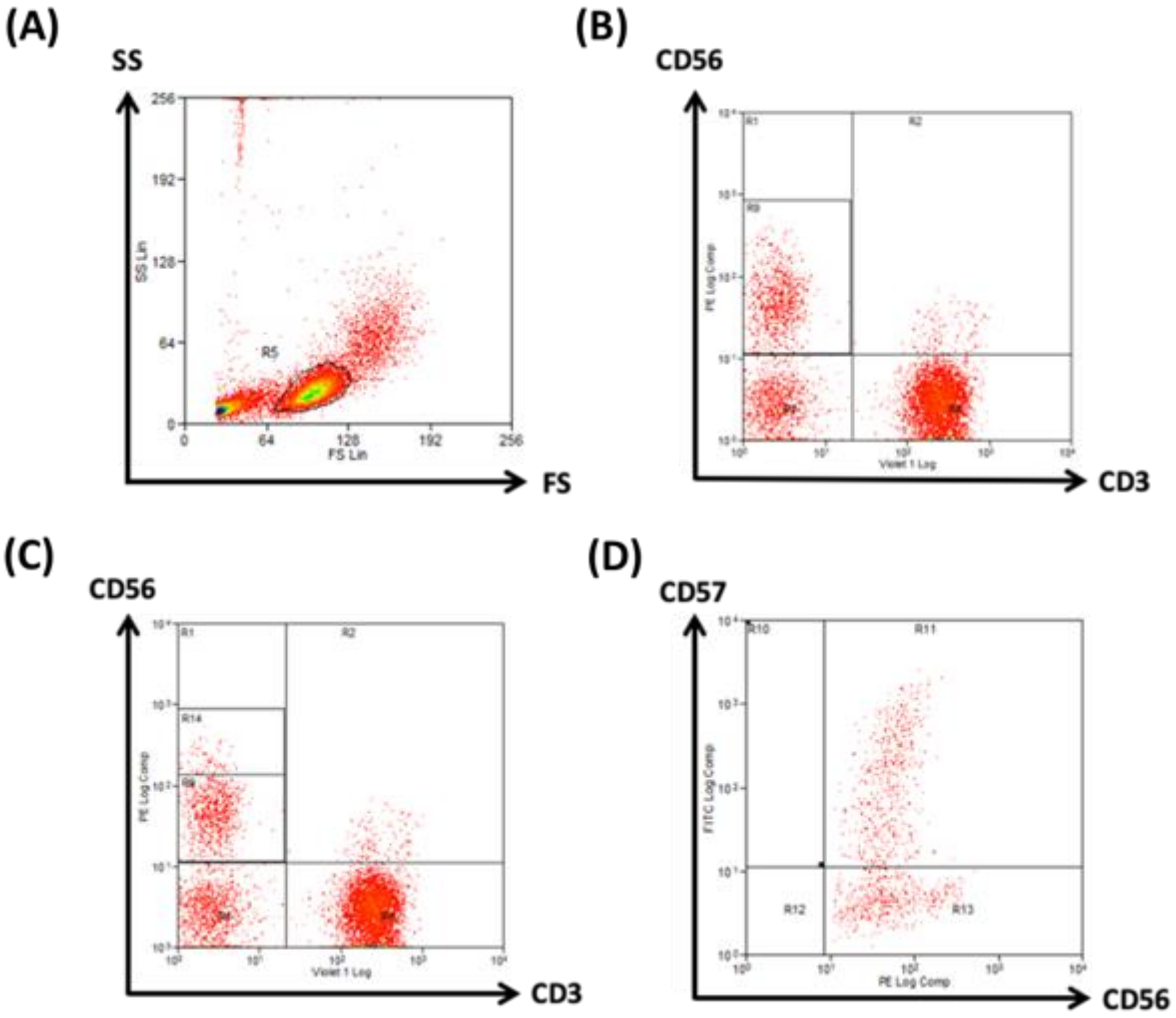

Figure 2.1**: Gating strategy for NK cell phenotyping from PBMCs**

(A) 10,000 cells were gated using a FS/SS flow cytometry plot to analyse the composition of the circulating lymphocyte pool. **(B)** NK cells were identified by a CD3^{-}CD56^{+} surface phenotype examined on a Violet 1 Log/PE log comp flow cytometry plot. **(C)** Based on differential expression of CD56, NK cells were further categorised into two distinct subpopulations: CD56^{DIM} and CD56^{BRIGHT}. **(D)** Afterwards, NK cell subsets and total NK cells were assessed for CD57 surface expression, which allowed identification of four distinct NK cell subgroups: CD56^{DIM}CD57^{-}, CD56^{DIM}CD57^{+}, CD56^{BRIGHT}CD57^{-} and CD56^{BRIGHT}CD57^{+}.

2.4.2 Measurement of ERK activation by flow cytometry

To analyse activation of the ERK1/2 signalling pathway in response to stimulation with PMA, NK cells (2 x 10_6ml) were treated with 25nM PMA for 5 minutes (37°C, 5% CO_2). Untreated NK cells were used to determine basal ERK1/2 expression. Post stimulation, NK cells were washed with 300 µl of ice cold PBS at 250 x *g* for 5 minutes at 4°C, after which supernatants were discarded and cells fixed with 300 µl of 1.6% (w/v) PFA for 30 minutes in the dark at RT. Following fixation, NK cells were washed with 300 µl of ice cold PBS at 250 x *g* for 5 minutes at 4°C, and subsequent pellets permeabilised with 300 µl of ice cold methanol for 20 minutes in the dark at RT. Cells were washed with 2 ml of ice cold PBS at 250 x *g* for 5 minutes at 4°C and re-suspended in 95 or 97.5 µl of ice cold PBS in preparation for intracellular staining. NK cells were stained with 2.5 µl of APC anti-ERK1/2 phosphospecific mouse antibody or 5 ul of APC isotype mouse IgG2α κ antibody for 30 minutes in the dark at 4°C. Afterwards, cells were washed with 500 µl of ice cold PBS at 250 x *g* for 5 minutes at 4°C; supernatants were discarded and NK cells re-suspended in 300 µl of ice cold PBS for flow cytometric analysis on a CyAn$_{ADP}$ cytometer using Summit v4.3 software. 10,000 NK cells were counted and the percentage of APC positive NK cells recorded along with the corresponding median fluorescence intensity (MFI) value.

2.5 Antibody stimulation of NK cells

As described by Hazeldine *et al*., 96-well flat-bottomed Enzyme-Linked Immunosorbent Assay (ELISA) Nunc plates (BD Falcon™) were coated with 100 µl of mouse monoclonal antibodies targeted against activating receptors NKG2D and NKp30 diluted from stock with carbonate-bicarbonate buffer [54]. After overnight incubation at 4°C, plates were washed 3X with 200 µl of PBS and blocked with 100 µl

of 10% (v/v) HIFCS/PBS for 30 minutes at RT. Post incubation, the blocking solution was decanted and plates washed 3X with 200 µl of PBS in preparation for NK cell stimulation.

150,000 freshly isolated NK cells were added to the wells and samples incubated with the NKG2D and NKp30 agonistic antibodies for 2, 5, 10, 30, 60, 90, 120 and 180 minutes (37°C, 5% CO_2).

To prepare cell lysates for Western blotting, 20 µl of hot 2x SDS sample buffer was added to the wells and lysates transferred to 0.5 ml Eppendorf's. Samples were boiled at 100°C for 10 minutes and stored at -20°C. Untreated NK cells served as basal controls.

For measurement of perforin secretion, receptor-stimulated NK cells were transferred to 0.5 ml Eppendorf's and centrifuged at 1500 x *g* for 2 minutes at 4°C. Post spin, cell-free supernatants were collected and stored at -80 until use.

2.6 Measurement of perforin release by stimulated NK cells

Perforin concentrations were determined using a commercial perforin (PRF1) human *in vitro* ELISA kit. Briefly, 80 µl of complete medium was added to thawed samples and 100 µl aliquots dispensed into wells of a microtiter plate pre-coated with a monoclonal antibody specific for perforin. Following incubation for 1 hour at RT, wells were aspirated and washed 3X with 1x wash buffer (diluted 200-fold from 200x wash buffer concentrate with ddH_2O). Post wash, 50 µl of 1x biotinylated anti-perforin (diluted from stock with biotinylated antibody diluent) was added into wells and the plate incubated for 1 hour at RT. Afterwards, the liquid was decanted and wells washed 3X with 1x wash buffer prior to incubation with 100 µl of 1x streptavidin-HRP solution (streptavidin-

HRP concentrate diluted with HRP-diluent) for 30 minutes at RT. Following liquid aspiration, wells were washed 3X with 1x wash buffer and incubated with 100 µl of chromogen TMB substrate solution for approximately 5 minutes in the dark at RT, after which 100 µl of stop reagent was dispensed into wells and the absorbance read on a spectrophotometer with a primary wavelength of 450 nm and a reference wavelength of 630 nm using Gen5 software. Perforin concentrations were determined using a standard curve that was created using values obtained from standards of known perforin concentrations via GraphPad Prism® software.

2.7 Assessment of MAPK signalling in receptor-stimulated NK cells by Western blotting

Frozen NK cell lysates were placed on a heating block for 8 minutes at 100°C and separated on 10% SDS-polyacrylamide gels for 90 minutes at 150V with a current of 35 amps. Proteins were transferred onto PVDF membranes for 90 minutes at 100V with a limit of 0.45 amps, after which membranes were blocked with 5% (w/v) BSA in 1x Tris buffered saline (TBS) (diluted 10-fold from 10x TBS (pH 7.5)) containing 0.1% (v/v) Tween-20; hereafter referred to as TBST) for 1 hour at RT. Blots were probed with rabbit monoclonal antibodies targeted against phospho-P38 or phospho-p44/42 MAPK (ERK1/2) (both diluted 1:1000 with TBST containing 2.5% (w/v) BSA) overnight on a shaker at 4°C. The following day, primary antibodies were removed and blots washed 3X for 15 minutes with TBST at RT before being probed with HRP conjugated-anti-rabbit IgG antibody (diluted 1:4000 in TBST; GE Healthcare) for 1 hour at RT on a shaker. Post incubation, blots were washed 3X for 10 minutes with TBST, after which protein levels were determined by ECL using ChemiDoc technology and ImageLab software.

Total loading controls were prepared to confirm equal amounts of protein expression across samples. In brief, blots were stripped with mild stripping buffer for 20 minutes at RT with some agitation to remove bound antibodies for subsequent primary antibody probing. Blots were washed twice with PBS for 10 minutes on a shaker at RT, followed by two more washes with TBST for 5 minutes on a shaker at RT. After the final wash, blots were washed for 15 minutes with TBST and blocked with 5% (w/v) BSA in TBST for 1 hour at RT. Afterwards, blots were probed with rabbit monoclonal antibodies targeted against P38 and p44/42 MAPK (ERK1/2) (diluted 1:1000 with 1x TBS containing 2.5% (w/v) BSA). The following day, blots were washed 3X for 15 minutes with TBST and probed with anti-rabbit IgG antibody conjugated with HRP for 1 hour at RT on a shaker. Blots were washed 3X for 10 minutes with TBST and protein levels were determined by ECL using ChemiDoc technology and ImageLab software. Densitometry analysis was performed using ImageJ software to quantify protein expression.

2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism® software. Data distribution was checked using a Kolmogorov-Smirnov test. For normally distributed data either a paired or unpaired student t-test was used, whereas a Wilcoxon-matched pairs signed rank test or a Mann Whitney U test was applied for non-normally distributed data. To assess differences between the means of P38 phosphorylation in unstimulated and receptor-stimulated NK cells, a repeated measures analysis of variance (ANOVA) was performed in addition to Dunnett's *post hoc* test. Statistical significance was accepted at $P \leq 0.05$.

3.0 RESULTS

3.1 The effect of age on the composition of the lymphocyte pool

Immunostaining and flow cytometric analysis of PBMCs isolated from healthy young and old adults were used to determine the effect of age on the composition of the circulating lymphocyte pool. Differences in lymphocyte proportions were observed between the two groups with older adults displaying a significantly lower proportion of $CD3^{+}CD56^{-}$ T lymphocytes compared to young adults (P = 0.0218) (Figure 3.1A). Conversely, no age-related difference in the proportion of circulating NKT cells was found due to marked variation in the percentage $CD3^{+}CD56^{+}$ of NKT cells between older participants (P = 0.4100) (Figure 3.1B). Analysis of NK cells revealed a greater percentage of $CD3^{-}CD56^{+}$NK cells in the lymphocyte pool of older adults, which rose from 10.66% in younger adults to 15.77% in elders (P = 0.0008) (Figure 3.1C).

3.2 The effect of age on the percentage of NK cell subsets

NK cells can be categorised as $CD56^{DIM}$ or $CD56^{BRIGHT}$ based on differential surface expression of CD56 [14]. When analysed according to this criterion, we found that older adults had a significantly higher proportion of $CD3^{-}CD56^{DIM}$ NK cells (14.97%) in their lymphocyte pool compared to young adult controls (9.57%) (P = 0.0004) (Figure 3.2A). However, no age-related difference in the percentage of $CD3^{-}CD56^{BRIGHT}$ NK cells was observed (P = 0.2241) (Figure 3.2B).

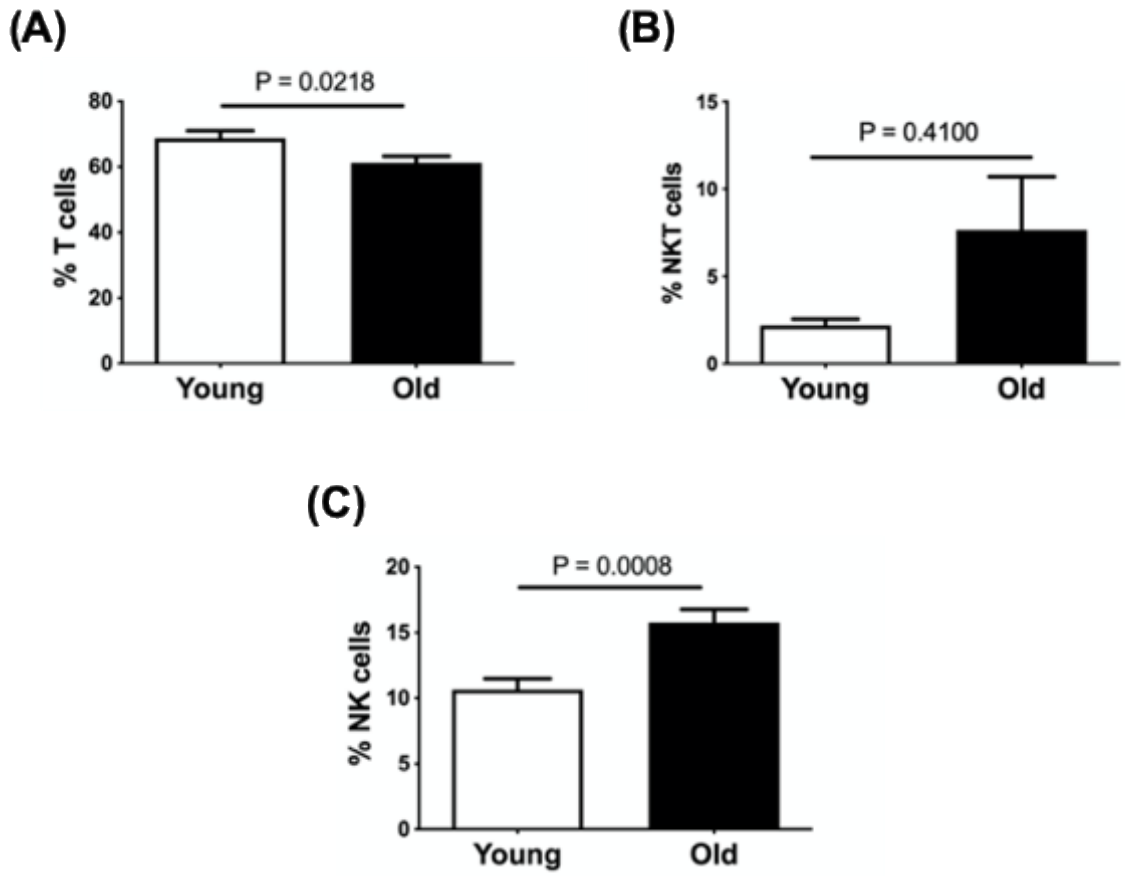

Figure 3.1: **Age-related changes in the composition of the lymphocyte pool**

(A-C) Comparison of the circulating percentage of T cells **(A),** NKT cells **(B)** and NK cells **(C)** between young and old adults. Values represent mean ± standard error of the mean (SEM) of 10 young and 13 old donors **(A-B)** and 15 young and 18 old donors **(C)** with differences between the groups assessed using unpaired student t-tests and Mann Whitney U tests. Data are combined with previous unpublished work by L. Rimmer: young (n=5) and old (n=5) participants.

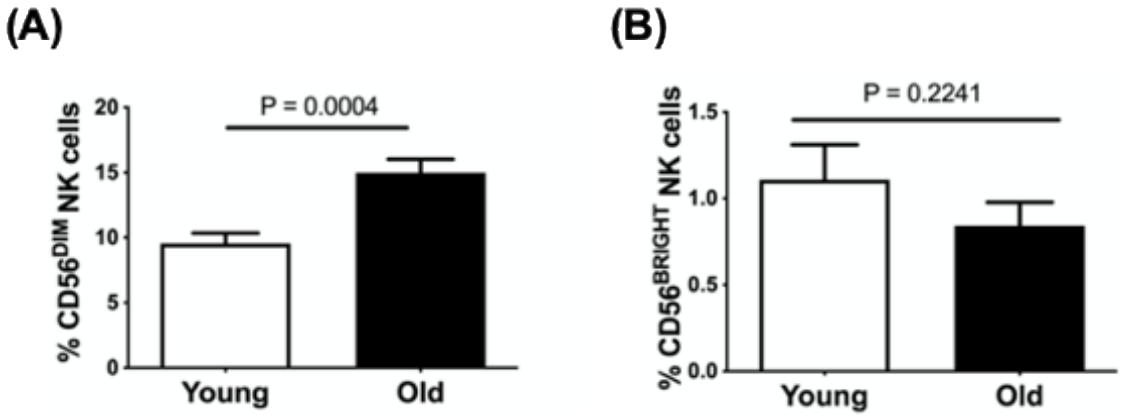

Figure 3.2: **Effect of age on the distribution of NK cell subsets**

(A-B) Comparison of the percentage of circulating $CD56^{DIM}$ **(A)** and $CD56^{BRIGHT}$ **(B)** NK cells between young and old donors taken as a proportion of total circulating lymphocytes. Values represent mean ± SEM of 15 young and 18 old donors. Differences between the two groups were assessed by an unpaired student t-test **(A)** and a Mann Whitney U test **(B)**. Data are combined with previous unpublished work by L. Rimmer: young (n=5) and old (n=5) participants.

3.3 The effect of age on NK cell maturity

One facet of immunesenescence is the accumulation of terminally differentiated immune cells, which is thought to drive immune dysfunction and may contribute to functional deterioration in NK cells, including reduced NKCC with age [52,142]. Thus, NK cells were assessed based on differential surface expression of CD57, a marker of cell maturity. We found that older adults expressed a significantly higher proportion of $CD57^{+}$ NK cells (55.73%) than their younger counterparts (38.46%) (P = 0.0017) (Figure 3.3A). When looking at CD57 expression in specific NK cell subsets, the percentage of $CD56^{DIM}CD57^{+}$ NK cells was significantly higher in older adults (P = 0.0232) (Figure 3.3B). However, we did not observe an age-related difference in the proportion of $CD56^{BRIGHT}CD57^{+}$ NK cells (P = 0.3434) (Figure 3.3C).

3.4 Perforin release by receptor-stimulated human NK cells

To determine the impact of age on perforin release by NK cells in response to recognition of senescent cells, perforin concentrations were compared between NK cells isolated from young and old donors following ligation of activating receptors NKG2D and NKp30. A time course of 2 and 3 hours was used as previous work by our laboratory showed perforin secretion is highest at these time points [unpublished work by M. Tariq; 54]. Unexpectedly, no differential effect for age was observed as NK cells from older adults released similar amounts of perforin at both time points compared with younger subjects (P = 0.1467 for the 2 hour time point) (Figure 3.4A and 3.4B).

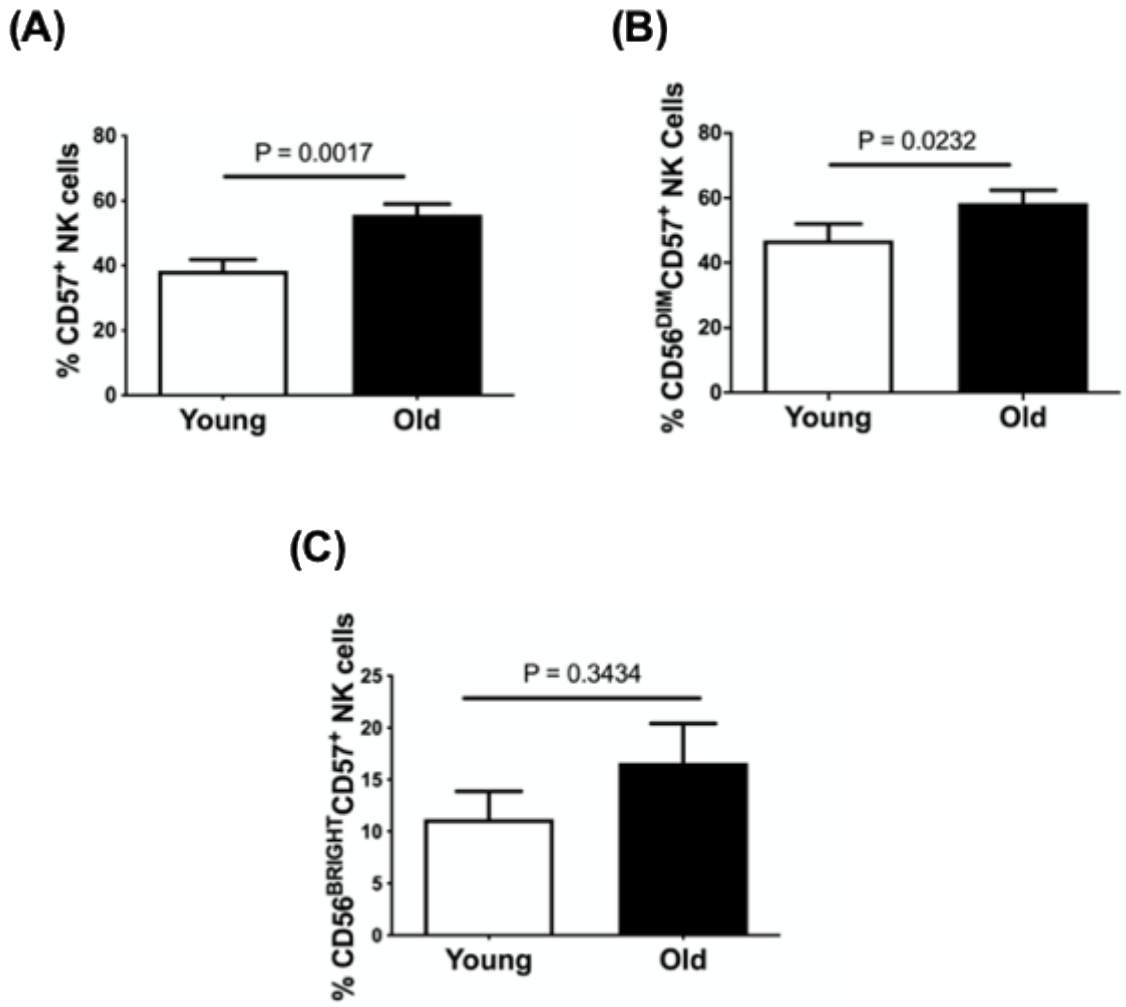

Figure 3.3: **Effect of age on NK cell maturity**

(A-C) Comparison of the percentage of $CD57^+$ total NK cells **(A)**, $CD56^{DIM}CD57^+$ NK cells **(B)** and $CD56^{BRIGHT}CD57^+$ NK cells **(C)** between young and old adults. Values represent mean ± SEM of 15 young and 18 old donors **(A)** and 10 young and 13 old donors **(B-C)**. Differences between the two groups were assessed by unpaired student t-tests and Mann Whitney U tests. **(A)** Data are combined with previous unpublished work by L. Rimmer: young (n=5) and old (n=5) participants.

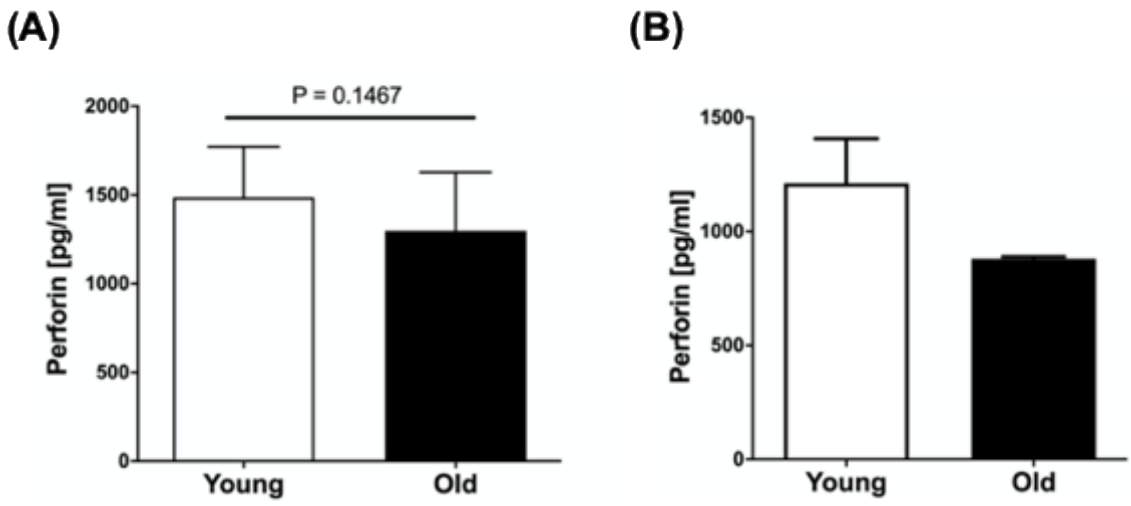

Figure 3.4: **Effect of age on perforin secretion by receptor-stimulated NK cells** **(A-B)** Comparison of perforin secretion by receptor-stimulated NK cells between young and old subjects after 2 **(A)** and 3 **(B)** hours of NKG2D and NKp30 activating receptor ligation. Data are mean ± SEM of 10 young and 3 old donors **(A)** and 10 young and 2 old donors **(B).** Differences between the two groups were assessed by an unpaired student t-test **(A)**.

3.5 P38 and ERK1/2 MAPK signalling in resting and PMA-treated NK cells

Constitutive MAPK activation has been shown previously to occur in NK cells from old donors [unpublished work by M. Tariq]. Since this age-related difference in P38 and ERK MAPK signalling has been shown to drive immunesenescence in aged T cells [141], constitutive activation of P38 and ERK MAPKs may also be implicated in reduced NKCC with age. To determine whether there are age-related differences in basal P38 and ERK1/2 MAPK signalling, P38 and ERK1/2 phosphorylation in resting human NK cells and those stimulated with 25nM of PMA were assessed by Western blotting and flow cytometry. We observed no increase in P38 signalling following PMA stimulation in both age cohorts (P = 0.1982 for young adults; P = 0.1141 for older adults) (Figure 3.5A-B). Additionally, there was no age-related difference in P38 phosphorylation in unstimulated NK cells (P = 0.3147) (Figure 3.5C).

Using flow cytometric analysis, we observed a significant increase in the percentage of NK cells that stained positive for phosphorylated ERK following PMA stimulation (P < 0.0001 for young adults; P = 0.0014 for older adults) (Figure 3.6A), which corresponded to a higher staining intensity of phosphorylated ERK (P = 0.0008 for young subjects) (Figure 3.6B). Nonetheless, no age-associated difference in either the percentage of NK cells positive for phosphorylated ERK or the staining intensity for this protein was found in resting (P = 0.3485 for % ERK_+ NK cells; P = 0.3762 for pERK MFI) and PMA-treated NK cells (P = 0.3762 for % ERK_+ NK cells; P = 0.5048 for pERK MFI) (Figure 3.6A and Figure 3.6B). Similarly, no statistical change was witnessed when upregulation of phosphorylated ERK in response to PMA stimulation was compared between the two groups (Figure 3.6C and Figure 3.6D). Analysis by Western

blotting revealed that PMA stimulation rapidly induced ERK1/2 signalling in NK cells from young and old donors (Figures 3.7A and 3.7B). However, there was no age-related difference in the expression of phosphorylated ERK1/2 in resting (P = 0.0865) or PMA-stimulated NK cells (P = 0.1331) (Figure 3.7C). This pathway is therefore intact with age in response to PMA.

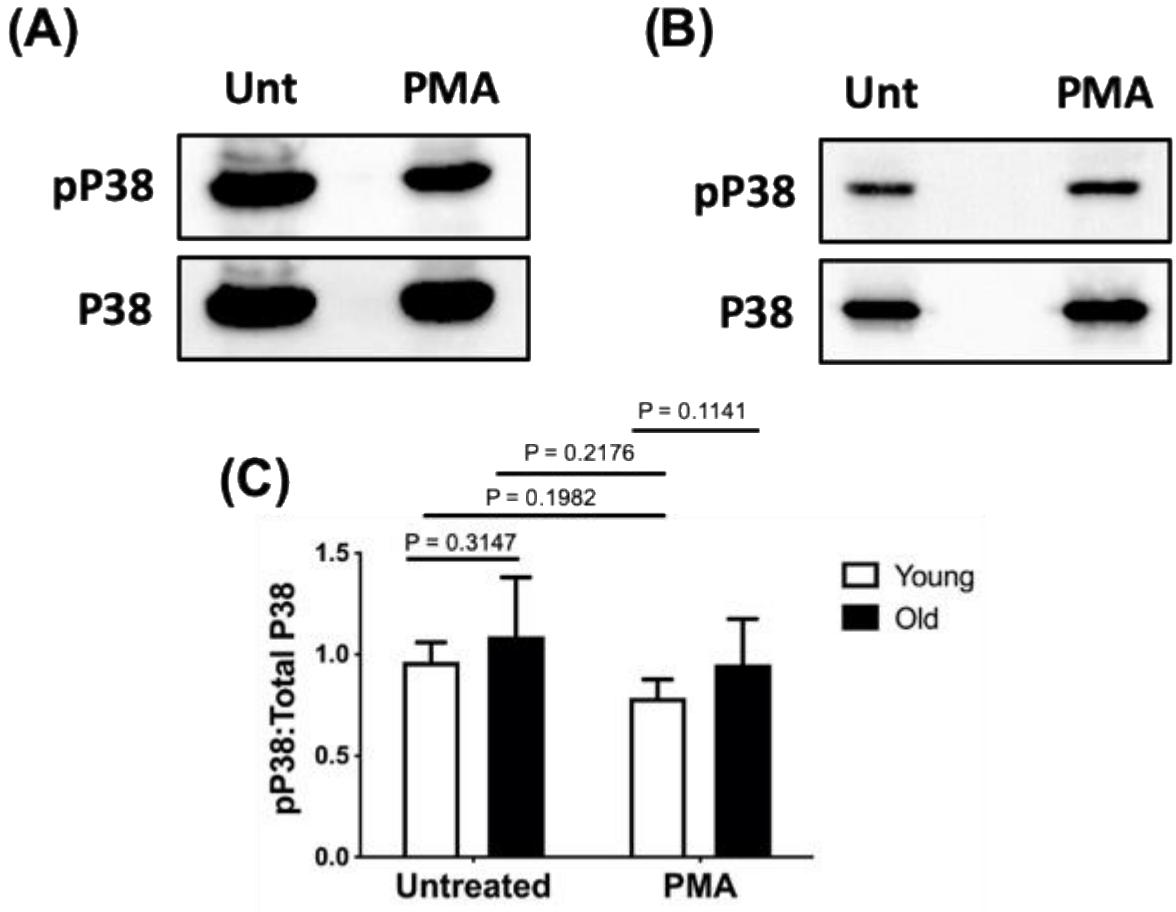

Figure 3.5: **Activation of P38 in resting NK cells**

(A-C) Comparison of phosphorylated P38 expression in resting and PMA-stimulated NK cells between young and old donors. **(A-B)** Representative blots showing the phosphorylation kinetics of P38 in resting and PMA-stimulated NK cells from young **(A)** and old **(B)** donors. **(C)** Densitometry analysis of phosphorylated P38 expression. Data are mean ± SEM of 4 young and 6 old donors. For statistical analysis, paired t-tests, Mann Whitney U tests and Wilcoxon matched-pairs signed rank tests were used.

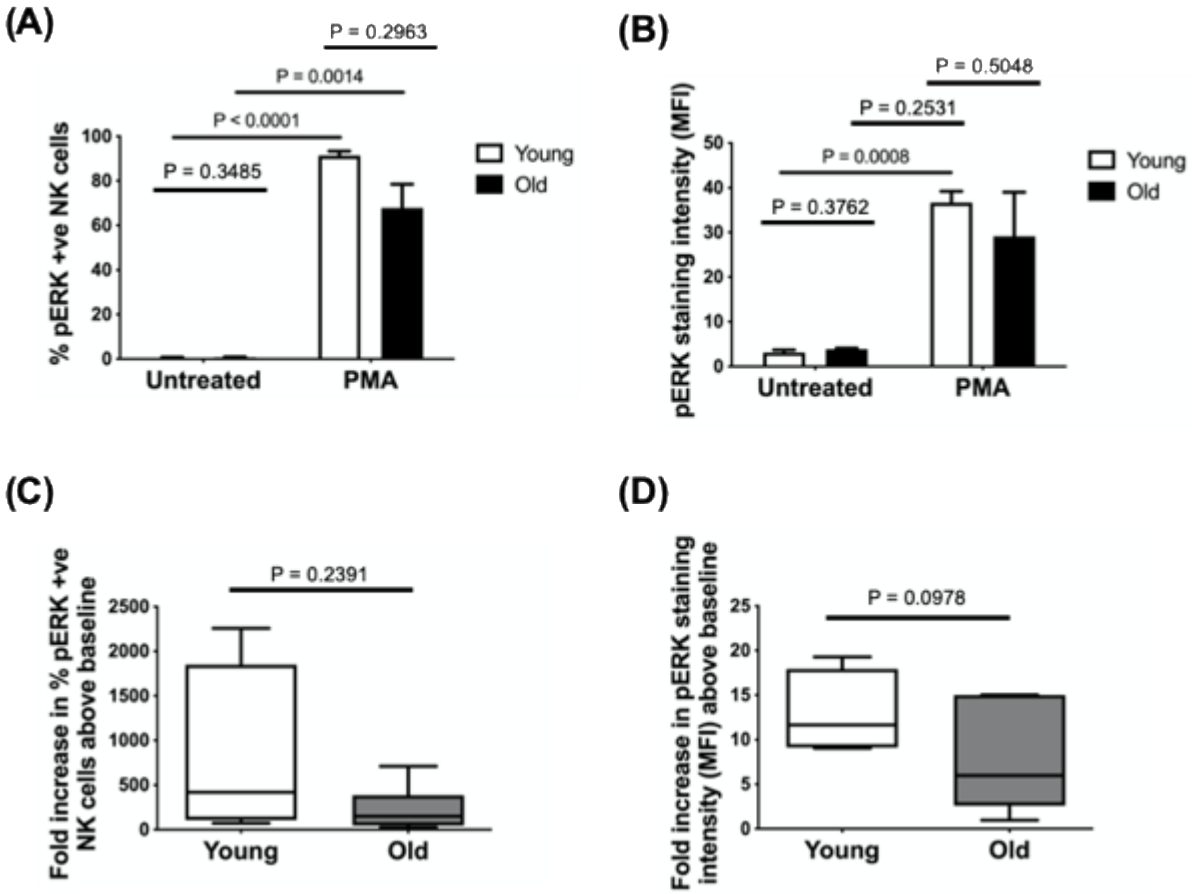

Figure 3.6: **Phosphorylated ERK staining in resting NK cells**

(A-D) Intracellular staining of phosphorylated ERK1/2 in resting and PMA-stimulated NK cells isolated from 4 young and 6 old donors. **(A)** Percentage of NK cells positive for phosphorylated ERK. **(B)** Phosphorylated ERK staining intensity (MFI). Fold increase above baseline in the percentage of pERK positive NK cells **(C)** and MFI values **(D)**. Data are mean ± SEM with differences between groups assessed by paired and unpaired student t-tests, a Wilcoxon matched-pairs signed rank test and Mann Whitney U tests.

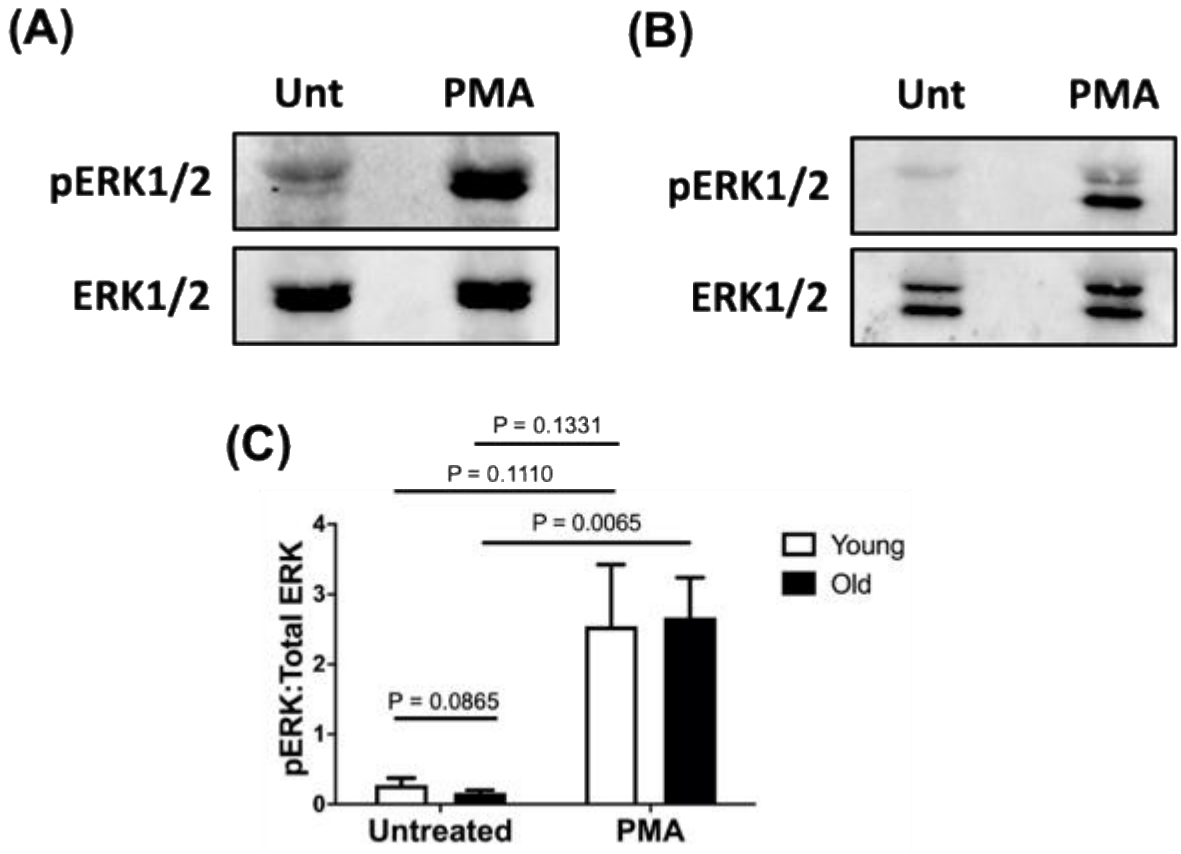

Figure 3.7: **Activation of ERK1/2 in resting NK cells**

(A-C) Freshly isolated NK cells were treated with 25 nM PMA for 5 minutes, after which phosphorylated ERK1/2 expression was assessed by Western blot analysis with Total ERK1/2 serving as a loading control. **(A-B)** Representative blots depicting the phosphorylation kinetics of ERK1/2 in resting and PMA-stimulated NK cells from young **(A)** and old **(B)** donors. **(C)** Densitometry analysis of phosphorylated ERK1/2 protein expression. Data are mean ± SEM of 3 young and 6 old donors. For statistical analysis, paired t-tests, Wilcoxon matched-pairs signed rank tests and Mann Whitney tests were used.

3.6 P38 and ERK1/2 MAPK signalling in receptor-stimulated NK cells

Given that MAPK signalling has been shown to be crucial for NKCC and granule exocytosis and was positive in response to PMA at least for ERK activation [31,36,37], we sought to determine whether ageing is associated with aberrant P38 activation in response to receptor ligation. To address this, human NK cells were incubated with agonistic antibodies targeted against NKG2D and NKp30 activating receptors over a 90-minute period, after which P38 and ERK1/2 protein phosphorylation was assessed by Western blotting. Unexpectedly, P38 phosphorylation decreased over the stimulation period and was significantly lower 90 minutes after receptor cross-linking in young adults ($P \leq 0.01$) (Figure 3.8), no difference in P38 phosphorylation was seen in receptor-stimulated NK cells from old donors (Figure 3.9).

NKG2D and NKp30 receptor ligation also did not rapidly induce ERK1/2 activation in NK cells isolated from young adults as indicated by low protein phosphorylation. Instead, stimulated NK cells expressed comparable amounts of phosphorylated ERK1/2 at each time point to that in unstimulated NK cells (Figure 3.10). Time did not allow for the study to be repeated in old adults.

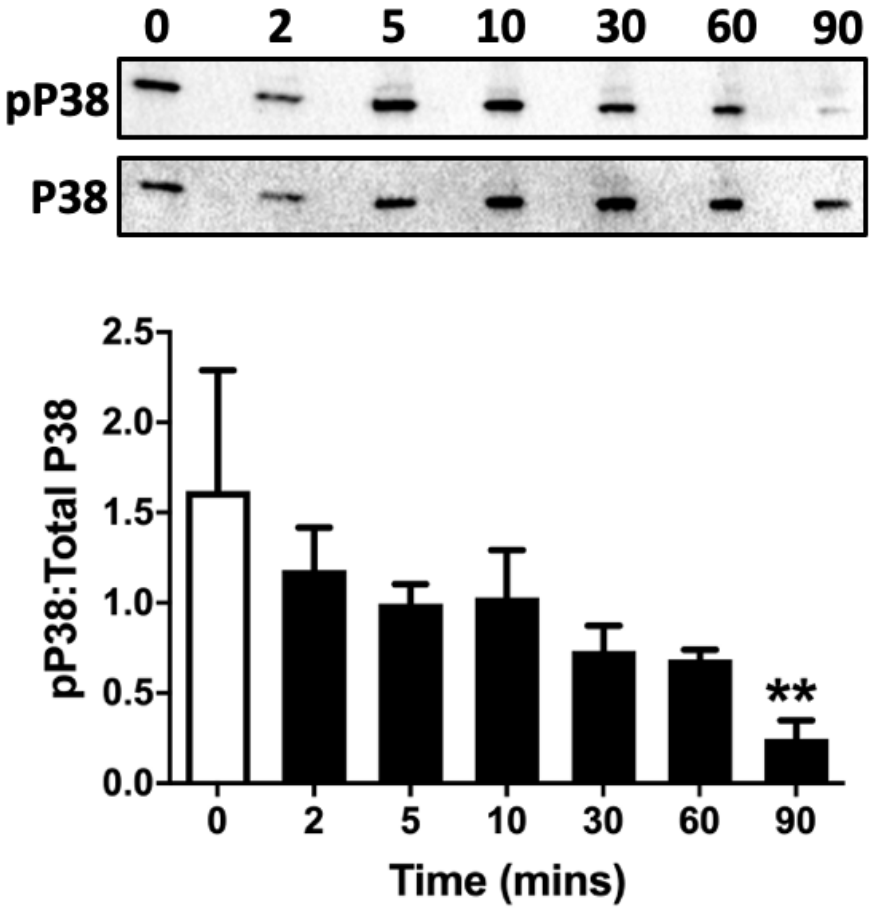

Figure 3.8: **P38 activation in NK cells isolated from young adults following NKG2D-NKp30 receptor ligation**

Phosphorylated P38 expression in human NK cells stimulated with 5 μg/ml of agonistic antibodies against NKG2D and NKp30 receptors over a 90-minute time course. Top panel shows a representative blot of 4 independent experiments for P38 stimulations. Bottom panel represents densitometry analysis of P38 MAPK activation. Data are mean ± SEM (n=4) with differences from each time point compared with 0 minute controls. For statistical analysis, a repeated measures ANOVA test was performed followed by Dunnett's *post hoc* test. ** indicates $p \leq 0.01$.

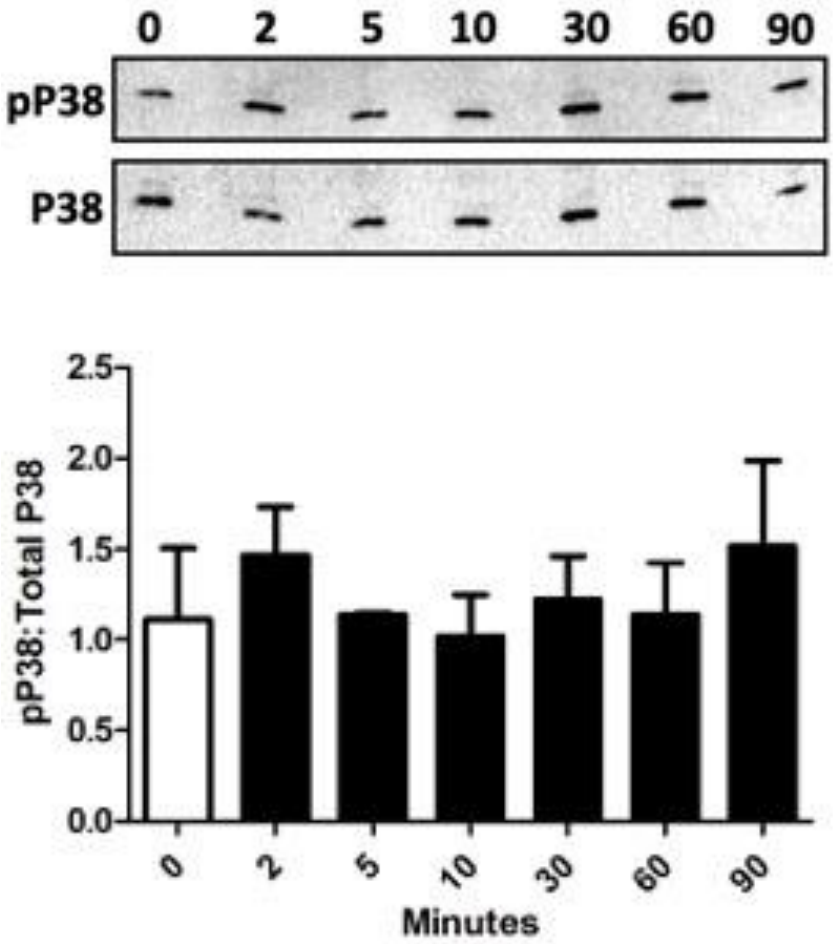

Figure 3.9: **P38 activation in NK cells isolated from older adults following NKG2D-NKp30 receptor ligation**

Human NK cells were crosslinked with agonistic antibodies targeted against NKG2D and NKp30 activating receptors for the indicated time points. Cell extracts were prepared and Western blotting analysis performed to determine phosphorylated P38 and Total P38 protein expression. Top panel: Representative Western blot, depicting P38 phosphorylation. Bottom panel: Represents densitometry analysis of P38 MAPK activation. Data are mean ± SEM (n=2).

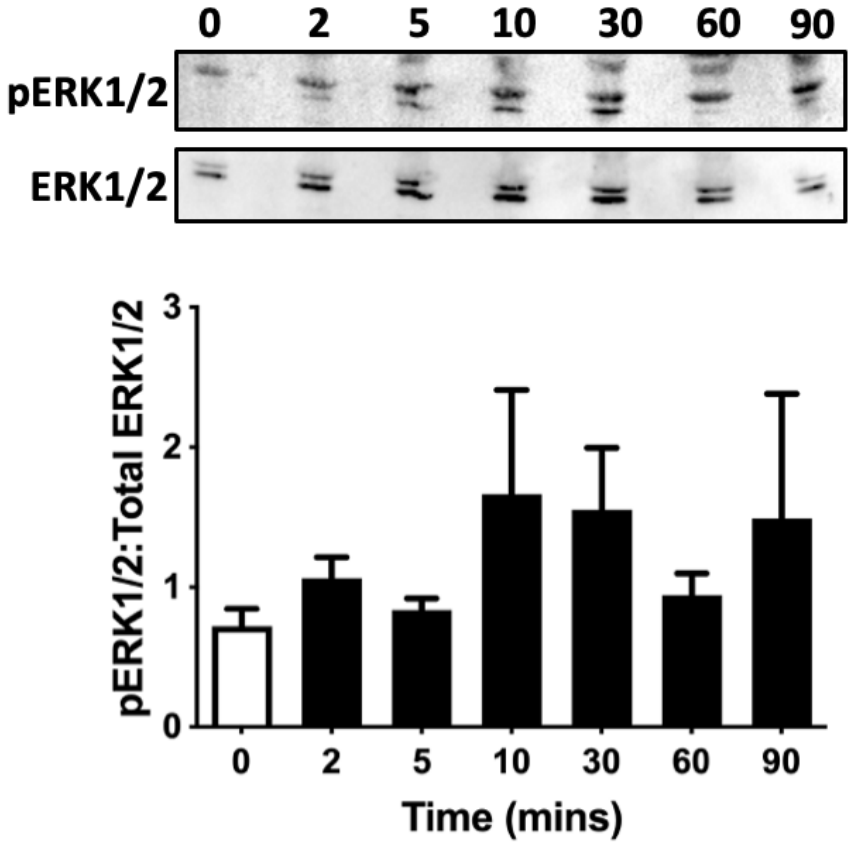

Figure 3.10: **ERK1/2 activation in NK cells isolated from young adults following NKG2D-NKp30 receptor ligation**

Phosphorylated ERK1/2 expression in human NK cells stimulated with 5 μg/ml of agonistic antibodies against NKG2D and NKp30 receptors over a 90-minute time course. Top panel shows a representative blot of 4 independent experiments for ERK1/2 stimulations. Bottom panel represents densitometry analysis of ERK1/2 MAPK activation. Data are mean ± SEM with differences from each time point compared with 0 minute controls. For statistical analysis, a repeated measures ANOVA was performed along with Dunnett's *post hoc* test.

4.0 DISCUSSION

Cellular senescence is a central component of ageing that has been shown to contribute to the pathology of numerous age-related diseases [107,122-126]. Normally, senescent cells are recognised and eliminated by various immune cell types, including NK cells [115]; however, physiological ageing is accompanied by remodelling of the immune system, termed immunesenescence, that affects both innate and adaptive immunity and leads to immune incompetence [5,7,8]. Decreased killing is a well-documented feature of NK cell immunesenescence that may contribute to the age-related accumulation of senescent cells across tissues [54,134]. Studies that have investigated the effect of age on NK cell killing have reported an age-associated decline in NKCC at the single level that is associated with reduced lytic activity and impaired granule exocytosis [53,93,94]. Underlying this impairment appears to be an age-related decrease in perforin secretion by NK cells that has been attributed to reduced lytic granule polarisation towards the immunological synapse [54]. Nevertheless, the mechanism(s) responsible for this defect have yet to be unearthed; therefore, the aim of this thesis was to elucidate the causes underlying compromised NKCC with age.

Here we show that ageing is accompanied by marked alterations in the composition of peripheral NK cells. Specifically, we observed an increased proportion of $CD56^{DIM}CD57^{+}$ NK cells with age that caused a shift in the maturity status of the circulating NK cell pool towards terminal differentiation. Given that acquisition of CD57 expression is associated with functional deterioration in other immune cells [89,90,143], we propose that an increased in $CD56^{DIM}CD57^{+}$ NK cells may contribute to reduced NKCC with age. We assessed perforin in response to NKG2D and NKp30

receptor ligation but found no reduced perforin release with age. Analysis of intracellular signalling pathways in activated NK cells revealed no age-associated difference in P38 or ERK MAPK phosphorylation in response to PMA, but we also found no activation of P38 in response to receptor ligation, though ERK was activated in young donor NK cells. We did not investigate ERK activation in response to receptor ligation in old donor NK cells. Thus, defects in the activation of MAPKs such as ERK1/2, which plays a prominent role in lytic granule polarisation [37,38,40], may underlie the age-related decline in NKCC and further research with higher sample numbers is required.

4.1 NK cell phenotype and percentage

In agreement with previous studies [51,53,54], our results showed ageing to be accompanied by marked alterations in the composition of the circulating lymphocyte pool. Compared with young subjects, older adults presented with a lower proportion of $CD3^+$ lymphocytes but a substantially higher percentage of $CD3^-CD56^+$ NK cells. This increased percentage suggests the existence of long-lived NK cells in the periphery [53,54,89]. Interestingly, the proportion of $CD3^+CD56^+$ NKT cells was unaltered with age, which contrasts with earlier observations that show a significant decrease in the proportion of NKT cells in elderly individuals [59-62]. Possible reasons for this discrepancy are differences in gender and the use of specific NKT cell markers. For instance, Molling *et al*. demonstrated that in both young and old age groups, males have a lower proportion of NKT cells compared with females and that the age-associated decrease in NKT cells is more prominent in males than in females [62]. Contrary to earlier reports [59-62], our study did not age- and gender-match donors or

specifically stain for NKT cell markers, which may explain why we did not witness an age-related difference in the proportion of NKT cells.

Based on differential surface expression of CD56, human NK cells can be categorised into two functionally distinct subgroups, $CD56^{DIM}$ and $CD56^{BRIGHT}$ NK cells, whose distribution changes with age [14]. In contrast with other reports [54,55,91], our results did not reveal a difference in the proportion of $CD56^{BRIGHT}$ NK cells between young and old adults. However, we did observe an age-associated increase in the $CD56^{DIM}$ NK cell percentage that was consistent with earlier studies that noted a profound increase in the percentage of $CD56^{DIM}$ NK cells in elderly individuals [54,55,91]. Whilst contributing to the age-associated increase in the circulating NK cell proportion, $CD56^{DIM}$ NK cell abundance may serve to counterbalance the age-associated decline in NKCC observed at the single cell level [54]. Originally thought to be solely responsible for direct cytotoxicity, $CD56^{DIM}$ NK cells, like $CD56^{BRIGHT}$ NK cells, are a major source of pro-inflammatory cytokines and chemokines that have various immunomodulatory effects in tissues [19,20,54,144]. Given that this subgroup rapidly produces greater amounts of pro-inflammatory modulators compared with $CD56^{BRIGHT}$ NK cells following target cell recognition [20], an increased proportion of $CD56^{DIM}$ NK cells in the periphery may therefore amplify ongoing inflammatory responses through the recruitment and overstimulation of immune effector cells [54], thereby contributing to immunesenescence and inflammageing.

In addition to redistribution of NK cell subpopulations, ageing is accompanied by a shift in the maturity status of the circulating NK cell pool towards terminal differentiation. Our findings revealed older adults possess a greater proportion of NK cells that stain positive for CD57 in comparison with younger subjects. This observation was reflected

by an age-associated increase in the proportion of $CD56^{DIM}CD57^{+}$ NK cells, whereas the percentage of $CD56^{BRIGHT}CD57^{+}$ NK cells was not affected by age. As a marker of cell maturity, CD57 expression on NK cells increases with age and predominantly affects $CD56^{DIM}$ NK cells [54,89,90]. Importantly, CD57 is commonly associated with senescence in human T cells [89,90,143], and may therefore contribute to NK cell immunesenescence in older adults [54]. Mature $CD56^{DIM}CD57^{+}$ NK cells display distinct phenotypical and functional differences in comparison with immature $CD56^{DIM}$ NK cells; these include decreased responsiveness to cytokine stimulation and poor proliferative capacity that correlates with an increased inhibitory KIR proportion [89,90,144]. Although $CD56^{DIM}CD57^{+}$ NK cells exhibit greater lytic activity following interaction with IgG-coated targets and become more frequent with age [89], older adults display similar levels of ADCC in comparison with young individuals, indicating ADCC may be impaired at the single cell level [54]. Similarly, acquisition of CD57 expression by $CD56^{DIM}$ NK cells may also explain why an age-related increase in the proportion of $CD56^{DIM}$ NK cells does not translate into enhanced NKCC.

4.2 Perforin release in response to receptor ligation

As previously mentioned, ageing is accompanied by a decline in NKCC at the single cell level that is characterised by impaired granule exocytosis [54,93,94]. Hazeldine *et al*. has also shown NKCC towards senescent cells to be reduced with age (unpublished). This author showed that despite comparable conjugate formation, NK cells from older adults exhibit reduced lytic activity towards tumour cells and impaired perforin secretion as a consequence of reduced secretory granule polarisation towards the immunological synapse [54]. Although this work looked at NK cell responses towards tumour cells, NK cells utilise the same pathway used to remove tumour cells

to also eliminate senescent cells. Importantly, NK cells recognise senescent cells via interactions between the NKG2D activating receptor and MICA/B ligands expressed on the senescent cell surface. Thus, to further investigate the mechanisms underlying reduced NKCC with age, we stimulated NK cells isolated from young and old donors with NKG2D and NKp30 receptor agonists for 2 and 3 hours, after which perforin concentrations were determined by ELISA. However, given the small sample size, we were unable to show a significant difference in perforin release by receptor-stimulated NK cells. Instead, our data suggested the concentration of perforin was comparable between young and old subjects after 2 and 3 hours of receptor ligation, which contrasts with previous data [54]. A possible explanation for these opposing observations is differences in NK cell stimulation. Whereas we only used two antibodies to stimulate two activating receptor types (i.e. NKG2D and NKp30), Hazeldine *et al*. used whole tumour cells to induce perforin secretion; thus, in this setting, there are more receptor-ligand interactions, which may result in a more prominent activating stimulus for the NK cell. Additionally, perforin secretion was not assessed in resting NK cells; therefore it may be that these antibodies are not agonistic or active in this model. Consequently, future work should aim to repeat this experiment using whole senescent cells, a larger sample size and resting NK cells as controls to study the effect of age on NKCC towards senescent cells.

4.3 MAPK signalling

MAPK activation is required for MTOC polarisation and lytic granule secretion during granule exocytosis, and ultimately NKCC [36-40]. Whilst no study to date has investigated the impact of age on MAPK signalling in NK cells, Lanna *et al*. showed constitutive activation of P38, ERK and JNK to be associated with increased sMAC

formation in aged T cells [141]. Although sestrins are stress-sensing proteins known for their anti-ageing properties, this group found that sestrins contribute to T cell ageing by promoting constitutive MAPK signalling and T cell dysfunction [141]. More recently, preliminary data from our group had found constitutive activation of ERK1/2 in resting NK cells from older adults, and that this was also associated with increased sestrin expression [unpublished work by M. Tariq]. Since there is compelling evidence that suggests NK cells from older adults exhibit reduced granule polarisation [54], we investigated the effect of age on P38 and ERK1/2 MAPK signalling in resting and receptor-stimulated NK cells. In contrast with previous unpublished work by M. Tariq, our data showed resting NK cells from young and older adults displayed constitutive P38 activity, but not ERK1/2. However, P38 phosphorylation did not differ between young and old donors, suggesting that there is no age-associated difference in MAPK activation in resting NK cells. Therefore, we concluded that the mechanisms driving functional deterioration in aged NK cells may differ to that observed in aged and stressed T cells.

With regards to MAPK activation in receptor-stimulated NK cells, NK cells from young adults were less responsive to receptor ligation compared with older subjects. In young subjects, receptor-stimulated NK cells exhibited significantly lower P38 activity in comparison with unstimulated NK cells at 90 minutes, which was unexpected. Conversely, P38 signalling did not differ between unstimulated and receptor-stimulated NK cells from older adults. Thus, we were unable to determine an age-associated difference in P38 activation in NK cells. Instead, we concluded that other MAPK signalling pathways such as ERK may be implicated in reduced NKCC with age. Assessment of ERK1/2 signalling in young adults revealed increased phosphorylated

ERK1/2 in receptor-stimulated NK cells, but this was only n=2. No studies were performed in old adult cells.

4.4 Future work

A proposed follow up study would be to determine whether there is an age-associated difference in ERK activation in receptor-stimulated NK cells given the significance of ERK in MTOC polarisation and perforin release. If an age-associated difference in ERK activation is found, it would be interesting to study PKC delta (PKCδ) activation in NK cells upon receptor ligation, since PKCδ (an upstream regulator of ERK) has been shown to be play a major role in lytic granule polarisation in $CD8^+$ T cells [145]. For example, Ma *et al*. previously demonstrated that upon stimulation, diacylglycerol (DAG) rapidly phosphorylates PKCδ, which localises to lytic granules and polarises them towards the immunological synapse for release [146].

Additionally, it would be interesting to compare perforin secretion between $CD57^-$ and $CD57^+$ NK cells to determine whether an increased percentage of $CD56^{DIM}CD57^+$ NK cells drives reduced NKCC with age. It is also important to repeat the perforin release studies, increasing the numbers for the old NK cell group with the inclusion of controls. Given that we only examined the impact of age on the proportion of circulating NK cells, it would also be interesting to determine whether age also affects the frequency of NK cells.

4.5 Conclusion

In summary, this study has shown that ageing is accompanied by marked alterations in the composition of the circulating NK cell pool, namely an increased proportion of $CD3^-CD56^+$ NK cells attributed to accumulation of long-lived $CD56^{DIM}CD57^+$ NK cells

in the periphery. We propose that this shift in the maturity status of the NK cell pool may contribute to reduced NKCC with age given that terminal differentiation is associated with senescence in T cells. We were unable to show reduced perforin secretion by receptor-stimulated NK cells with age, and did not find ageing to be associated with constitutive activation of P38 or ERK1/2 in resting NK cells. NK cells from older adults exhibited a similar level of ERK signalling to that of young subjects in response to PMA. Moreover, stimulating human NK cells with NKG2D and NKp30 receptor agonists rapidly induced ERK MAPK signalling in young adults, but did not increase P38 signalling in both age groups. Taken together, these results suggest that reduced perforin secretion is not affected by P38 signalling with age, but may be the result of defects in the activation of MAPK signalling pathways such as ERK in $CD56_{DIM}CD57^{+}$ NK cells. Therefore, future work should look to determine the role of ERK signalling in the age-related decline in perforin release by NK cells.

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